Identification of Hepatitis C Virus Core Protein Residues Critical for the Interaction with the Cellular DEAD-box Helicase DDX3 and their Functional Relevance

by

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A thesis presented for the degree of Doctor of Philosophy in the Faculty of Biomedical and Life Sciences at the University of Glasgow

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June 2007

Summary

Hepatitis C virus (HCV) is a single-stranded RNA virus belonging to the *Flaviviridae* and infects approximately 170 million people worldwide. Unlike other known RNA viruses, HCV causes a persistent infection in the majority of infected people and can lead to cirrhosis of the liver and hepatocellular carcinoma. For these reasons, HCV is rightly classified as a major human pathogen.

HCV core protein is believed to form, by analogy with other members of the *Flaviviridae* family, the nucleocapsid of the virus. As well as this, core has been shown to modulate many cellular processes via interactions with numerous host-cell proteins. One such protein shown to interact with HCV core is the DEAD-box RNA helicase DDX3. In cells expressing either HCV core alone, or as part of the full length HCV polyprotein, DDX3 is redistributed from its normal diffuse cytoplasmic localisation to lipid droplets where it colocalises with core. The cellular function of DDX3 is still unknown although it has been suggested to be involved in processes such as splicing, translation and RNA transport.

The aim of this study was to investigate the role of DDX3 in the life cycle of HCV. This was aided by the recent discovery of a fully infectious HCV genotype 2a clone (strain JFH-1), allowing previously inaccessible aspects of the virus life cycle to be studied such as particle assembly and release. A library of HCV core mutants (residues 1-59 only) was produced by error-prone PCR and subsequently expressed in bacteria and analysed for their ability to bind bacterially expressed DDX3 using a rapid, high throughput ELISA screen. Six HCV core residues, conserved throughout all genotypes, were identified as being critical for interaction with DDX3. These residues were confirmed as being critical for the interaction by transfection of mutant core (together with E1 and E2 to ensure

correct processing of core) into Huh7 cells. None of the 6 mutant core proteins were able to redistribute cellular DDX3.

In order to study the effects of abolishing the core-DDX3 interaction in terms of a fully infectious HCV life cycle, the 6 critical residues were individually mutated to alanine in the cell culture infectious strain JFH-1 genome. All 6 mutant JFH-1 RNAs were capable of replication and being translated. Further investigation however, suggested that replication rate of mutant JFH-1 RNA was >50-fold lower than that of wild type JFH-1 RNA replication. Mutant core proteins colocalised with the lipid droplet marker ADRP, indicating correct subcellular localisation of the viral protein. Western-immunoblot analysis of mutant cores also confirmed that core proteins of same molecular weight to that of wild type core were produced, suggesting mutant cores were correctly processed. Of the 6 mutant JFH-1 clones analysed, 5 of them were capable of secreting infectious HCV particles that could subsequently infect naïve Huh7 cells, as detected by immunofluorescence and RT-PCR. However, one mutant, in which residue 33 of core had been changed from glycine to alanine, was initially unable to produce infectious particles. Upon passaging of cells electroporated with this mutant, infectious particles were eventually produced. The production of infectious particles consistently coincided with the presence of a second mutation in the surrounding area of the originally mutated residue 33. However, JFH-1 RNA containing both the mutation at residue 33 and the second identified mutation nearby, was unable to produce infectious particles upon electroporation, suggesting another lesion elsewhere in the HCV genome may also be required in order to overcome the effect of mutating residue 33.

A recent report has indicated that DDX3 may be a nucleo-cytoplasmic shuttling protein, utilising the CRM1 export pathway. To confirm this, DDX3 localisation was analysed in the presence of the CRM1 inhibitor leptomycin B (LMB). In the absence of LMB, DDX3

was seen to have a diffuse cytoplasmic localisation while a small proportion was also seen in the nucleus. In the presence of LMB however, a build-up of DDX3 was seen in the nucleus, confirming that DDX3 uses the CRM1 pathway to shuttle from the nucleus to the cytoplasm.

The results of this study indicate that the interaction of the cellular DEAD-box helicase DDX3 with core protein is not essential for the life cycle of HCV. It has been shown here however, that the replication rates of mutant HCV RNA are lower than that of wild type, suggesting that DDX3 may enhance either replication itself, or translation (which in turn provides the machinery required for viral RNA replication). Investigating this possibility is the subject of our future work. The identification of glycine 33 of core protein as being essential for production of infectious virus particles (without abolishing replication) will provide the basis for further studies on the production of infectious particles and the role that core protein plays in this process. The panel of JFH-1 core mutants will also be useful in studying the core-DDX3 interaction in a much wider context involving the role of DDX3 in normal cells.

This study has uncovered important details regarding the interaction between core and DDX3 and, together with the reagents produced throughout this investigation, should enable further successful study into the role of DDX3 in the life cycle of HCV.

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Acknowledgements

Many thanks to -

Professor Duncan McGeoch for providing the opportunity to study at the MRC Virology Unit.

My Supervisor, Dr. Arvind Patel, for his excellent guidance throughout my studies and also during the writing of this thesis.

All members of Lab 106A both past and present for their support, advice and patience.

Dr. Derek Gatherer for his assistance with structural analysis.

Dr. Russell Thompson, for never failing to answer the technical question of the day, not to mention his invaluable friendship.

The Students, for the tea breaks, laughter and fun over the last 4 years.

My parents, whithout whose continued support this would not have been possible.

Lastly, but by no means least, Susan. Thanks for all your love and support, but most importantly for becoming my wife.

Author's Declaration

This work has been completed at the University of Glasgow between October 2003-2006 and has not been submitted for another degree. The work is original and unless otherwise stated in the text has been completed by the author.

Signed

Date

Definitions

aa	amino acid
APS	ammonium persulphate
ARFP	alternative reading frame protein
ATP	adenosine triphosphate
BVDV	bovine viral diarrhoea virus
bp	base pairs
BSA	bovine serum albumin
C-	carboxy terminus
CAP-Rf	core associated protein-RNA helicase full-length
cDNA	complementary DNA
CTL	cytotoxic T lymphocyte
dH_2O	distilled water
DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleoside triphosphates
ds	double stranded (DNA/RNA)
ECL	enhanced chemiluminescence
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
eIF	eukaryotic initiation factor
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FCS	foetal calf serum
FITC	fluorescin isothiocyanate
g	gravitational force
hrs	hours
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCVcc	HCV cell culture
HCVpp	HCV pseudo particle
HIV	human immunodeficiency virus
HRP	horse-radish peroxidase
Huh7	human hepatoma 7
HVR	hypervariable region
IgG	immunoglobulin G
IFN	interferon
IL	interleukin
IPTG	isopropyl β-D-1-thiogalactopyranoside
IRES	internal ribosome entry site
ISDR	interferon sensitivity determining region
kDa	kilodaltans
l	litre
LB	Luria-Bertani
LT-βR	lymphotoxin-β receptor
Luminol	3-aminophthalhydrazide
M	molar
mAb	monoclonal antibody
mg	milligram
min	minutes

ml	millilitre
mM	millimolar
mRNA	messenger RNA
N-	amino terminus
NCR	non-coding region
NEAA	non-essential amino acids
NH_4OAc	ammonium acetate
nt	nucleotide
OD	optical density
ORF	open reading frame
pAb	polyclonal antibody
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffer solution
PCR	polymerase chain reaction
RdRp	RNA dependent RNA polymerase
RNA	ribonucleic acid
Rnase	ribonuclease
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
ss	single stranded
TEMED	N,N,N,N’-tetra-methyl-ethlene diamene
TMB	3,3’5,5’-tetramethyl benzidine
TMD	transmembrane domain
TNF-R1	tumour necrosis factor- receptor 1
TP	tryptose phosphate
TRIS	2-amino-2-(hydroxymethyl)-1,3-propandiol
Tween 20	polyoxyethylene-sorbitan mono laurate
µg	microgram
µl	microlitre
UTR	untranslated region
UV	ultraviolet
V	volts
wt	wild type
YFV	yellow fever virus

1. Introduction

1.1 HCV Background

1.1.1 Discovery of Hepatitis C Virus

Hepatitis C virus infects approximately 170 million people worldwide (WHO, 2000). The virus was discovered after specific diagnostic tests for both hepatitis A virus (HAV) and hepatitis B virus (HBV) revealed that most cases of post-transfusion hepatitis were not linked to HAV or HBV (Bradley, 1999). However, it was only after many years of attempting to isolate the agent responsible for this so-called post-transfusion, non-A, non-B hepatitis (NANBH) that, in 1989, with the aid of modern techniques of molecular cloning and phage display, a new RNA virus, termed hepatitis C virus (HCV), was isolated (Choo *et al.*, 1989). HCV causes a persistent infection in the majority of infected people and can lead to cirrhosis of the liver and hepatocellular carcinoma (HCC) (Houghton, 1996, Saito *et al.*, 1990, Shimotohno, 2000). For this reason, and the high prevalence of infection worldwide, HCV is rightly classed as a major human pathogen.

1.1.2 Classification

HCV, a positive sense, single-stranded RNA virus, has been categorised as a member of the *Hepacivirus* genus within the *Flaviviridae* by genome analogy with other members of this family (Takamizawa *et al.*, 1991, Choo *et al.*, 1991, Choo *et al.*, 1989). This family also includes the flaviviruses such as dengue virus and Japanese encephalitis virus (Rice, 1996), the pestiviruses such as bovine viral diarrhoea virus and classic swine fever virus (Rice, 1996), and the recently discovered GBV-A and B viruses (Simons *et al.*, 1995) and hepatitis G virus (Linnen *et al.*, 1996). GBV-A and B were discovered after inoculum from a patient (initials GB) with acute sporadic hepatitis was used to successfully infect tamarins and the viral genomes subsequently cloned from serum of an infected animal. The HCV genome encodes a polyprotein of approximately 3011 amino acids (Choo *et al.*, 1989, Choo *et al.*, 1991), which is comparable in size to other members of the *Flaviviridae* such as the flavivirus yellow fever virus (YFV; ~3460 aa), and the pestivirus bovine viral diarrhoea virus (BVDV; ~3960 aa). The structural proteins of both the flavi- and pestiviruses are located at the N termini of their polyproteins, beginning with a small, basic nucleocapsid protein (Collett *et al.*, 1989, Hahn *et al.*, 1988). The N-terminus of the HCV polyprotein is also highly basic (Choo *et al.*, 1991). Furthermore, HCV, flavi- and

pestiviral polyproteins all share similar hydrophobic characteristics. Alignment of the HCV genome with other genomes of the *Flaviviridae* shows regions of sequence homology as well as comparable genomic organisation (Miller and Purcell, 1990). For example, one region, from amino acids 1230-1500, contains many residues identical to a putative NTP-binding helicase encoded by human flaviviruses, animal pestiviruses and plant potyviruses (Choo *et al.*, 1991). Also, upstream from this lies a region sharing residues conserved among the putative trypsin-like serine proteases (Choo *et al.*, 1991), thought, by comparative sequence analysis with trypsin-like molecules, to be encoded by flaviviruses and pestiviruses (Gorbalenya *et al.*, 1989a). The nucleotides of the HCV 5' non-coding region (NCR), show similarity to those of pestiviruses. Several tracts of identical 5'NCR sequence have been identified between HCV and two pestiviruses, BVDV and classical swine fever virus (CSF) (Han *et al.*, 1991). Unlike the flaviviruses which bind ribosomes via 5'-cap structures (Rice, 1996), HCV and the pestiviruses have, within their 5'NCR, an internal ribosome entry site (IRES), which directs cap-independent translation of the open reading frame (ORF) (Poole *et al.*, 1995, Tsukiyama-Kohara *et al.*, 1992). The 5'NCR of HCV has also been shown to share a large, conserved stem-loop structure with the 5'NCR of both BVDV and CSF (Brown *et al.*, 1992), suggesting that HCV is more closely related to the pestiviruses. Despite similarities between HCV and both flaviviruses and pestiviruses, significant differences also exist, leading to the proposal of a third *Flaviviridae* genus, the hepaciviruses (Robertson *et al.*, 1998).

Within a single patient, a population of variant HCV species (termed quasispecies) can be isolated (Gomez *et al.*, 1999). Investigators in Japan were the first to suggest that HCV was a genetically diverse virus (Enomoto *et al.*, 1990), with subsequent studies identifying at least 6 major genetic groups. To classify these groups, all presently known HCV isolates have been placed into one of 6 clades containing all 11 HCV subtypes (Fig. 1.1) (Robertson *et al.*, 1998).

The virus was shown, by filtration, to be between 30 and 60 nm (He *et al.*, 1987) with a low buoyant sucrose density between 1.18-1.21 g/ml (Carrick *et al.*, 1992). It is believed to be enveloped (derived from host cell membranes) as evidenced by its sensitivity to chloroform (Feinstone *et al.*, 1983). Electron microscopy (EM) studies on HCV positive plasma samples using specific monoclonal and polyclonal antibodies allowed visualisation of spherical particles of diameter 55-70 nm (Prince *et al.*, 1996, Kaito *et al.*, 1994), in agreement with the filtration data.

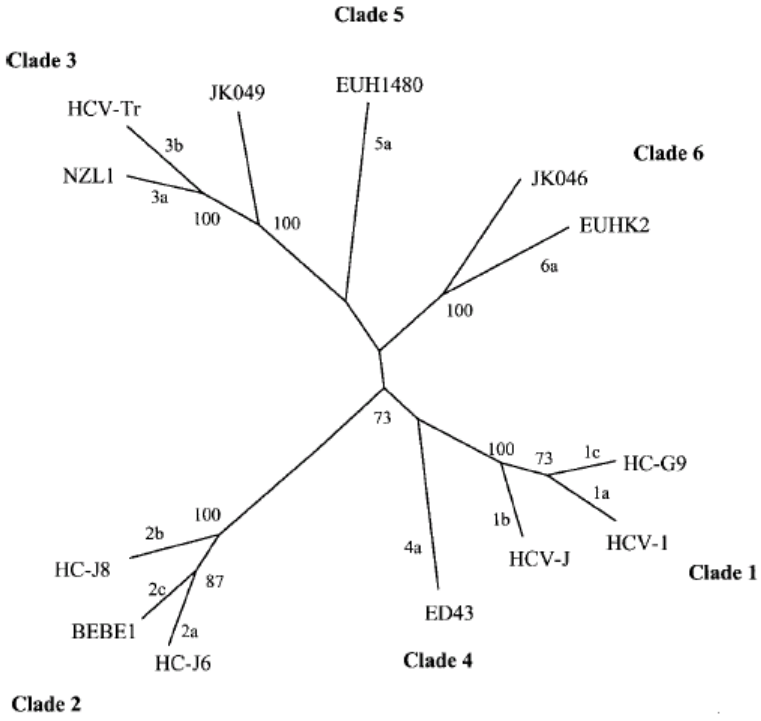


Figure 1.1: Classification of HCV genotypes. Phylogenetic analysis of whole representative genomes, allowing delineation of 6 separate clades for HCV (taken from Robertson *et al.*, 1998).

1.1.3 Epidemiology and Transmission of HCV

Despite being endemic worldwide, there is high geographic variability in the distribution of HCV. Africa and Asia have the highest reported prevalence rates while the lowest are found in industrialised countries such as North America, Australia and those in Northern and Western Europe (World Health Organisation guide, 2002) (Fig. 1.2). The highest reported seroprevalence rate is in Egypt where approximately 22% of the population are HCV seropositive, thought to be due to contaminated glass syringes used in nationwide schistosomiasis treatment campaigns from 1960 to 1987 (Frank *et al.*, 2000).

The most common factors responsible for HCV transmission worldwide are blood (transfusion from unscreened donors), intravenous drug abuse, unsafe therapeutic injections and other healthcare related procedures (Shepard *et al.*, 2005). Within developed countries, the introduction of blood-screening tests for HCV (Kuo *et al.*, 1989, Huber *et al.*, 1996) has effectively eradicated transmission by blood transfusion. Instead, injection drug use has been the predominant mode of transmission in recent times (Lavanchy, 1999). In the developing world however, unsafe therapeutic injections and blood transfusions are still major modes of transmission (Shepard *et al.*, 2005). HCV transmission via occupational, perinatal or sexual exposure is much less common. Perinatal transmission is estimated to occur in 2.7–8.4% of infants born to HCV infected mothers, with higher rates in those born to HIV/HCV co-infected mothers (Thomas *et al.*, 1998, Yeung *et al.*, 2001). Sexual mode of transmission has been reported (Alter *et al.*, 1989), however it is far less efficient than for other sexually transmitted viruses.

1.1.4 Clinical Manifestations and Natural History of HCV Infection

HCV infection is very often clinically silent with most acute infections symptom-free and only a small number showing signs of jaundice (Houghton, 1996). Rapid, fulminant hepatitis associated with acute HCV infection has been reported in Japan (Yoshiba *et al.*, 1994), however this is not common elsewhere. This may reflect differences in genotype distribution as genotypes 2 and 3 are most prevalent in Japan while genotype 1 is prevalent in North America (Yoshiba *et al.*, 1994). Infection becomes chronic in approximately 75% of patients, as confirmed by persistence of HCV RNA in serum (Shimotohno, 2000). Chronically infected patients may present with vague symptoms such as fatigue and joint aching, however it is more common for patients to be unaware until complications of

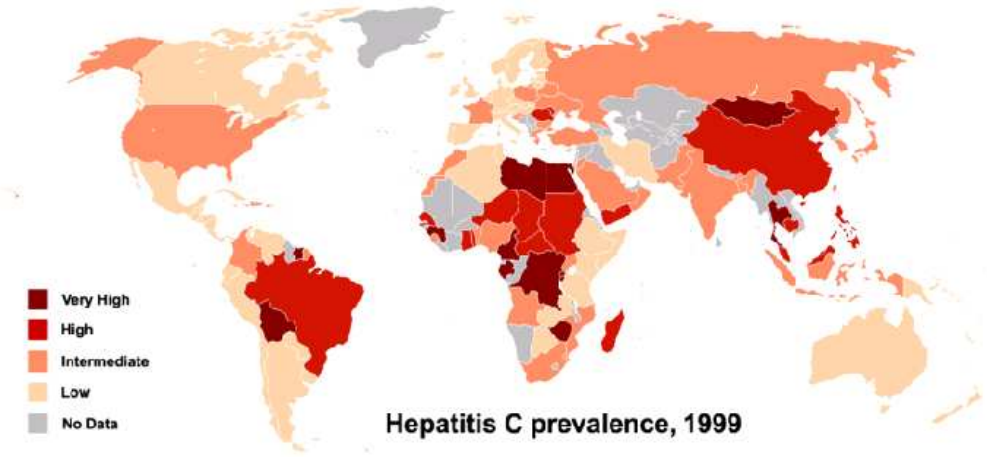


Figure 1.2: Estimated prevalence of HCV infection by WHO region (WHO guide 2002).

chronic liver disease occur, often decades following infection (Di Bisceglie *et al.*, 1991). Almost all chronically infected patients develop histological features of chronic hepatitis such as portal inflammation, interface hepatitis and lobular injury (Di Bisceglie, 1998). Up to 20% of patients develop cirrhosis within the first two decades of HCV infection (Yano *et al.*, 1996). A wide range of factors can influence the development of cirrhosis, but it seems that being male, aged over 50 at time of infection and high alcohol intake increase susceptibility to cirrhosis (Poynard *et al.*, 1997). Complications of chronic liver disease include liver failure and hepatocellular carcinoma (HCC). In patients with established cirrhosis, HCC may develop in up to 4% per year and up to 4% of patients infected with HCV may go on to develop HCC during their life (Di Bisceglie, 1997).

1.1.5 Immune Response

Innate and acquired immune responses both play a role in HCV infection. The critical period, in terms of determining the outcome of infection appears to be the acute phase (Gremion and Cerny, 2005). Natural killer (NK) cells (part of the innate immune response), may play a vital role as chimpanzees with asymptomatic HCV infection can eliminate the virus without any detectable HCV-specific T-cell response (Thomson *et al.*, 2003). Within 4 weeks of infection, the majority of patients show seroconversion, indicated by the presence of antibodies against both structural and non-structural proteins (Rubin *et al.*, 1994). It is still not completely understood whether or not antibodies neutralise HCV infectivity. HCV infection of chimpanzees can be neutralised by *in vitro* treatment with antibodies (Farci *et al.*, 1994), however naturally acquired antibodies failed to prevent reinfection of immune chimpanzees or humans (Farci *et al.*, 1992, Lai *et al.*, 1994). Clearance of infection is associated with a strong $CD4^+$ and $CD8^+$ T-cell response (Thimme *et al.*, 2001, Diepolder *et al.*, 1996). However, the cellular immune response may occur at the expense of a long-lasting inflammatory reaction resulting in liver cirrhosis and HCC (Gremion and Cerny, 2005). During acute infection, the ability to generate detectable $CD4^+$ and $CD8^+$ T-cell responses seems to be important in predicting the outcome of infection, with weak T-cell responses resulting in poorly controlled viraemia resulting in progression to persistence (Thimme *et al.*, 2001). While successful $CD8^+$ responses generally target multiple major histocompatibility complex (MHC) class I-restricted epitopes in structural and non-structural HCV proteins (Cooper *et al.*, 1999, Lechner *et al.*, 2000), infection leading to chronicity usually correlates with low frequencies of CTLs targeting few epitopes (Wedemeyer *et al.*, 2002). In those patients that do proceed to

chronic infection, HCV specific CD8+ T-cells may partially control continuing viral replication as well as contributing to progressive liver disease.

1.1.6 Therapy

Initially, HCV infection was treated with interferon-α. Beneficial effects of interferon-α were noticed before the discovery of HCV itself (Hoofnagle *et al.*, 1986). Interferon-α has potent antiviral activity, not by acting directly on the virus or replication cycle, but instead by inducing interferon-stimulated genes (ISGs) which in turn promote a non-virus-specific antiviral state within the infected cell (Sen, 2001). Interferon-α therapy alone has limited success with only 16-20% of patients producing sustained responses after 12 months of treatment (Di Bisceglie and Hoofnagle, 2002). The addition of the antiviral agent Ribavirin more than doubled the response rate seen with interferon-α alone (McHutchison and Poynard, 1999). The mechanism by which Ribavirin acts is still poorly understood. Ribavirin is a guanosine analogue that is intracellularly phosphorylated to the mono-, di- and triphosphate forms. Misincorporation of the triphosphate form by the viral RNA dependent RNA polymerase results in early chain termination and inhibition of replication (Maag *et al.*, 2001). However, if ribavirin resulted in inhibition of replication, a reduction in viraemia would be expected in the early treatment phase. Ribavirin alone led to only a slight transient reduction in HCV viraemia in early stages compared to interferon-α, suggesting chain termination is not the main mechanism of action (Pawlotsky *et al.*, 2004). Other mechanisms have been suggested including inosine-monophosphate-dehydrogenase inhibition, immunomodulation and modulation of interferon signalling pathways. Ribavirin monophosphate is a competitive inhibitor of inosine-monophosphate-dehydrogenase (IMPDH) leading to a depletion of cellular GTP (required for viral RNA synthesis). However, the addition of excess guanosine only partly reverses the effects of ribavirin, suggesting this may contribute to, but not be completely responsible for, the antiviral activity of ribavirin (Lau *et al.*, 2002). Ribavirin has also been shown to alter the T_H1/ T_H2 balance towards a T_H1 response (Tam *et al.*, 1999), an early response of which has been shown to lead to viral clearance (Rehermann and Nascimbeni, 2005). It is also possible that ribavirin somehow augments or stabilises the action of interferon-α, thus enhancing its effects. Today, pegylated interferon, in which polyethylene glycol (PEG) is covalently attached to recombinant interferon-α, in combination with Ribavirin, is the treatment of choice. PEG interferon-α has a longer half-life, better pharmacokinetics and a better rate of virological response compared to interferon-α (Zeuzem *et al.*, 2000, Glue *et al.*, 2000). The

outcome of treatment can be grouped into one of three categories. Sustained virological response (SVR) is defined as the loss of detectable HCV RNA during, and continuing for 6 months after, the end of therapy. End-of-treatment response and relapse is defined as a transient response followed by relapse, while non-response, which occurs in approximately one-third of chronically infected patients, defines those who never become HCV RNA negative. Although benefits can be seen with pegylated interferon-α/ribavirin treatment, therapy does result in side effects such as fatigue, influenza-like symptoms, haematological abnormalities and neuropsychiatric symptoms (Fried, 2002).

Recently, research has focussed on new targets for HCV treatment. Agents have been discovered which inhibit specific processes in the virus life cycle including inhibitors of HCV enzymes as well as nucleic acid based molecules that interfere with the viral RNA. Small molecule inhibitors have been identified that block essential viral enzymes such as the NS3/4A protease and the NS5B polymerase (De Francesco and Migliaccio, 2005). Nucleic acid based antiviral agents have also been discovered recently including antisense oligonucleotides and small interfering RNAs (siRNAs). A major target of these nucleic acid based antivirals is the HCV IRES due to the wealth of knowledge on this region and its conservation throughout HCV genotypes. SiRNAs have been shown to completely eradicate HCV from more than 98% of HCV replicon-bearing cells (Randall *et al.*, 2003). Potential has also been shown by novel immunomodulatory agents as candidates for treatment of HCV. Synthetic agonists of Toll-like receptors (TLRs) 7 and 9 have shown anti-HCV activity by stimulating the production and release of tumour necrosis factor-α (TNF-α), interleukin (IL)-12 and interferon-α from plasmacytoid dendritic cells (Lee *et al.*, 2003, Horsmans, 2004).

The drawback with many of these proposed therapies is the appearance of mutations within the viral genome conferring resistance to the antiviral agent. Resistance is easily acquired to NS5B nucleoside analogues (Olsen *et al.*, 2004, Migliaccio *et al.*, 2003) and a single mutation is enough to confer resistance to BILN 2061, an NS3/4A protease inhibitor (Lin *et al.*, 2004, Lu *et al.*, 2004). Development of BILN 2061 has now been halted due to the occurrence of cardiac toxicity in laboratory animals (Reiser *et al.*, 2005).

1.2 HCV Genome Structure

1.2.1 Genome Organisation

HCV has a single stranded, positive sense genome of approximately 9.6kb. The genome contains one long open reading frame (ORF) encoding a polyprotein of approximately 3011 amino acids, flanked by 5' and 3' non-coding regions (NCRs) (Fig 1.3) (Choo *et al.*, 1991). The highly conserved 5'NCR (Bukh *et al.*, 1992) is 341 nucleotides in length (Han *et al.*, 1991) and contains extensive secondary structure (Tsukiyama-Kohara *et al.*, 1992). Similarities in structure between the HCV 5'NCR and that of picornaviruses led to the conclusion that HCV had an internal ribosome entry site (IRES), required for translation initiation (Tsukiyama-Kohara *et al.*, 1992). The ORF encodes a single polyprotein which is processed co- and post-translationally by host and viral proteases to produce at least 10 viral proteins (Lindenbach and Rice, 2001). The structural proteins (core, E1, E2 and p7) are located within the amino-terminal third of the polyprotein, while the non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) are found within the carboxy-terminal two-thirds (Kato *et al.*, 1990, Lindenbach and Rice, 2001). Processing of the polyprotein involves both host and viral proteases, the structural proteins being processed by host proteases and the non-structural proteins by viral proteases (Shimotohno *et al.*, 1995). The 3' NCR is a tripartite structure consisting of a conventional 3' end, a poly(U) tract and a recently discovered 3' X-tail (Kolykhalov *et al.*, 1996). This X-tail is highly conserved and forms an elaborate stem-loop structure, suggesting a possible role in replication (Tanaka *et al.*, 1995a, Kolykhalov *et al.*, 1996), a theory proven by the requirement of the 3' X-tail for replication of the HCV replicon (Friebe and Bartenschlager, 2002).

1.2.1.1 5' UTR

Depending on isolate, the HCV 5'UTR consists of approximately 341 nt (Han *et al.*, 1991). Sequence analysis of this region shows sections up to 14 nt long that have perfect identity to that of the pestiviruses BVDV and CSF (Han *et al.*, 1991). Sequence analysis also shows regions of high conservation between HCV isolates (Han *et al.*, 1991, Bukh *et al.*, 1992) as well as regions of heterogeneity (Bukh *et al.*, 1992, Smith *et al.*, 1995). The secondary structure of the 5'UTR has been predicted, indicating the presence of 4 highly structured domains (Fig. 1.4) (Honda *et al.*, 1999b). While members of the flavivirus genus tend to

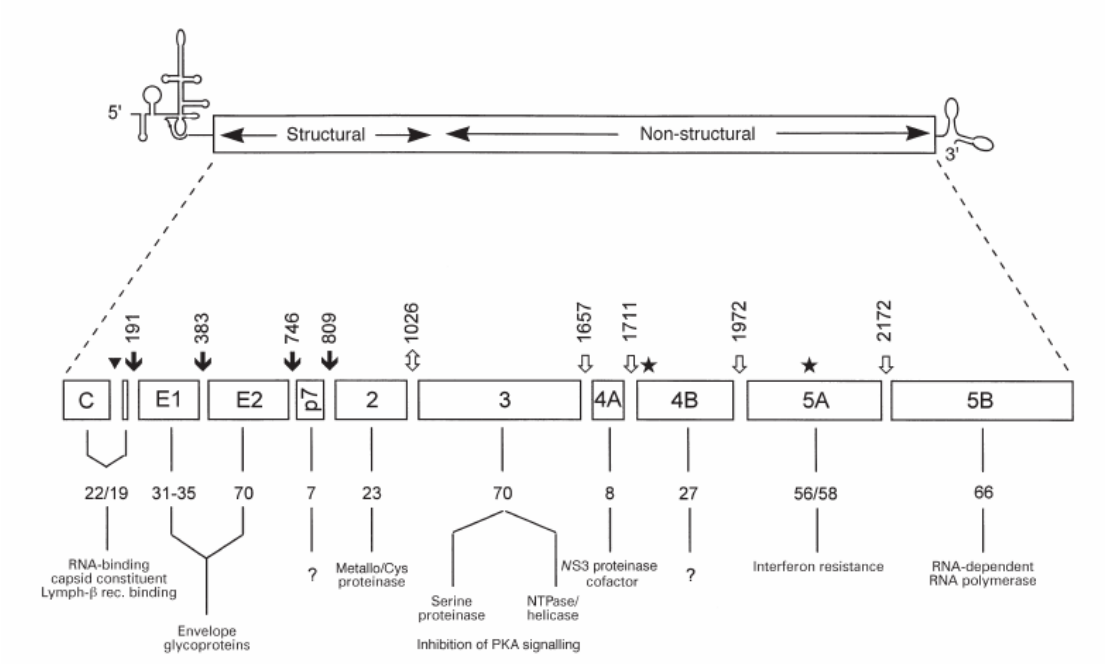


Figure 1.3: General features of the HCV genome, polyprotein processing and properties of individual cleavage products. 5' and 3' NCRs are separated by a single (ORF) which encodes all HCV proteins. Structural proteins core, E1, E2, and p7 are grouped at the N-terminus, followed by the non-structural proteins (NS2-5B). Cleavage sites for host cell signal peptide peptidase (▼) and signalase (⬇), the NS2-3 proteinase (⇳) and the NS3-4A (⇩) are highlighted. Approximate molecular weights (in kDa) and properties of each protein are indicated (taken from Bartenschlager, 1999).

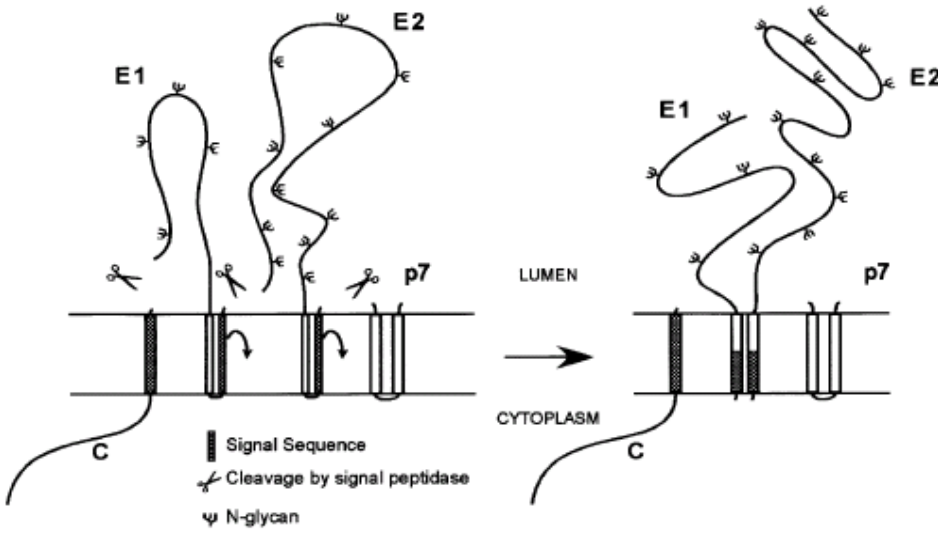


Figure 1.4: Topology of the TMDs of the HCV envelope proteins before and after signal sequence cleavage (taken from Op de Beeck *et al.*, 2001).

have smaller 5'UTRs and undergo 5' cap-dependent translation (similar to that of eukaryotic mRNAs), HCV, similar to the pestiviruses (Poole *et al.*, 1995), undergoes internal initiation of translation via an internal ribosome entry site (IRES) in a cap-independent manner (Wang *et al.*, 1993, Tsukiyama-Kohara *et al.*, 1992). The HCV IRES spans domains II, III and IV of the 5'UTR, together with the first 24-40 nucleotides of core-coding sequence (Honda *et al.*, 1996, Reynolds *et al.*, 1996). Translation efficiency from the IRES has been shown to vary between different genotypes (Collier *et al.*, 1998). The IRES therefore, is proposed to provide a structure capable of directing ribosomes to the initiation codon at position 342 (Clarke, 1997). The interaction between IRES and ribosome is likely to require several protein co-factors. Interestingly, a number of cellular proteins have been shown to bind the 5'UTR. Polypyrimidine tract-binding protein (PTB) has been shown to bind the 5'UTR at at least 3 sites. Immunodepletion of PTB from translation lysates completely inhibits translation from the HCV 5'UTR (Ali and Siddiqui, 1995). The same group have also shown an interaction between the 5'UTR and the RNA binding protein La (Ali and Siddiqui, 1997). Addition of La to an *in vitro* translation reaction enhanced HCV translation. The open reading frame initiation codon is required for binding La, suggesting that La may be involved in initiation of translation. Finally, poly(C)-binding proteins (PCBs) 1 and 2 have been shown to bind the 5'UTR (Spangberg and Schwartz, 1999). Depletion of PCBs from HeLa S10 extract resulted in abrogation of poliovirus IRES dependent translation, suggesting PCBs may also be involved in translation initiation at the HCV IRES.

1.2.1.2 Core

The general properties, putative pathogenic roles and interactions of core protein are discussed in detail in section 1.5.

1.2.1.3 E1 and E2

E1 and E2 are the envelope glycoproteins. These proteins are thought to be involved in host-cell entry by binding to cell surface receptors and fusing with host-cell membranes (Penin *et al.*, 2004). E1 and E2 are both type I transmembrane (TM) glycoproteins, having short C-terminal transmembrane domains (TMDs) and N-terminal ectodomains, and form

non-covalent heterodimers, the proposed virion components (Deleersnyder *et al.*, 1997). Both glycoproteins are modified by N-linked glycosylation with E1 containing up to 6 and E2 potentially 11 glycosylation sites (Op De Beeck *et al.*, 2001). The TMDs of E1 and E2, composed of 2 short stretches of hydrophobic residues separated by a short polar segment, have many important functions including being essential for heterodimerisation (Fig. 1.5) (Op De Beeck *et al.*, 2000) as well as being required for glycoprotein retention in the ER (due to at least one charged residue within the polar segment) where virion assembly likely takes place (Cocquerel *et al.*, 1998, Cocquerel *et al.*, 1999, Cocquerel *et al.*, 2000). Early interaction between the TM domains (possibly before insertion into the endoplasmic reticulum) has been shown to improve the stability of E2 insertion into the membrane (Cocquerel *et al.*, 1998). Calnexin, an ER chaperone protein involved in disulphide bond formation (Bergeron *et al.*, 1994), rapidly associates with both E1 and E2 whereby folding and heterodimer formation occur, with assembled glycoprotein complexes being retained in a pre-*trans*-golgi compartment (Dubuisson and Rice, 1996).

The development of retroviral particles bearing unmodified HCV E1E2 (called HCV pseudoparticles or HCVpp) (Bartosch *et al.*, 2003a) has enhanced the understanding of both HCV entry and particle neutralisation. HCV pseudoparticles have a preferential tropism for hepatic cells (Bartosch *et al.*, 2003a) and are specifically neutralised by anti-E2 monoclonal antibodies as well as sera from HCV infected patients, thus confirming the correct assembly of the glycoproteins in this system (Bartosch *et al.*, 2003a, Kato *et al.*, 1990, Weiner *et al.*, 1991). A 27 amino acid region at the N-terminus of E2 (amino acids 384-410), termed hypervariable region-1 (HVR-1) is a target for anti-HCV neutralising antibodies. This region is highly tolerant of amino acid substitutions and is subject to strong positive selection pressure (Kato *et al.*, 1990, Weiner *et al.*, 1991). The biological role of HVR-1 is unknown, however it has been suggested to act as a decoy to the immune system during acute infection (Ray *et al.*, 2000). Antibodies against HVR-1 are neutralising *in vitro* and are capable of protecting chimpanzees after *in vitro* neutralisation (Farci *et al.*, 1996). However, in contradiction to these findings, virus lacking the HVR-1 (ΔHVR-1) was capable of infecting chimpanzees and causing hepatitis, although this virus initially replicated at low levels until the appearance of compensatory mutations, indicating that ΔHVR-1 virus is attenuated but HVR-1 is not essential for HCV viability (Forns *et al.*, 2000). As both groups used HCV H77 strain in their studies, it is difficult to understand the differences between their results. One possibility however, is that their experimental methods may have had different functional effects on E2, with neutralisation of the HVR1 region having a more detrimental effect on E2 function than the deletion of HVR1. It is also possible that there is an alternative, less efficient, method of HCV entry in which

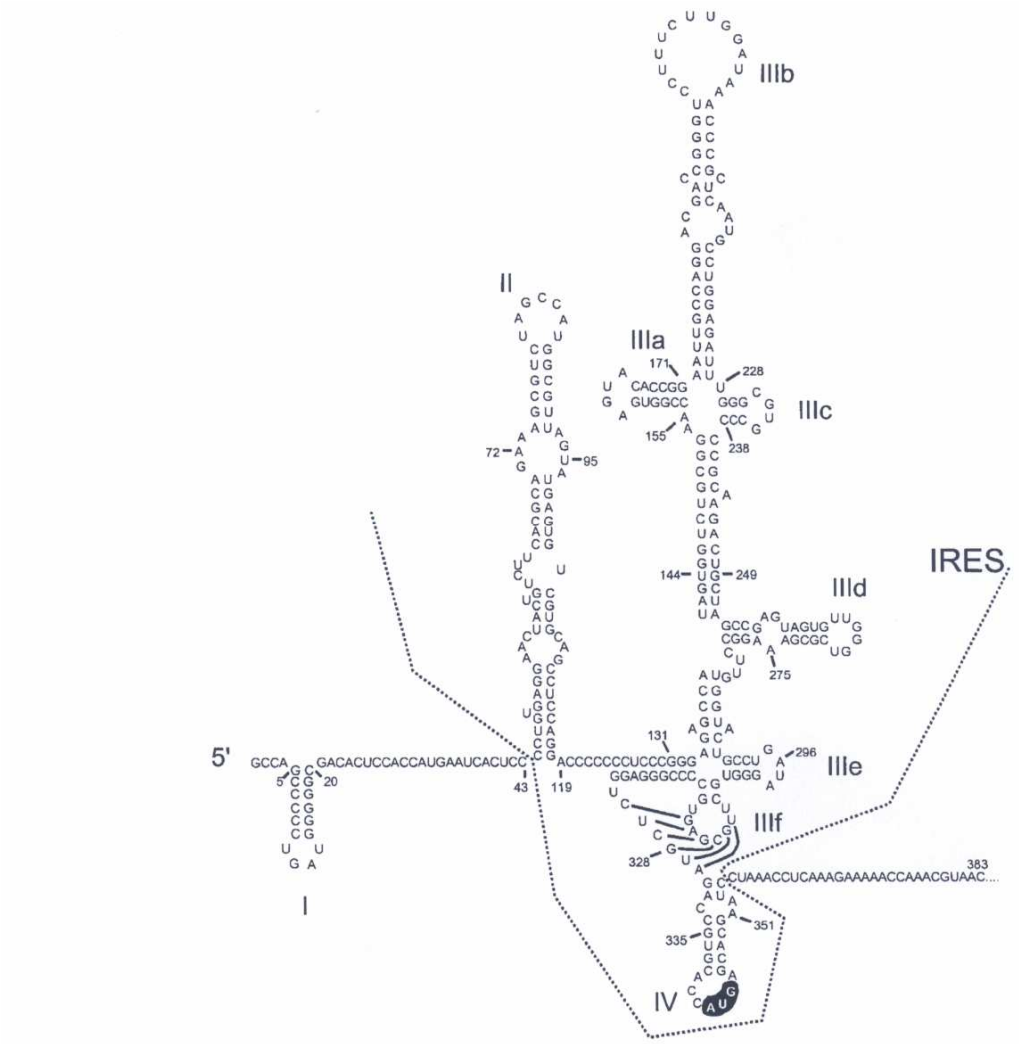


Figure 1.5: Proposed secondary and tertiary structure of the HCV 5'NCR, including a small portion of the core coding sequence, from a genotype 1b infectious clone (HCV-N). Major structural domains are labelled with Roman numerals and the initiator codon within stem-loop IV is highlighted. Circled nucleotides indicate differences with the genotype 1a HCV-H strain (taken from Honda *et al*,. 1999).

HVR1 is not required, thus explaining the attenuated phenotype seen by Forns *et al*. Although the amino acid sequence of HVR1 is highly variable, the conformation is well conserved and the sequence made up of basic residues, possibly required for interaction with negatively charged molecules such as lipids, proteins and glycosaminoglycans (Penin *et al.*, 2001). The HCVpp model has been used to study the neutralising potential of the mouse monoclonal antibody AP33, which binds E2 downstream of HVR1 between residues 412 and 423. Retroviral pseudoparticles incorporating a panel of full-length E1E2 clones from genotypes 1-6 were assessed for their potential to be neutralised by AP33. The monoclonal antibody was able to potently neutralise HCVpp entry no matter what genotype (Owsianka *et al.*, 2005), a phenomenon that has also been confirmed using the recently described JFH-1 HCVcc system (Tarr *et al.*, 2006), suggesting the linear epitope recognised by AP33 may be a suitable HCV vaccine target. Four discontinuous residues within this epitope have been reported to be required for neutralisation (Tarr *et al.*, 2006), however, it still remains to be determined whether or not this antibody will have such potent neutralising effects on patient derived virus and if it can also neutralise in animal model systems.

As well as HVR1, two other hypervariable regions have been identified within E2, firstly between positions 474 and 482 (HVR2) (Hijikata *et al.*, 1991a), and secondly between positions 431 and 466 (HVR3), part of which has been proposed to be exposed at the surface of E2, suggesting it may play a role in immune recognition and/or host-cell receptor binding (Troesch *et al.*, 2006). The sequence variations concentrated within these regions surely point to some functional importance in receptor binding and cell entry, however the exact functional relevance has yet to be elucidated.

1.2.1.4 p7

The identification of two distinct forms of N-deglycosylated E2 led to the discovery of a small hydrophobic protein between E2 and NS2 termed p7 (Lin *et al.*, 1994a). Cleavage of p7 from the polyprotein is mediated by a host signal peptidase, which, due to the presence of an E2-p7-NS2 precursor and stable E2-p7 form, has been shown to be processed post-translationally and that cleavage is sub-optimal due to a weak signal peptide cleavage site (Isherwood and Patel, 2005, Lin *et al.*, 1994a). Analysis of the amino acid composition of p7 shows that it is made up of mostly uncharged polar or hydrophobic residues interspersed by a few conserved charged residues, thus allowing a double membrane-

spanning conformation, separated by a few charged residues on the cytoplasmic side and with the N- and C-termini facing the luminal side (Lin *et al.*, 1994a).

The precise role of p7 in the life cycle of HCV is still unknown. It has been shown to be dispensable for viral RNA replication, as replicons lacking p7 replicate efficiently (Lohmann *et al.*, 1999), while viral RNA with the p7 region deleted was non-infectious in chimpanzees (Sakai *et al.*, 2003), suggesting p7 is not required for efficient HCV genome replication but is required for the production of infectious virus. A recent report has shown that p7 may be involved in assembly and/or release of infectious virus particles as a single mutation in p7 increased the yield of infectious virus from an H77S/JFH-1 chimeric clone (Yi *et al.*, 2007). Pestiviruses, such as BVDV, also have a p7 protein showing similar hydrophobicity and incomplete cleavage pattern (Harada *et al.*, 2000). A large in-frame p7 deletion within the BVDV genome does not affect replication, however, similar to the finding by Sakai *et al* (2003), no infectious virus is produced (Harada *et al.*, 2000). As p7 is not thought to be incorporated into the closely related pestivirus particle (Elbers *et al.*, 1996), p7 may be involved in protecting the infectivity of the newly assembled particles before and/or during release (Yi *et al.*, 2007). Studies using recombinant expression plasmids suggest p7 localises to the ER (due to an ER retention signal) (Carrere-Kremer *et al.*, 2002) and mitochondrial membranes (Griffin *et al.*, 2005), while a fraction is transported to the cell surface, suggesting it plays multiple roles in the secretory pathway (Carrere-Kremer *et al.*, 2002). A recent report however suggests that HA-tagged p7, expressed from full-length infectious JFH-1 RNA, localises solely to the ER (Haqshenas *et al.*, 2007). The fact that p7 has been reported to localise to both the ER and mitochondria suggests multiple populations of p7 exist and that p7 may have more than one role in the life cycle of the virus. One such role of p7 is that of an ion channel, possibly regulating the flow of calcium ions from the ER to the cytoplasm (Griffin *et al.*, 2003, Griffin *et al.*, 2004). Recently, the same group has revealed the heptameric structure of this channel, providing further evidence for its function as an ion channel in the virus life cycle (Clarke *et al.*, 2006). As more is known about the properties of p7 it is becoming clear that this is an important factor in the virus life cycle, presumably at a post-replication stage. This makes p7 an interesting target for future antiviral studies as it may be possible to block the proposed ion channel and thus inhibit the production of infectious particles.

1.2.1.5 NS2

The NS2 region (in conjunction with the N-terminal end of NS3) contains a zinc-dependent metalloproteinase, responsible for cleavage at the NS2/3 junction (Grakoui *et al.*, 1993c, Hijikata *et al.*, 1993). This protease overlaps with the serine protease located at the N-terminal end of NS3, however the function of the NS2 protease does not rely on its NS3 counterpart (Grakoui *et al.*, 1993c, Hijikata *et al.*, 1993). Residues His 952 and Cys 993 have been shown to be essential for NS2 protease function but have no effect on downstream cleavage events (Grakoui *et al.*, 1993c), presumably because downstream cleavage is carried out by the NS3 protease. Upon processing, NS2 is inserted into the ER membrane where it resides as a transmembrane protein (Santolini *et al.*, 1995), with possibly 4 TM domains (Yamaga and Ou, 2002). NS2 is not essential for replication as shown by replication of sub-genomic replicons lacking the NS2 gene (Lohmann *et al.*, 1999), however an interaction has been described between processed NS2 and NS3 (Dimitrova *et al.*, 2003, Kiiver *et al.*, 2006) and NS2 plays an essential role in the phosphorylation of NS5A (Liu *et al.*, 1999), suggesting NS2 has other functions as well as NS2/3 cleavage which are not directly involved in replication. The crystal structure of the catalytic domain of NS2-3 has been solved (Lorenz *et al.*, 2006), showing that the monomeric form has 2 subdomains connected by an extended linker, while the dimeric form resembles a “butterfly” in which the C-terminal subdomain of one molecule interacts with the N-terminal subdomain of the other and *vice versa*. The protease activity of NS2 is required for its cleavage from the polyprotein, however as it is dispensible for replication, its true function remains unknown but is presumably required for some accessory role in the virus life cycle as shown by its role in the phosphorylation of NS5A and its association with NS3.

1.2.1.6 NS3

NS3 is a multifunctional protein of 68 kDa, containing a serine protease domain within its N-terminal third (Bartenschlager *et al.*, 1993, Grakoui *et al.*, 1993b, Hijikata *et al.*, 1993, Tomei *et al.*, 1993) and an NTPase/helicase domain within its C-terminal two-thirds (Kim *et al.*, 1995, Suzich *et al.*, 1993). The two functional domains are not cleaved from each other (Bartenschlager *et al.*, 1993) yet act independently of each other (Kim *et al.*, 1995). The serine protease domain is required for 4 cleavage events, acting in *cis* to release itself from the HCV polyprotein and in *trans* to produce the N-termini of NS4B, NS5A and

NS5B (Grakoui *et al.*, 1993a, Bartenschlager *et al.*, 1993, Tomei *et al.*, 1993, Lin *et al.*, 1994b). In addition to the N-terminal third of NS3, a C-terminal domain of NS4A has been described as an NS3 co-factor, required for efficient cleavage of the downstream polyprotein, especially at the NS4B/5A cleavage site (Failla *et al.*, 1994, Bartenschlager *et al.*, 1994), resembling that seen in flaviviruses and pestiviruses. Cleavage of dengue virus (type 4) polyprotein at NS2A/2B and NS2B/NS3 requires NS2B (Falgout *et al.*, 1991), while cleavage at NS4B/5 of yellow fever virus by the NS3 protease requires association with NS2B (Chambers *et al.*, 1991). The NS3/4A protease has also been shown to control host cell antiviral defences by disrupting pathways that lead to activation of interferon regulatory factor 3 (IRF3) and subsequent induction of type I interferon. As an antiviral defence mechanism, cells possess molecules which are specifically activated by dsRNA. One such protein, the DexD/H-box RNA helicase retinoic acid inducible gene I (RIG-I), contains a C-terminal helicase domain that binds a pathogen-associated molecular pattern (PAMP) embedded within the 5' or 3' NCR of HCV. In doing so, the conformation of RIG-I is altered, thus allowing recruitment of downstream interacting partners (Sumpter *et al.*, 2005). One such interacting partner of RIG-I is mitochondrial antiviral signalling protein (MAVS; also known as IPS-1, VISA, and CARDIF). Interaction between RIG-I and MAVS leads to activation of IRF-3 via phosphorylation by the kinases TBK and IKKε, which in turn leads to nuclear translocation of IRF-3 and subsequent transcriptional activation of promoters of IFN-β, IFN-stimulated genes ISG56 and ISG15, and RANTES genes. To overcome this antiviral signalling cascade, HCV possesses a tactic for blocking this pathway. In studying the effects of HCV replication and polyprotein expression on Sendai virus-induced IRF-3 translocation, it was shown that HCV NS3/4A specifically blocks expression of IRF-3-activated genes (Foy *et al.*, 2003). Specifically, NS3/4A cleaves MAVS at cys-508, resulting in dislocation of the N-terminus of MAVS from the mitochondria and subsequent inhibition of the signalling pathway (Johnson *et al.*, 2007, Li *et al.*, 2005), a catalytic event requiring the protease domain of NS3 and a minimal NS4A co-factor but not NS3 helicase activity (Foy *et al.*, 2003, Foy *et al.*, 2005, Johnson *et al.*, 2007). HCV has therefore developed a method for counteracting the antiviral immune response imposed by the host, possibly resulting in progression to chronic infection. Similarly, GBV-B, the phylogenetically closest relation of HCV, has recently been shown to disrupt the RIG-I signalling pathway in the same manner (Chen *et al.*, 2007). Inhibitors of the HCV NS3 protease have been shown to restore the normal mitochondrial distribution of MAVS and in doing so reduce viral protein abundance (Johnson *et al.*, 2007), thus indicating a novel role for HCV protease inhibitors in the battle against HCV infection.

NS4A is thought to localise NS3 to the ER membrane in an NS3/4A complex, increasing the stability of NS3 (Wolk *et al.*, 2000). NS5B has also been shown to associate with this NS3/4A complex, suggesting this complex could function as part of the HCV replication machinery (Ishido *et al.*, 1998), while the recent identification of an interaction between NS3 and NS2 (section 1.2.1.5) suggests NS2 may function as an anchor to retain NS3 at the ER membrane until cleavage of NS4A (Kiiver *et al.*, 2006).

The C-terminal two-thirds of NS3 possess NTPase and helicase activity. Polynucleotide-stimulated NTPase activity, capable of hydrolysing all NTPs and dNTPs, has been shown (Suzich *et al.*, 1993), while RNA helicase activity, requiring ATP and a divalent ion, has also been identified (Kim *et al.*, 1995). Emphasising its role as a functional helicase, the NS3 C-terminal domain is capable of unwinding RNA-RNA, RNA-DNA and DNA-DNA substrates and does so in a 3'-5' direction (Tai *et al.*, 1996). According to amino acid homology, the helicase domain of NS3 resembles that of the DExH-box NTPase/helicases, which includes elongation initiation factor (eIF) 4A , thus classifying NS3 as a member of this family (Gorbalenya *et al.*, 1989b). Regarding its function within the viral life cycle, the helicase domain of NS3 has been shown to interact with the 3' terminal sequence of both positive and negative HCV RNA (Banerjee and Dasgupta, 2001), similar to that shown for poliovirus NTPase/helicase 2C (Banerjee *et al.*, 1997), while an active NS3 helicase domain has recently been reported to be required for replication of an HCV subgenomic replicon (Lam and Frick, 2006), thus suggesting a role in unwinding viral RNA during replication. However, the precise role of the NS3 helicase domain during replication is still not fully understood. NS3 has been shown to bind many cellular factors including protein kinase A, protein kinase C, tumour suppressor p53 and histones H2B & H4 (reviewed in (Tellinghuisen and Rice, 2002) and has effects on various processes such as cell metabolism, differentiation and tumour promotion (Borowski *et al.*, 1996, Sakamuro *et al.*, 1995). The multifunctionality of NS3, together with its interactions with numerous cellular factors, indicates that this protein plays a pivotal role in the life cycle of HCV, not just in terms of replication of the viral RNA but also by interacting with host-cell proteins which may result in some of the pathogenic effects associated with HCV infection. Due to its enzymatic activities, NS3 is a possible antiviral target. The design of such NS3 protease/helicase inhibitors has been helped by the solving of the structure of NS3 by X-ray crystallography (Yao *et al.*, 1999).

1.2.1.7 NS4A

Non-structural region NS4 of HCV is a hydrophobic region that is cleaved by the NS3 protease to produce NS4A and NS4B (Grakoui *et al.*, 1993d). As described in section 1.2.2.5, NS4A is a co-factor of the NS3 protease, the central hydrophobic domain of which (amino acids 21-32) is essential for NS3 protease co-factor function (Lin *et al.*, 1995, Tomei *et al.*, 1996). NS4A also helps to stabilise NS3 by anchoring it to the ER membrane while also associating with NS4B, NS5A and NS5B to form a complex structure, possibly involved in viral replication (Tanji *et al.*, 1995a). As well as this, NS4A (and NS4B) have been shown to inhibit protein synthesis at the translation stage, with NS4A function mediated by the first 40 amino acids (Kato *et al.*, 2002, Florese *et al.*, 2002). This effect was negated when NS4A was co-expressed with NS3, suggesting conformational differences before and after cleavage may be important in this process (Florese *et al.*, 2002). As with bovine viral diarrhea virus protein NS5A (Johnson *et al.*, 2001), HCV NS4A has been shown to interact with eukaryotic elongation factor (eIF) 1A (Liu *et al.*, 2005, Kou *et al.*, 2006), further suggesting an involvement in protein synthesis inhibition. It is therefore possible that NS4A is involved in the switch from translation of the HCV polyprotein to replication of viral RNA once sufficient protein production/processing has occured.

In conjunction with NS4B, NS4A has been suggested to play a role in abrogating host defences via inhibition of protein traffiking. The precursor protein NS4A/4B has been shown to slow the rate of ER-Golgi traffiking in a similar manner to that of Poliovirus 3A protein (Doedens and Kirkegaard, 1995), however this is only seen when NS4A/4B is expressed in isolation and not when NS4A or NS4B are expressed on their own (Konan *et al.*, 2003). If ER-Golgi traffiking is slowed by NS4A/B, the virus would require an alternative route of exit. Rotaviruses exit the cell via a non-classical secretory route that bypasses the Golgi apparatus (Jourdan *et al.*, 1997). It is possible that HCV exits via a similar non-Golgi route, thus inhibition of the secretory pathway downstream of the ER would not affect virus maturation but would affect host defences, such as traffiking of MHC antigen presentation molecules to the cell surface (Konan *et al.*, 2003), therefore providing a possible role for NS4A/B in inhibition of host-cell defenses. Finally, phosphorylation of NS5A by cellular kinases has also been shown to be enhanced in the presence of NS4A (Kaneko *et al.*, 1994, Reed *et al.*, 1997, Tanji *et al.*, 1995b).

1.2.1.8 NS4B

The relatively hydrophobic NS4B protein is the least well-understood HCV protein. The 27 kDa protein is exclusively present as a fine membraneous network in the cytoplasm (Park *et al.*, 2000), localising to the ER where it colocalises with other non-structural proteins (Hugle *et al.*, 2001). Localisation to the ER is independent of other HCV proteins, indicating the presence of endogenous signals for ER targeting and membrane anchorage (Hugle *et al.*, 2001). NS4B is thought to have at least 4 TM domains, with a fifth occurring upon predicted translocation of the N-terminus to the lumenal side of the ER (Lundin *et al.*, 2006, Lundin *et al.*, 2003). This translocation is impaired by NS5A, suggesting NS4B has different functions during the viral life cycle (Lundin *et al.*, 2006). Alterations to membrane structure (termed the membranous web) have been described in cells expressing NS4B and also in cells expressing full length HCV polyprotein (Egger *et al.*, 2002). The membranous web may comprise the HCV replication complex and has been shown to harbour all HCV structural and non-structural proteins in UHCVcon-57.3 cells (Egger *et al.*, 2002). Within the ER membrane, NS4B is mobile but becomes less mobile at small foci within this membranous web (termed membrane associated foci, (MAFs)) (Gretton *et al.*, 2005). If these MAFs are the site of HCV replication complexes then it could be suggested that the formation of oligomeric complexes incorporating NS4B increase the bulk of TM domains and thus retard movement (Gretton *et al.*, 2005). Palmitoylation of NS4B, the attachment of fatty acids to cysteine residues of membrane proteins, has recently been described. Cysteine residues 257 and 261 have been shown to be palmitoylated, with mutation of these residues affecting interaction between NS4B and NS5A (Yu *et al.*, 2006). Abolishing palmitoylation at cys261 was also shown to completely inhibit replication of a sub-genomic replicon (Yu *et al.*, 2006). A nucleotide-binding motif has also been identified in NS4B, genetic disruption of which impairs GTP binding/hydrolysis and inhibits RNA replication in a replicon system (Einav *et al.*, 2004). The importance of this nucleotide-binding motif for replication would suggest that NS4B has other roles in replication as well as simply providing structural scaffolding for the replication complex.

Expression profiles of HeLa genes in the presence of NS4B showed that 34 genes were up-regulated and 56 genes down-regulated, including genes involved in oncogenesis, tumour suppression, cell receptors, adhesion, transcription and translation, and cellular stress (Zheng *et al.*, 2005). Similarly, Park *et al.* showed that NS4B, co-expressed with HA-*ras*, led to loss of contact inhibition, morphological alterations and anchorage-independent

colony formation in NIH3T3 cells (Park *et al.*, 2000), indicating NS4B may also be involved in HCV pathogenesis.

1.2.1.9 NS5A

NS5A is a predominantly hydrophilic protein containing no transmembrane helices and is associated with the ER membrane via an amphipathic alpha helix within its N-terminal 30 amino acids (Brass *et al.*, 2002). There are two phosphorylated forms of NS5A, a basally phosphorylated 56 kDa form and a hyperphosphorylated 58 kDa form (Tanji *et al.*, 1995b, Kaneko *et al.*, 1994). As described previously (section 1.2.1.7), NS4A is required for hyperphosphorylation of the 56 kDa NS5A protein (Kaneko *et al.*, 1994) and the N-terminal portion of NS5A has been shown to be required for both NS4A binding and resultant NS5A phosphorylation (Asabe *et al.*, 1997). The kinases responsible for phosphorylation of NS5A are thought to be serine/threonine kinases from the CMGC (CDK, MAPK, GSK and Cdc-like) kinase family although specific kinases have not yet been identified (Reed *et al.*, 1997). NS5A/NS5 serine/threonine phosphorylation has been shown for flavivirus (yellow fever virus), pestivirus (bovine viral diarrhea virus) and hepacicvirus (HCV) genera of the *Flaviviridae*, suggesting NS5A/NS5 may play an important role in the *Flaviviridae* life cycle (Reed *et al.*, 1998). Mutation resulting in the disruption of the amphipathic alpha helix of NS5A completely inhibited replicon replication, suggesting that NS5A membrane association is essential for HCV RNA replication (Elazar *et al.*, 2003). Human vesicle-associated membrane protein-associated protein-A (hVAP-A) is thought to function as a docking site for assembly or localisation of the HCV replication complex (Tu *et al.*, 1999). The same group showed that hVAP-A binds NS5A, leading to the postulation (Evans *et al.*, 2004) that the hyperphosphorylated form of NS5A represents a “closed” conformation within the replication complex, unable to bind hVAP-A and thus inhibiting replication whereas the hypophosphorylated form of NS5A represents an “open” conformation, allowing interaction with hVAP-A and viral replication, thus regulating the HCV life cycle.

A 40 amino acid region of NS5A has been reported to contain residues that confers resistance to interferon-α treatment (Enomoto *et al.*, 1995, Enomoto *et al.*, 1996). This region has been termed the interferon sensitivity determining region (ISDR) (Enomoto *et al.*, 1995). Interferon induces cellular antiviral responses, mediated in part by the dsRNA-activated protein kinase (PKR). PKR is transcriptionally activated from low levels of

expression upon cellular exposure to interferon. Upon activation by dsRNA, PKR phosphorylates the α-subunit of eukaryotic translation initiation factor 2 (eIF-2α), resulting in cessation of protein synthesis and therefore viral replication (Merrick and Hershey, 1996). NS5A, via its ISDR, has been shown to interact and inhibit interferon-induced PKR (Gale *et al.*, 1997, Gale *et al.*, 1998), although in a separate study, no interaction between NS5A and PKR could be detected (Podevin *et al.*, 2001). The discrepancy between these two reports may be accounted for by the different cell lines used by the two groups. Gale *et al.* used the fibroblast Cos-1 cell line while Podivin *et al.* used the more relevant hepatoma Huh7 cell line. Podevin *et al.* did however see an impact of NS5A on the regulation of interferon-α antiviral efficacy, suggesting a PKR-independent mechanism (Podevin *et al.*, 2001). One such mechanism suggested involves the cytokine interleukin (IL)-8, the expression of which has been reported to be induced by NS5A (Girard *et al.*, 2002, Polyak *et al.*, 2001). IL-8 can attenuate the antiviral properties of interferon-α (Khabar *et al.*, 1997), thus suggesting a theory of PKR-independent inhibition of interferon-α. Recent reports have however suggested that NS5A does not contain an ISDR (Aus dem Siepen *et al.*, 2005, Brillet *et al.*, 2007). NS5A genes, isolated from interferon-α responders and non-responders (infected with the same HCV strain), were inserted into the backbone of the Con1 replicon. No significant differences in sensitivity of HCV RNA replication to interferon-α treatment were seen (Aus dem Siepen *et al.*, 2005). Similarly, no significant changes were seen in NS5A amino acid sequence between interferon-α responders and non-responders 24 hrs post-administration, suggesting interferon-α does not select interferon-resistant variants with specific NS5A sequences (Brillet *et al.*, 2007). The argument over whether or not NS5A has an ISDR is difficult to assess, possibly due to genetic differences within human hosts, thus resulting in some patients being susceptible to interferon resistant variants while others are not.

NS5A has also been suggested to modulate mitogenic signalling via mitogen-activated protein kinase (MAPK) pathways, inhibit apoptosis and induce HCC via perturbation of phosphatidylinositol 3-kinase-mediated signalling pathways (Macdonald and Harris, 2004), indicating it too may be involved in HCV pathogenesis.

1.2.1.10 NS5B

Replication of HCV RNA involves synthesis of a complementary negative-strand RNA molecule from the genomic template strand, followed by synthesis of new genomic

positive-strand RNA copies from the negative-strand intermediate RNA template. The protein responsible for this in HCV is NS5B. Containing motifs common amongst all RNA-dependent RNA polymerases, NS5B was confirmed as the HCV RdRp by presence of enzymatic activity capable of copying HCV RNA (Behrens *et al.*, 1996, Lohmann *et al.*, 1997). Since then the crystal structure has been resolved and classical “fingers”, “palm” and “thumb” sub-domains identified (Ago *et al.*, 1999, Bressanelli *et al.*, 1999, Lesburg *et al.*, 1999). NS5B has been shown to be an integral membrane protein with a cytosolic orientation, associating with membranes via a highly hydrophobic 21 aa C-terminus, thus being a member of the tail-anchored protein family (Schmidt-Mende *et al.*, 2001). In functional assays, bacterially expressed, purified NS5B can copy full-length HCV RNA in the absence of a primer, presumably using the 3’ ends of both positive and negative strand HCV RNA as templates (Oh *et al.*, 1999). Indeed, a specific interaction between NS5B and the 3’ end of viral RNA has been reported (Cheng *et al.*, 1999). Having no cellular equivalent, HCV RdRp, like NS3, is an attractive target for antiviral therapy (Huang *et al.*, 2006).

1.2.1.11 3’ UTR

The 3’ UTR is a tripartite structure consisting of a short (28-42 nt), poorly conserved sequence (variable region), followed by a poly(U)/polypyrimidine tract of variable length and a highly conserved 98 nt sequence termed the X-tail (Fig 1.6) (Tanaka *et al.*, 1996, Kolykhalov *et al.*, 1996, Blight and Rice, 1997). Despite the significant heterogeneity in the poly(U)/polypyrimidine tract between isolates, all isolates carry this tract, suggesting some importance in replication (Friebe and Bartenschlager, 2002). The X-tail is highly conserved even between the most divergent HCV isolates (Tanaka *et al.*, 1996), suggesting some functional importance. The secondary structure of the 3’UTR has been determined (Blight and Rice, 1997, Kolykhalov *et al.*, 1996) and includes two stem-loops upstream of the poly(U)/polypyrimidine tract and another 3 within the X-tail. Structural determination indicated one stable stem-loop within the terminal 46 nts of the X-tail (3’SL1), and possibly two other unstable stem-loops within the remaining 52 nts of the X-tail (3’SL2 and 3’SL3) (Blight and Rice, 1997, Kolykhalov *et al.*, 1996). Several studies have indicated regions of the 3’UTR that are required for both replication and translation. Two *in vivo* studies showed that deletion of the X-tail destroyed the ability of infectious RNA to replicate in chimpanzees (Kolykhalov *et al.*, 2000, Yanagi *et al.*, 1999) while Yanagi *et al.* took this study further and showed that deletion of any large segment of the 3’UTR (except

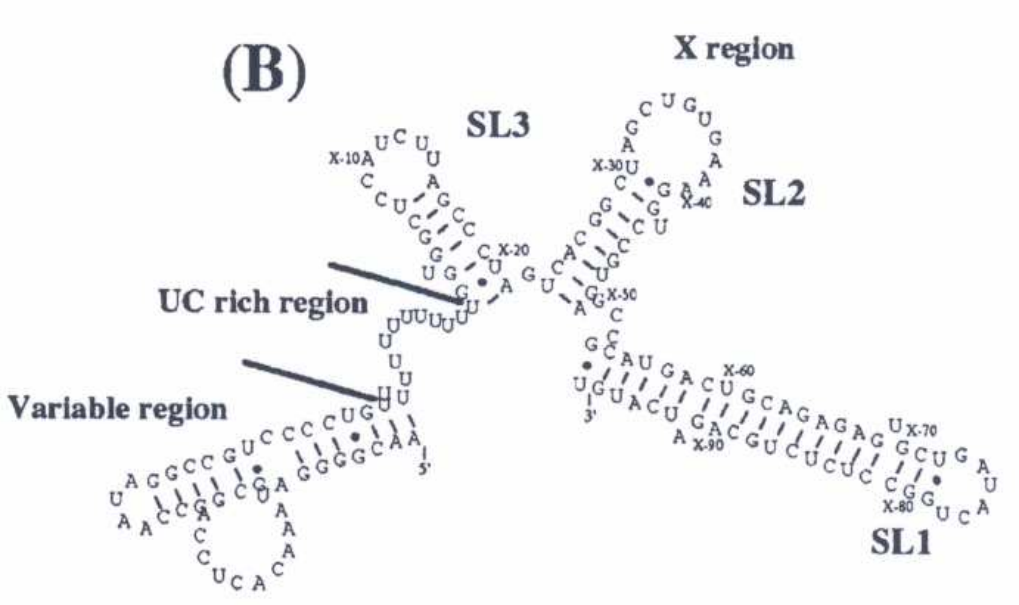


Figure 1.6: Proposed secondary and tertiary structure of the HCV 3'NCR. The 3'UTR is a tripartite structure consisting of a variable region, a poly(U)/polypyrimidine tract of variable length and a highly conserved X-tail which forms 3 stem loops (taken from Ito & Lai, 1997).

for the variable region) abolished the replicative ability of the HCV RNA (Yanagi *et al.*, 1999). These *in vivo* studies were confirmed by studies using HCV replicons showing that the X-tail and poly(U)/polypyrimidine tract are indispensable for replication, while deletion of the complete variable sequence reduces replication efficiency (Friebe and Bartenschlager, 2002). Replication was also shown to be abolished when any of the 3 putative X-tail stem-loops were deleted, and similarly, replication was abolished on deletion of the complete poly(U)/polypyrimidine tract (Yi and Lemon, 2003). In this study, replication was only sustainable in the presence of at least 50 nts of poly(U)/polypyrimidine tract. Translation of the HCV polyprotein is also thought to be influenced by the 3'UTR in that the X-tail specifically enhances IRES-dependent translation from the 5'-end of HCV viral RNA (Ito *et al.*, 1998a), while more recently it was shown that all regions of the 3'UTR contribute to translation stimulation and that this stimulation was stronger in hepatoma cell lines (Huh-7 and HepG2) compared to non-hepatoma lines (BHK and HeLa) (Song *et al.*, 2006).

A number of studies have shown an interaction between the 3'UTR and host-cell proteins, for example polypyrimidine tract-binding protein (PTB). PTB has been reported to bind the poly(U)/polypyrimidine tract and the 5'-end of the X-tail of the 3'UTR (Tsuchihara *et al.*, 1997, Gontarek *et al.*, 1999, Luo, 1999). This interaction is of interest as it has previously been shown that PTB can also bind the 5'UTR (Ali and Siddiqui, 1995) and since PTB is capable of dimerisation, it is conceivable that PTB is required to bring the 5' and 3'UTRs together, possibly during replication. Other cellular proteins shown to bind the 3'UTR include La, heterogeneous nuclear ribonucleoprotein C (hnRNPC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal proteins L22, L3 and S3, as well as the mitochondrial homologue of L3 (mL3) (Wood *et al.*, 2001, Gontarek *et al.*, 1999, Petrik *et al.*, 1999, Spangberg *et al.*, 1999). Finally, in agreement with the theory of the 3'UTR being involved in replication, both NS5B and the helicase domain of NS3 have been shown to specifically interact with the 3'UTR (Cheng *et al.*, 1999, Banerjee and Dasgupta, 2001). The array of host cell proteins shown to bind the 3'UTR, along with the enzymatic NS5B and NS3 suggest that a major complex of proteins may form around the 3'UTR thus contributing to the viral RNA replication process.

1.3 HCV Replication

Until the recent discovery of an HCV clone fully infectious in cell culture (Wakita *et al.*, 2005), the study of HCV replication was severely hampered. Using comparative analysis and characterisation of recombinant HCV proteins however, a hypothetical model of HCV replication has been proposed (Fig. 1.7) (Bartenschlager and Lohmann, 2000). The detection of hepatitis C-specific antigens in liver biopsies of chronic HCV carriers (Blight *et al.*, 1994) has led to the identification of the liver as the primary site of virus replication (Bartenschlager and Lohmann, 2000). Extrahepatic sites of replication have also been suggested however. Peripheral blood mononuclear cells (PBMC) are permissive for HCV replication although replication levels are low (Cribier *et al.*, 1995), while viral particles have also been identified within an HCV infected human B lymphoblastoid cell line (Serafino *et al.*, 1997). Human T-cells have also been suggested to be capable of sustaining HCV replication (Shimizu *et al.*, 1992) and more recently negative strand HCV RNA (indicative of replication) has been detected in the central nervous systems of patients with recurrent HCV infection after liver transplantation, suggesting HCV is neuroinvasive (Vargas *et al.*, 2002). While the liver is clearly the primary site of HCV replication, it is possible that extrahepatic sites of replication do exist, however the role these infections play in the pathogenesis of HCV has yet to be ascertained.

1.3.1 Attachment and Entry

To initiate its life cycle, a virus must bind to the host cell. Binding occurs via a specific interaction between a host cell receptor and an attachment protein on the surface of the virus. Glycoproteins E1 and E2 are present on the surface of the HCV particle and are believed to be responsible for virus attachment and entry. Antibodies against E2 and the HVR1 of E2 have been shown to block virus attachment in vitro (Zibert *et al.*, 1995) and neutralise infectious virus both *in vivo* (Farci *et al.*, 1996) and in infectious cell culture system (Lindenbach *et al.*, 2005). Initially, due to the lack of reproducible cell culture model, virus-like particles (VLPs) were used for the study of HCV binding. VLPs were produced in insect cells by recombinant baculovirus containing cDNA of HCV structural proteins (Baumert *et al.*, 1998), however these particles were non-infectious and were retained within intracellular compartments making it difficult to evaluate how closely they resembled real virus particles. A better model for studying HCV binding is the

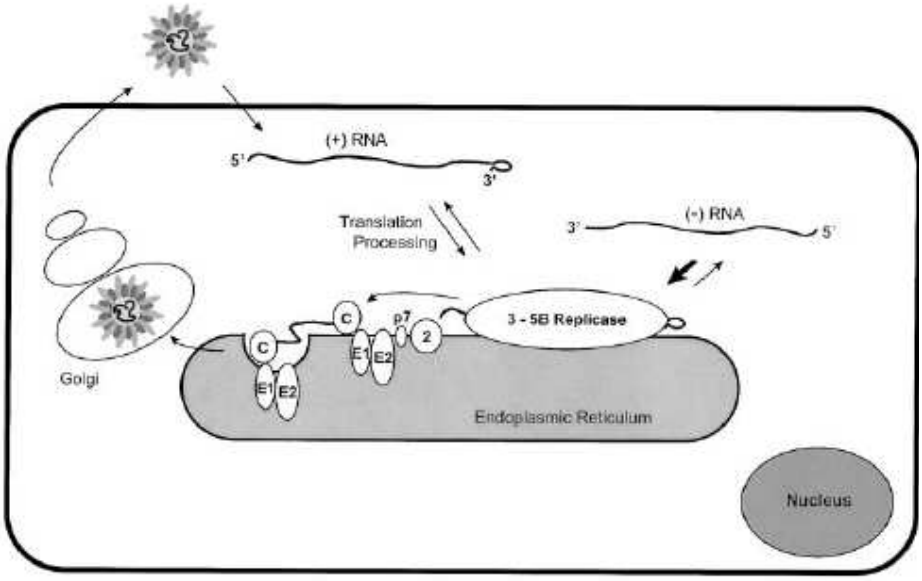


Figure 1.7: Putative model for the HCV replication cycle. Upon attachment and entry into the host cell, positive-sense, single-stranded genomic RNA is released into the cytoplasm and translated. A single polypeptide is produced which is subsequently processed by both host and viral proteases. Formation of a membrane-bound replication complex occurs, consisting of viral proteins NS3-5B which generates negative-sense replication intermediate HCV RNA that is then used to as a template for production of more genomic HCV RNA. Progeny RNA is encapsidated by core protein and resultant nucleocapsids are enveloped by budding into the ER lumen, prior to egress via the secretory pathway (taken from Bartenschlager & Lohmann, 2000).

pseudoparticle (HCVpp) model. Pseudoparticles are retroviral or lentiviral core particles displaying unmodified HCV envelope glycoproteins (Hsu *et al.*, 2003, Bartosch *et al.*, 2003a). These particles mimic the early stages of infection, have a preferential tropism for the liver and are neutralised by anti-E2 monoclonal antibodies and HCV positive patient sera (Hsu *et al.*, 2003, Bartosch *et al.*, 2003a, Lavillette *et al.*, 2005, Owsianka *et al.*, 2005). While these model systems have helped elucidate some of the mechanisms behind cell tropism and entry, it is only with the production of infectious HCV particles from a cloned viral genome (Wakita *et al.*, 2005), that these mechanisms can be clearly understood in terms of infectious virus particles.

The first molecule to be suggested as a putative HCV receptor was CD81 (Pileri *et al.*, 1998). CD81 is a 25 kDa cell-surface molecule involved in many processes including cell adhesion and signal transduction and has 4 transmembrane domains, giving rise to 2 extracellular loops (termed large and small) and categorising it as a member of the tetraspanin superfamily. Evidence for CD81 being a receptor for HCV came from the competitive inhibition of binding of anti-CD81 to CD81-expressing cells by recombinant E2, with E2 specifically binding to the large extracellular loop of CD81 in protein immunoblots (Pileri *et al.*, 1998). Recombinant GST-fusion polypeptides encompassing the large extracellular loop of human CD81, pre-incubated with HCVpp, specifically precipitated E1-E2 complexes and was shown to neutralise HCVpp infection of Huh-7 cells (Bartosch *et al.*, 2003a), while HCVpp infection was also inhibited by monoclonal antibodies against CD81 as well as by knockdown of CD81 expression by siRNA (Zhang *et al.*, 2004). Finally, evidence from infectious cell culture systems suggests an involvement of CD81 since infectivity was blocked by pre-incubation with soluble recombinant CD81 large extracellular loop molecules (Lindenbach *et al.*, 2005) while anti-CD81 monoclonal antibodies were able to neutralise infectious virus (Wakita *et al.*, 2005). Koutsoudakis *et al.* recently added to this by showing that anti-CD81 inhibits HCV JFH-1 infection at a post-binding stage, suggesting CD81 is required after an initial binding step (Koutsoudakis *et al.*, 2006) while susceptibility to HCV infection depends on a critical quantity of cell surface CD81 molecules (Koutsoudakis *et al.*, 2007). A study comparing the large extracellular loop of human CD81 with that of African Green Monkey (AGM) (having no affinity for E2), showed only 4 differences in amino acid sequence (Flint *et al.*, 1999) while mutagenesis of these 4 residues indicated that residues 163 and 186 were important for E2 binding (Higginbottom *et al.*, 2000). CD81 mutant T163A increased binding affinity for E2 while mutant F186L completely abolished interaction with E2. In a similar study, residues 162, 182, 184 and 186 were identified by random mutagenesis as being critical for E2 binding (Drummer *et al.*, 2002). The region of E2 involved in binding

CD81 has been studied by site-directed mutagenesis and subsequent functional analysis of CD81 binding and infectivity in the context of HCVpp. From this, it has been suggested that E2 residues 420, 527, 529, 530 and 535 are important in binding since mutation led to >90% reduction in binding to recombinant CD81. Interestingly, while those mutants showing >90% reduction in CD81 binding showed concomitant poor infectivity, some mutants that were able to bind CD81 also showed poor infectivity, indicating that CD81 binding is necessary but not sufficient for HCVpp infectivity (Owsianka *et al.*, 2006). In a similar study, residues 436-438 and 441-443 were also shown to be important for both CD81 binding and HCVpp infectivity (Drummer *et al.*, 2006). In agreement with the theory that CD81 binding is not sufficient for infectivity, a study of which cell types were permissive for HCVpp infection showed that while all human cell lines studied (with the exception of HepG2 and HH29) expressed CD81, only 4 (Huh-7, Huh-7.5, PLC/PR5 and Hep3B) were permissive to HCVpp infection, suggesting CD81 alone is not sufficient to confer susceptibility to infection (Zhang *et al.*, 2004).

The low-density lipoprotein (LDL) receptor has also been suggested as a putative HCV receptor (Agnello *et al.*, 1999). Analysis of HCV particles with heterogenous densities led to the identification of an association between HCV and low density β-lipoproteins (Thomssen *et al.*, 1992), suggesting β-lipoprotein associated particles may enter host cells via the LDL receptor. While anti-LDL receptor antibody could inhibit HCV infection in a dose-dependent manner, increasing cell surface LDL receptor levels increased the percentage of cells positive for HCV infection (Agnello *et al.*, 1999). Biochemical inhibitors of LDL endocytosis (heparin sulphate and EGTA) inhibited LDL receptor mediated endocytosis of HCV particles, suggesting LDL receptor mediates endocytosis of HCV (Agnello *et al.*, 1999). Further evidence for the requirement of the LDL receptor for HCV entry came from the inhibition of particle-binding to human fibroblasts by purified LDL and the reporting that HCV only binds COS-7 cells when transfected with LDL receptor cDNA (Monazahian *et al.*, 1999). LDL and HCV particles have been shown to co-localise on the surface of Molt-4 cells, however, LDL was unable to completely block HCV binding (Wunschmann *et al.*, 2000), suggesting other receptors may be involved. Recently, Wunschmann *et al.* reported an increase in E2 binding to Molt-4 and Huh-7 cells when LDL was incubated with E2 prior to contact with cells. A dose-dependent reduction in binding was also seen when cells were pre-incubated with anti-LDL receptor antibody (Wunschmann *et al.*, 2006). Interestingly, binding of E2 to 3T3 cells was only seen when cells expressed human CD81, with no increase in binding seen in the presence of LDL suggesting a requirement for human LDL receptor for this increase in binding (Wunschmann *et al.*, 2006).

A third putative HCV receptor is human scavenger receptor class B type I (SR-BI) (Scarselli *et al.*, 2002). SR-BI, which selectively uptakes cholesterol from the hydrophobic cores of lipoproteins, belongs to the CD36 superfamily that includes cell-surface membrane proteins that bind chemically modified lipoproteins (Acton *et al.*, 1996). SR-BI is a high-density lipoprotein (HDL) receptor which has 2 transmembrane domains with short N- and C-termini located in the cytoplasm and a large extracellular loop (Acton *et al.*, 1994) and is expressed primarily in the liver and steroidogenic tissue (Acton *et al.*, 1994, Landschulz *et al.*, 1996). HepG2 cells, which do not express CD81, efficiently recognise recombinant E2, suggesting the presence of an alternative attachment molecule. By reversible cross-linking with E2 and subsequent cell lysis, an 82 kDa, glycosylated molecule was isolated and identified as SR-BI (Scarselli *et al.*, 2002). HVR1 of E2 is required for recognition of SR-BI as mutant E2 lacking HVR1 is unable to bind SR-BI (although still able to recognise CD81) (Scarselli *et al.*, 2002). Using HCVpp, Bartosch *et al.* confirmed the requirement of SR-BI for HCV infection, however both CD81 and LDLr were also required (Bartosch *et al.*, 2003b). Alternative HCV models have also confirmed a role for SR-BI in HCV infection. The tree shrew, *Tupaia belangeri*, is closely related to primates and can be infected with HCV (Xie *et al.*, 1998). Anti-tupaia SR-BI antibodies inhibited the binding of HCV VLPs and soluble E2 to primary tupaia hepatocytes in a concentration dependent manner, providing more evidence for an involvement of SR-BI in HCV binding (Barth *et al.*, 2005). However, inhibition of E2/SR-BI interaction during infection studies using HCV RNA positive serum did not prevent infection, suggesting other receptors may also play a role in HCV entry (Barth *et al.*, 2005). High-density lipoproteins (HDLs) have been suggested to enhance SR-BI mediated HCV cell entry. Increased entry of HCVpp in the presence of HDLs has been reported (Voisset *et al.*, 2005, Bartosch *et al.*, 2005) and this enhancement shown, by RNAi knockdown of SR-BI, to be dependent on SR-BI, while drugs inhibiting the transfer of HDL cholesteryl ester have also been shown to reduce HCVpp entry (Voisset *et al.*, 2005). SR-BI binds HDLs and lipopolysacharrides (LPS), allowing entry to the cell via intracellular compartments such as the golgi complex or endocytotic compartment (Vishnyakova *et al.*, 2003). HCV may therefore utilise this entry pathway in order to avoid the classical degradation pathway. Also, fusion-activation of HCV glycoproteins is pH-dependent, suggesting HCV may use SR-BI to traffic itself to endosomal compartments in which low pH could activate the fusion properties of the glycoproteins (Bartosch *et al.*, 2003b).

Other molecules thought to bind HCV particles include the asialoglycoprotein receptor (ASGP-R) (Saunier *et al.*, 2003) and the liver (L)- and dendritic cell (DC)-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrins (L-SIGN and DC-

SIGN) (Gardner *et al.*, 2003, Pohlmann *et al.*, 2003, Lozach *et al.*, 2003). Anti-ASGP-R antibody was shown to inhibit binding of virus-like particles to Molt-4 and HepG2 cells, while binding to 3T3 cells was only possible in the presence of transfected ASGP-R (Saunier *et al.*, 2003). L-SIGN and DC-SIGN are type II integral membrane proteins with short N-terminal cytoplasmic domains and a C-terminal calcium dependent lectin domain (Gardner *et al.*, 2003). L-SIGN is expressed in the liver (although not on hepatocytes) (Pohlmann *et al.*, 2003) while DC-SIGN is found on dendritic cells. L-SIGN and DC-SIGN are known to bind HIV gp120 with high affinity and transfer virus particles to adjoining CD4-positive cells (Geijtenbeek *et al.*, 2000). These molecules have also been shown to bind E2, VLPs and infectious HCV from human serum (Gardner *et al.*, 2003, Lozach *et al.*, 2003, Pohlmann *et al.*, 2003), while co-culture of DC-SIGN/L-SIGN expressing cells (pre-incubated with HCVpp) with Huh-7 cells permits transmission of infectious HCVpp to Huh-7 cells (Cormier *et al.*, 2004a, Lozach *et al.*, 2004). L-SIGN is expressed on liver sinusoidal endothelial cells which form vessels that separate hepatic blood from hepatocytes, leading to the suggestion that these cells could be involved in trans-infection, transferring virus from the blood to hepatocytes in the liver (Lozach *et al.*, 2004, Lozach *et al.*, 2003).

Several putative receptors have now been suggested for HCV binding and entry. It is possible that the virus requires more than one receptor, possibly using one for initial binding followed by binding to a second co-receptor with or without some conformational change (Cormier *et al.*, 2004b, Bartosch *et al.*, 2003b). However, until the recent discovery of the infectious cell culture system, the exact function, if any, of these receptors in the HCV life cycle has been difficult to confirm. Initial analysis of potential HCV receptors relied on soluble, truncated E2, however it is not certain whether this model fully represents the corresponding E2 structures on the HCV virion. Owsianka *et al.* showed ligand-dependent differences in monoclonal antibody inhibition of E2-CD81 when comparing soluble, truncated E2, full-length E1E2 and virus-like particles (Owsianka *et al.*, 2001), indicating care is required when interpreting results from these experimental systems.

Upon initial binding to the cell surface, enveloped viruses enter the host either by fusion at the plasma membrane or by receptor-mediated endocytosis. Receptor-mediated endocytosis results in fusion of the viral envelope with the endosomal membrane, triggered by low pH within the endosome. Flaviviruses, such as West Nile virus and tick-borne encephalitis virus, undergo pH-dependent fusion with membranes, indicating they enter cells via receptor-mediated endocytosis (Vorovitch *et al.*, 1991, Gollins and Porterfield,

1986). Hsu *et al.* has shown that HCVpp infectivity is reduced by >90% in the presence of inhibitors of vacuolar acidification (ammonium chloride or concanamycin A) (Hsu *et al.*, 2003), while Blanchard *et al.* showed similar results using the inhibitors bafilomycin and chloroquine in the HCVcc system (Blanchard *et al.*, 2006), thus indicating that HCV requires a pH-sensitive route of entry. A dose-dependent inhibition of infectivity using the HCVcc system was seen even if the inhibitor concanamycin was added up to 3 hrs post-binding (Koutsoudakis *et al.*, 2006). HCV pH-dependent entry is thought to be clathrin-mediated, a theory that was confirmed by siRNA knockdown of clathrin as well as by chlorpromazine inhibition of clathrin-coated pit assembly, both of which reduced HCVcc entry (Blanchard *et al.*, 2006). Fusion has also been shown to be dependent on E1 and E2, occurs optimally at pH 5.5 and is enhanced by cholesterol (Lavillette *et al.*, 2006). pH-dependent viruses usually synthesise their glycoproteins in an inactive form, preventing premature fusion with internal cellular membranes (Hsu *et al.*, 2003). Interestingly, Op de Beek *et al.* reported conformational changes in E1E2 of HCVpp at low pH (Op De Beeck *et al.*, 2004) and pre-exposure to low pH decreased fusion ability (Lavillette *et al.*, 2006), indicating that some conformational change takes place within E1E2 which is required at the point of fusion. Some viruses encode an ion channel (viroporin) thought to facilitate a pH change within the virion to allow uncoating (Fischer and Sansom, 2002). Recently, HCV p7 was identified as a putative member of the viroporin family (Pavlovic *et al.*, 2003, Griffin *et al.*, 2003), suggesting that this may be a mechanism for pH-induced fusion and uncoating of the HCV virion.

1.3.2 Viral RNA Transcription, Replication and Translation

In common with all other positive-sense, single stranded RNA viruses, HCV RNA translation and replication is thought to take place in the cytoplasm of infected cells. Being a positive sense RNA virus, the viral RNA acts as mRNA and is therefore directly translated. Unlike cellular mRNAs which are capped, allowing translation by the scanning ribosome mechanism, HCV RNA translation is cap-independent and instead requires an IRES located in the 5'UTR for translation initiation (Tsukiyama-Kohara *et al.*, 1992), (See section 1.2.2.10). Translation by the scanning ribosome mechanism involves binding of the ribosomes to the 5' end of the mRNA and scanning until the authentic AUG codon is found. However, the HCV IRES directs the ribosome to a site in close proximity to the initiator AUG. No cellular factors are required for HCV RNA translation (Pestova *et al.*, 1998), although cellular factors such as PTB and La have been shown to bind to the IRES

and influence translation (Ali and Siddiqui, 1995, Ali and Siddiqui, 1997). Translation of HCV RNA occurs at the rough ER and produces a single polyprotein which is cleaved co- and post-translationally by both viral and cellular proteases, to produce the structural and non-structural proteins (Grakoui *et al.*, 1993d, Hijikata *et al.*, 1993), (See section 1.2). Many positive-sense, single stranded RNA viruses induce distinct membrane alterations within their host and the same has been shown for HCV. Changes in membrane structure, termed the membraneous web, have been seen in HCV infected cells and is thought to be induced by NS4B (Gosert *et al.*, 2003, Shi *et al.*, 2003, Egger *et al.*, 2002, El-Hage and Luo, 2003). All HCV proteins interact with cellular membranes directly or indirectly and all have been shown to associate with the membraneous web, as has replicating HCV RNA, indicating the membraneous web may harbour the HCV replication complex (Egger *et al.*, 2002, El-Hage and Luo, 2003, Gosert *et al.*, 2003, Shi *et al.*, 2003). These membrane structures are resistant to detergent treatment and can be co-fractionated with the lipid-raft associated caveolin-2, suggesting that the replication complexes are situated within lipid-raft type membrane structures (Shi *et al.*, 2003).

HCV replicates via negative strand replication intermediates as shown by the presence of negative strand HCV RNA in serum and the liver of infected patients (Fong *et al.*, 1991). As with all positive-sense RNA viruses, once sufficient translation of HCV RNA has occurred, the virus must switch the template role of genomic RNA from translation to replication (Ahlquist *et al.*, 2003). A “copy-back” replication mechanism has been suggested for HCV (Behrens *et al.*, 1996, Lohmann *et al.*, 2000), in which the high complimentarity of the 3’UTR sequence allows the 3’-terminal nucleotides to intramolecularly base-pair, thus generating a primer/template molecule which can then be used by the NS5B RNA-dependent RNA polymerase. NS5B is able to copy long templates without additional viral or cellular factors and has no apparent template specificity, being capable of copying homologous and heterologous templates (Lohmann *et al.*, 1997, Lohmann *et al.*, 2000), thus confirming its capability to replicate molecules such as the HCV genome.

1.3.3 Assembly, Maturation and Release

Nucleocapsid formation of virus particles probably involves multimerisation of core protein and an interaction with viral RNA. With this in mind, several studies have indicated an interaction between core and the HCV 5’UTR. Residues 1-75 of core,

containing 4 clusters of basic amino acids, have been shown to have RNA binding capacity (Santolini *et al.*, 1994), while a number of groups have identified HCV RNA sequences capable of binding core. HCV RNA from nucleotide 1-2327 was detected by northern blot after immunoprecipitation by anti-core antibody (Shimoike *et al.*, 1999), with further delineation of this region mapping specific sequences within the 5'UTR (Tanaka *et al.*, 2000). Surface plasmon resonance (SPR) was used to confirm a stable interaction between core and the HCV 5'UTR. Core was found to preferentially bind sequences with high G content. Loop IIId domain of the 5'UTR (Fig. 1.4), having a high G content, was identified as the main core-binding region. Interestingly, loops IIIe and IIIf also have high G content yet do not bind core, suggesting that the hairpin loop and internal bulge loop of domain IIId are essential secondary structures required for core interaction (Tanaka *et al.*, 2000). The secondary structure of loop IIId is highly conserved across all genotypes and also in the related pestiviruses, suggesting this may be an essential structure in nucleocapsid formation (Brown *et al.*, 1992). Residues 1-20 of core have been shown to inhibit translation from the HCV IRES with this inhibition depending on both a minimum concentration of core and a high core:RNA ratio (Li *et al.*, 2003). This suggests that core may be involved in the switch from translation of RNA to replication and resultant packaging of viral RNA.

Multimerisation of core, requiring homotypic interaction, would be expected if core protein is to form capsid structures. Yeast-2-hybrid studies have led to various regions of core being suggested as homotypic interaction sites, with the hydrophilic region encompassing amino acids 1-115 of core shown to be sufficient for core-core interaction, although no interaction was detected using full-length core protein, possibily due to the hydrophobic domain of core affecting the nuclear transport of the fusion proteins in yeast (Matsumoto *et al.*, 1996). Residues 1-115 of core are rich in basic residues and are highly conserved, indicating essential properties (Bukh *et al.*, 1994). The binding domain was tentatively mapped to residues 36-91 by yeast-2-hybrid analysis, overlapping the predicted ribosome and RNA binding domains (Santolini *et al.*, 1994). However, residues 36-91 were insufficient for efficient core-core binding suggesting surrounding areas may be required (Matsumoto *et al.*, 1996). In agreement, the interaction domain was reported to be within residues 82-102 of core (Nolandt *et al.*, 1997). Again, this was only seen using C-terminal truncated core protein and no interaction was seen using full-length core protein. These results also bring up the possibility that the homotypic interacting domain may be masked in full-length core and some conformational change is required to allow core-core interaction. Interestingly, a conformational change in core has been identified upon interaction with tRNA (Kunkel and Watowich, 2002). The N-terminus of core is

proteinase-sensitive and the C-terminal end is required to stabilise free core protein. However, core complexed with RNA or assembled into nucleocapsid particles was proteinase-resistant, suggesting conformational changes in core occur upon binding RNA and assembling into nucleocapsids, possibly in order to protect core from cellular degradation (Kunkel and Watowich, 2002). Other domains of core have also been suggested to be involved in homotypic interaction including residues 122-172 (Nolandt *et al.*, 1997) and both the N- and C-termini (Yan *et al.*, 1998). In agreement with the N- and C-termini being involved in multimerisation, truncated core protein (residues 1-124) was shown to assemble into virus-like particles only in the presence of highly structured positive-strand 5'UTR RNA while full-length core underwent de novo assembly in the absence of RNA (Kim *et al.*, 2006), thus suggesting the N-terminus may be involved in multimerisation upon interaction with RNA while the C-terminus may initiate spontaneous multimerisation early in capsid formation. Other studies on HCV assembly have shown that the HCV structural proteins core, E1 and E2, encoded in baculovirus vectors and expressed in insect cells, assemble into VLPs with a lipid bilayer (Baumert *et al.*, 1998). These virus-like particles were shown to have E1 and E2 on their lipid bilayer surface and could be specifically labelled with anti-E1 and anti-E2 antibodies, as well as with anti-HCV human serum (Clayton *et al.*, 2002, Baumert *et al.*, 1998), while also preferentially encapsidating HCV RNA (Baumert *et al.*, 1998). Cell-free systems have also been used to show that core can multimerise into capsid structures. Capsid assembly in a cell-free system is independent of E1 and E2 (Klein *et al.*, 2004) and, similar to the results generated by yeast-2-hybrid studies (Matsumoto *et al.*, 1996), clusters of basic residues at the N-terminus are critical for assembly (Klein *et al.*, 2005).

Upon formation of the nucleocapsid, envelopment by cellular membranes containing viral glycoproteins is likely. Although the mechanism of budding for HCV is not understood, by comparison with the related flaviviruses, HCV is thought to acquire its envelope at the ER as the HCV glycoproteins localise predominantly to the ER (Deleersnyder *et al.*, 1997, Dubuisson *et al.*, 1994). HCV glycoproteins do not migrate further than the *cis* Golgi, suggesting that HCV budding may indeed occur in the ER (Dubuisson *et al.*, 1994). As flaviviruses and alphaviruses share similar structural features (Helenius, 1995), expression of HCV structural proteins within a semliki forest virus vector has been studied (Blanchard *et al.*, 2002). Major differences in ER structure were observed, with areas of convoluted membranes present in transfected cells. Self-assembly of HCV structural proteins was observed at these convoluted membranes by EM. At higher magnification, budding of virus-like particles of approximately 50 nm towards the dilated ER lumen was observed and these particles could be strongly labelled with anti-core and anti-E1 antibodies,

suggesting the HCV capsid acquires its envelope by budding through the ER membrane. Enforced expression of E1 and E2 (in *trans*) in replicon harbouring cells increases the formation frequency of replication complexes (Brazzoli *et al.*, 2006). In these cells, co-localisation of HCV structural proteins with all non-structural proteins and HCV RNA was observed, indicating this may be a hypothetical site for virus budding. No infectious particles were found in the supernatant however, and no data on structured particles within the cell has been reported (Brazzoli *et al.*, 2006). Envelopment and budding of the nucleocapsid is likely to require an interaction between core and the HCV glycoproteins. Indeed, an interaction between core and E1 has been reported (Ma *et al.*, 2002, Lo *et al.*, 1996). Since little, if any, of E1 is situated on the cytosolic side of the ER, the interaction is likely to occur within the ER (Ma *et al.*, 2002, Lo *et al.*, 1996).

Following particle formation at intracellular membranes as seems most likely with HCV, viral egress to the cell surface is expected. Flaviviruses commonly egress through utilisation of the host cell constitutive secretory pathway (Fields, 2001). The secretory pathway is a highly conserved route for proteins destined for the outer membrane of the cell. The pathway involves a complex series of membrane-bound subcellular compartments including the ER, intermediate compartment and Golgi. Most proteins traversing the secretory pathway are modified by addition of oligosaccharide side-chains. Interestingly, HCV glycoproteins are modified by N-linked glycosylation, with E1 containing up to 6 and E2 potentially 11 glycosylation sites (Op De Beeck *et al.*, 2001). Addition of these side-chains takes place in the lumen of the ER, which is also where the HCV glycoproteins are located. During transport through the secretory pathway, it is essential that the integrity and fusogenic form of the glycoproteins are maintained, so as to allow future host-cell attachment and entry. To prevent premature acid-induced inactivation of the glycoproteins, several viruses employ a viroporin to prevent acidification of vesicles. Influenza M2 protein is a viroporin which is suggested to play a role in regulating the pH of vesicles in the *trans*-golgi network, thus ensuring the correct maturation of the hemagglutinin glycoprotein during egress (Sugrue and Hay, 1991). HCV also encodes a viroporin in its p7 gene (Griffin *et al.*, 2003) and therefore may have a similar role to the M2 viroporin of influenza, ensuring premature acid-induced activation of the HCV glycoproteins is avoided. Members of the flavivirus genus adopt a mechanism of delayed cleavage, preventing irreversible conformational changes in the acidic compartment of the secretory pathway. Tick-borne encephalitis (TBE) virus employs its prM protein to form a stable heterodimeric complex with the envelope protein (E). Low pH in the acidic compartment of the secretory pathway induces a conformational change in prM, providing access to a furin-specific cleavage site. Furin is a membrane-bound,

calcium-dependent protease of the *trans*-golgi network (Bosshart *et al.*, 1994). Cleavage of prM by furin shortly before release from the cell results in formation of the mature virion (Stadler *et al.*, 1997), thus preventing premature exposure of the mature virion to low pH. It is possible that HCV employs a similar mechanism in order to retain the fusogenic properties of its glycoproteins. Interestingly, a recent report has described differences in buoyant densities between infectious intracellular virus particles and infectious extracellular particles, suggesting the biological composition of these two forms of infectious particles may differ (Gastaminza *et al.*, 2006). It is possible that this difference in buoyant density may be due to factors involved in avoiding premature fusion during viral egress.

1.4 Model Systems to Study HCV

Before the discovery of a fully infectious HCV clone capable of producing infectious particles in cell culture (Wakita *et al.*, 2005), it was difficult to study the virus's structure and assembly process. The following paragraphs describe the various model systems used to study HCV in the absence of a robust cell culture model, culminating in the description of the infectious JFH-1 cell culture system which has opened up new avenues in HCV research.

1.4.1 Comparative Studies with HCV-related Viruses

Much of the current knowledge of HCV has been derived from comparative studies. HCV has been shown to share similarity at the level of genomic organisation and amino acid sequence with the flaviviruses and, in particular, the pestiviruses (Miller and Purcell, 1990). The pestivirus BVDV generally provides the best model for HCV comparative studies due to the availability of infectious clones and efficient cell culture systems (Moormann *et al.*, 1996, Meyers *et al.*, 1996) and is also used as a surrogate model of HCV for the evaluation of antiviral agents (Buckwold *et al.*, 2003). GB virus-B, a hepatotropic virus of unknown natural host that causes acute and sometimes chronic hepatitis in tamarins and marmosets, has also been used as a surrogate model for HCV as it is the virus most closely related to HCV, with infection resulting in similar pathological features to those of HCV infection (Beames *et al.*, 2001, Muerhoff *et al.*, 1995). Domain 2 of GB

virus-B core protein shows ~41% sequence identity with that of domain 2 of HCV core protein (Hope *et al.*, 2002), suggesting these two domains may perform similar functions. Also, the putative envelope proteins of GB virus-B and HCV share common structural features while significant homology was observed among the NS3 serine protease, the NS3 RNA helicase and the NS5B RNA-dependent RNA polymerase regions as well as at predicted cleavage sites in the non-structural regions (Muerhoff *et al.*, 1995). The function and substrate specificities of the GB virus-B and HCV NS3 serine proteases are also similar (Scarselli *et al.*, 1997).

1.4.2 Animal Models

The only animal capable of being infected with HCV is the chimpanzee. HCV was shown to be transmissible to chimpanzees many years before the discovery of the virus in 1989. The chimpanzee model was used to characterise the physicochemical properties of the then unknown agent (Farci and Purcell, 1998) and later, HCV was cloned from plasma collected from a persistently infected chimpanzee (Choo *et al.*, 1989). RNA transcripts produced from full-length cDNA clones have been shown to be infectious upon injection into the liver of chimpanzees (Yanagi *et al.*, 1997). The chimpanzee is an ideal model for HCV infection, being more than 98.5% genetically identical to humans (Grakoui *et al.*, 2001). However, chimpanzees are limited in availability and are expensive to acquire and maintain, not to mention the ethical issues which arise from their use. To overcome the lack of suitable animal model, transgenic mice with humanised livers have been produced (Turrini *et al.*, 2006, Mercer *et al.*, 2001). These mice can be infected with HCV-positive human serum and support HCV replication within the human portion of their livers at clinically relevant titres. HCV is capable of long-term persistence and can be serially passaged from infected mouse to naïve transgenic mouse (Mercer *et al.*, 2001). Transgenic mice carrying HCV proteins have been used in many studies to investigate the pathogenic properties of these proteins (Honda *et al.*, 1999a, Moriya *et al.*, 1998, Moriya *et al.*, 1997). Tree shrews (*Tupaia*) have been shown to be capable of infection with HCV and produce anti-HCV antibodies. Efficiency of infection was increased by whole-body irradiation, suggesting immunosuppression may facilitate infection (Xie *et al.*, 1998). The feasibility of using this model to study HCV has yet to be investigated however.

1.4.3 Infection of cultured cells

HCV-positive patient sera has been used to infect certain monolayer cell lines and primary cell cultures of liver origin (Ito *et al.*, 1996), as well as blood mononuclear cells (although replication *in vitro* was at low levels) (Cribier *et al.*, 1995). A human T-lymphotropic virus (HTLV) type I infected cell line (MT-2) was reasonably sensitive to HCV infection and HCV replication was detected 10 days post-infection, however no infectious particles were produced (Kato *et al.*, 1995). As HCV can infect *Tupaia* (Xie *et al.*, 1998), Zhao *et al.* (2002) investigated the possibility of infecting primary *Tupaia* hepatocytes with HCV. Negative strand HCV RNA was detected in these cells and nuclease resistant RNA found in the culture medium. This culture medium was infectious for naïve primary *Tupaia* hepatocytes, indicating these cells were capable of HCV replication and production of progeny particles (Zhao *et al.*, 2002). More recently, a 3-D radial flow bioreactor (RFB) has been used to study HCV infection *in vitro*. This system allowed human liver cells to retain their differentiated hepatocyte functions and morphological appearance for longer periods of time (Matsuura *et al.*, 1998). FLC4 cells, grown in a 3-D RFB were susceptible to propagation of HCV RNA or infected serum and allowed production of infectious particles that could be immunogold labelled with anti-E1 antibody (Aizaki *et al.*, 2003). At present this system is relatively complex and expensive, providing an obstacle to its use as a research tool for HCV particle production.

1.4.4 HCV Pseudo-particles

In an attempt to study the process of HCV cell entry, infectious, genetically tagged HCV pseudo-particles harbouring unmodified E1 and E2 glycoproteins were developed (Bartosch *et al.*, 2003a). HCV glycoproteins E1 and E2 were assembled onto retroviral core proteins derived from murine leukaemia virus (MLV) by transfection of human 293T cells with expression vectors encoding E1E2 polyprotein (including the carboxyl terminus of core), MLV Gag-Pol core proteins and a packaging-competent MLV-derived genome encoding GFP as a marker. Expression in human 293T cells results in the packaging of the GFP marker-gene into MLV-core pseudo-particles, displaying unmodified E1 and E2 HCV glycoproteins. These pseudo-particles were released into the culture medium and shown to infect naïve hepatocytes, thus confirming that HCV pseudo-particles show a preferential tropism for hepatic cells while also leading to the discovery that they are specifically neutralised by anti-E2 monoclonal antibodies as well as HCV positive sera. The HCV

pseudo-particle system allows detailed study of the interactions between HCV glycoproteins and cell surface receptors. A similar system using lentivirus transfer and packaging constructs has also been described (Hsu *et al.*, 2003).

1.4.5 HCV Replicon System

A major breakthrough in HCV research came with the development of the HCV replicon system. The replicon system was based on the stable autonomous replication of subgenomic, selectable, HCV RNAs (Lohmann *et al.*, 1999). Deletion of the core-NS2 region, and insertion of a gene encoding the selectable marker neomycin phosphotransferase (neo) and the EMCV IRES, resulted in a bicistronic replicon with translation of neo driven by the HCV IRES and translation of the second cistron (NS3-5B) driven by the EMCV IRES. Upon transfection into Huh-7 cells and selection with neomycin sulphate (G418), colonies grew containing autonomously replicating HCV RNAs. HCV RNA replication levels in these cells were ~100,000 fold higher than in other *in vitro* systems (Bartenschlager and Lohmann, 2001), however limited numbers of G418-resistant colonies grew and cell culture adaptive mutations were required for enhancement (Blight *et al.*, 2000, Lohmann *et al.*, 2001, Krieger, 2001). Several mutations were identified and, in particular, mutation of aa 2884 in NS5B led to ~500 fold increase in replication (Lohmann *et al.*, 2001), while a cluster of mutations were found in the central region of NS5A (Blight *et al.*, 2000) and two others identified in NS3 (Krieger, 2001). The replicon system was further modified in 2002 when Pietschmann *et al.* generated selectable full-length HCV genomes in which the HCV structural proteins were efficiently expressed. Intruigingly however, no viral particles were produced from this system, suggesting that Huh-7 cells may lack some essential factors required for production of HCV particles (Pietschmann *et al.*, 2002) (a theory which has now been dispelled due to the production of JFH-1 infectious particles from these cells (Wakita *et al.*, 2005)). The replicon system has been an invaluable discovery in studying HCV replication and also in antiviral drug design given that all viral enzymes are present and replicating RNA can be propagated for years.

1.4.6 JFH-1 Infectious Clone

Recently, a major breakthrough occurred when an HCV genotype 2a clone (isolated from a Japanese patient suffering from fulminant hepatitis (Kato *et al.*, 2001)) that was fully infectious in cell culture was discovered (Wakita *et al.*, 2005). Since then, a number of

reports have confirmed these results (Lindenbach *et al.*, 2005, Zhong *et al.*, 2005). Initially, the aim was to construct a genotype 2a replicon construct as no 2a replicon had previously been made. Upon analysis of this replicon it was noted that efficient replication occurred without the need for cell culture-adaptive mutations (Kato *et al.*, 2003). *In vitro*-transcribed full-length JFH-1 RNA was transfected into Huh-7 cells and resulted in HCV replication and secretion of infectious particles (Wakita *et al.*, 2005). HCV RNA was shown to replicate to high levels by Northern hybridisation and virus could be serially passaged. Sucrose-density gradients showed that core protein and nuclease-resistant HCV RNA sedimented to the same density of 1.17 g/ml indicating the presence of cell culture-derived virus particles, while particles were also visualised by immuno-electron microscopy, being labelled with an anti-E2 antibody. Most importantly, the particles visualised were infectious for naïve Huh-7 cells. CD81-specific antibodies and HCV positive human sera were both capable of neutralising infectivity in a dose-dependent manner and culture medium was infectious for chimpanzees when injected intravenously (Wakita *et al.*, 2005). Shortly after this discovery, these results were confirmed and it was shown that virus titres could consistently reach 10^4-10^5 infectious units/ml (Zhong *et al.*, 2005). Using the non-structural genes of JFH-1, infectious particles were produced by a chimeric construct containing the structural proteins of genotype 2a J6 isolate, but not by a similar construct using genotype 1a H77 structural proteins, while both chimeras were replication competent (Lindenbach *et al.*, 2005). This suggests that interactions between the structural and non-structural proteins may be essential for production of infectious particles. Interestingly, although by equilibrium centrifugation in 10-40 % iodixanol, the peak fraction of HCV RNA was found to correspond to a density of 1.13-1.14 g/ml, this fraction had little infectivity. Instead the most infectious fraction was found to correspond to a density of 1.09-1.11 g/ml, suggesting most of the particles are non-infectious (Lindenbach *et al.*, 2005). The discovery of this new system should enable the study of previously inaccessible stages of the HCV life cycle.

1.5 HCV Core Protein

1.5.1 Maturation

Core is the N-terminal most product of the HCV polyprotein and is cleaved by host cell proteases (Grakoui *et al.*, 1993d, Hijikata *et al.*, 1991b), with three products being

described as a result of this processing. As the amino terminus of E1 has been mapped to residue 192 (Hijikata *et al.*, 1991b), it is believed that core is cleaved from the polyprotein at residue 191. A second cleavage event around residue 173 produces a smaller core protein, thought to be the mature form (Santolini *et al.*, 1994), however, other reports suggest this cleavage event occurs between residues 179 and 181 (Hussy *et al.*, 1996). There has been some confusion over the names of these two core molecules with some groups naming them P21 and P19 (Hussy *et al.*, 1996, Lo *et al.*, 1994, Lo *et al.*, 1995), however, in the remaining sections they will be termed P23 and P21 in accordance with more recent publications (McLauchlan, 2000). Isolate HCV-1 has a third cleavage site, producing a P16 form of core which is only found in genotype 1a isolates, resulting from a lysine residue at amino acid 9 of core. Mutation of this residue to arginine abolishes the P16 form (Lo *et al.*, 1994, Lo *et al.*, 1995). Generation of both P23 and P21 requires microsomal membranes, suggesting membrane associated proteases are responsible for cleavage of core (Santolini *et al.*, 1994, Hussy *et al.*, 1996). Cleavage at residue 191 is predicted to occur on the luminal side of the ER by the cellular signal peptidase complex (Santolini *et al.*, 1994, McLauchlan, 2000), while a membrane-bound signal peptide peptidase is responsible for the second cleavage event resulting in the mature P21 form of core, with residues 180, 183 and 184 of core being necessary for efficient processing (McLauchlan *et al.*, 2002, Hussy *et al.*, 1996). Amino acid sequence analysis shows that core is highly conserved among all genotypes (Bukh *et al.*, 1994), with 3 domains being identified from its hydropathicity pattern (Fig. 1.8). Residues 1- ~122 (domain 1) contain a high proportion of basic residues (23.4%) as well as 2 short hydrophobic domains. Domain 2 (amino acids 123-174) has a lower proportion of basic residues and is more hydrophobic than domain 1 while domain 3 (residues 175-191) is highly hydrophobic. Domain 3, acting as a signal sequence, is required to direct E1 to the ER (Santolini *et al.*, 1994), while removal of the C-terminus results in translocation of core to the nucleus (Suzuki *et al.*, 1995, Moradpour *et al.*, 1996).

1.5.2 Intracellular Distribution

Difficulty in detecting core in biopsy samples of HCV-infected patients has hampered the study of the subcellular localisation of core. However, core has been shown to have a cytoplasmic, granular localisation in some liver biopsies (Gonzalez-Peralta *et al.*, 1994, Yap *et al.*, 1994). Subcellular fractionations indicate that core is associated with membranes while immunofluorescence data from tissue culture systems has also shown

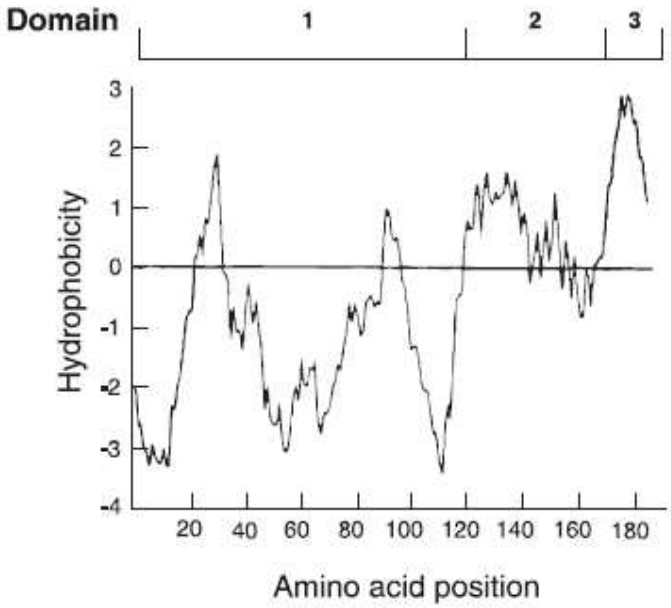


Figure 1.8: Hydropathicity pattern of HCV core protein. Domain 1 (residues 1-~122) contains a high proportion of basic residues (23.4%) as well as 2 short hydrophobic regions. Domain 2 (residues 123-174) has a lower proportion of basic residues and is more hydrophobic than domain 1, while domain 3 is highly hydrophobic (taken from McLauchlan 2000).

core to be cytoplasmic, and to associate with granular structures or reticular networks (Selby *et al.*, 1993, Santolini *et al.*, 1994, Barba *et al.*, 1997). These granular structures have been shown to be lipid droplets by EM and an accumulation of gold particles has been seen around lipid droplets and ER in liver biopsies of HCV-infected chimpanzees (Barba *et al.*, 1997). Domain D2 of core, consisting of two amphipathic alpha-helices separated by a hydrophobic loop, is responsible for targeting core to lipid droplets (Hope and McLauchlan, 2000a, Boulant *et al.*, 2006). No single structural motif within domain D2 is responsible for lipid droplet association. Instead, a combination of both amphipathic alpha-helices and the hydrophobic loop are required (Boulant *et al.*, 2006). While core has mostly been reported to be cytoplasmic, several reports have also suggested that a proportion may localise to the nucleus. C-terminally truncated core protein localised to the nucleus (Suzuki *et al.*, 1995), as does HCV-1 P16 core in the absence of E1 sequence (Lo *et al.*, 1995). A nuclear species of core was also reported to be conformationally distinct from the cytoplasmic species since antibodies could discriminate between core species in either compartment (Yasui *et al.*, 1998). However, the presence and relevance of this nuclear species of core has yet to be confirmed *in vivo*.

1.5.3 The Structural Role of Core

By analogy with other members of the *Flaviviridae*, core is thought to form the capsid of the HCV particle. However, due to the lack of robust cell culture (prior to the report of the JFH-1 infectious cell culture system), or small animal model and low detection levels in infected human or chimpanzee livers, little is known about core in this regard. Core protein has been detected in patient sera by fluorescent enzyme immunoassay (FEIA) (Tanaka *et al.*, 1995b, Kashiwakuma *et al.*, 1996), suggesting core is associated with the virus particle, while core protein of similar size to that of recombinant core (produced in mammalian cells) was found in virus-like particles produced from recombinant baculovirus (Baumert *et al.*, 1998). Residues 1-75 of core can bind RNA (Santolini *et al.*, 1994), as would be expected of a capsid protein, while the same region has been shown to be involved in core multimerisation (Matsumoto *et al.*, 1996) (section 1.3.3). A cell-free system has recently been described in which cellular events are reproduced in eukaryotic cell extracts (Klein *et al.*, 2004). In this system, the production of either P23 or P21 forms of core results in capsid assembly, with velocity sedimentation and buoyant density peaks similar to those of de-enveloped particles from patient sera. Transmission electron microscopy showed that particles produced in this cell-free system also have the same size range, size heterogeneity

and morphological appearance to those from patient sera (Klein *et al.*, 2004). Essential residues required for assembly in this system were shown to be within the first 68 residues of core (Klein *et al.*, 2004) and were further identified as being basic residues within this region (Klein *et al.*, 2005). Decreasing the density of basic residues in this region was shown to reduce the ability of core to assemble into capsids, while deletion of a region containing no basic residues (amino acids 27-38) has no effect on capsid assembly. By yeast-2-hybrid screen, residues 82-102 of core were shown to be required for core multimerisation (Nolandt *et al.*, 1997), while using a Semliki Forest virus replicon system, the aspartic acid at residue 111 of core was identified as being important for assembly (Blanchard *et al.*, 2003). In the cell-free system however, deletion of residues 82-102 nor mutation of residue 111 had any effect on capsid assembly (Klein *et al.*, 2005), suggesting that these model systems may not accurately reproduce *in vivo* capsid assembly. As described in section 1.4.6, the discovery of the JFH-1 infectious cell culture system has improved the study of infectious HCV particles and should lead to a better understanding of core protein and its role as capsid protein for HCV.

1.5.4 Core (+1) ORF/ARF/F Protein

The HCV genome contains a number of synonymous codons with highly conserved 3rd position nucleotides (Fig. 1.9) (Walewski *et al.*, 2001). In a study of 8 highly divergent HCV sequences, residues 33, 39 and 41 of core contained the same glycine codon (GCA). The probability that all 8 sequences contained the same glycine codon is 1:16,384, indicating that more genetic information may be contained than in a single ORF. A second coding region could therefore contribute to the excessive 3rd position conservation in the main HCV ORF (Walewski *et al.*, 2001). The HCV genome has been shown to contain an alternative reading frame, overlapping the core protein gene and encoding a recently described core+1 ORF/alternate reading frame (ARF)/frameshift (F) protein (Varaklioti *et al.*, 2002, Walewski *et al.*, 2001, Xu *et al.*, 2001). Synthesis of this alternative protein is thought to be due to ribosomal frameshift around codon 11 of core protein, within a region of 10 adenine nucleotides (Xu *et al.*, 2001). A similar overlapping ORF has been identified in GBV-B (Bukh *et al.*, 1999), and sera from HCV infected patients reacted with *in vitro* synthesised protein (Xu *et al.*, 2001, Walewski *et al.*, 2001), suggesting that this protein is produced during natural HCV infection. No function has yet been assigned to this protein, however it is not required for replication of the HCV genome as subgenomic replicons (lacking the structural genes) replicate efficiently (Lohmann *et al.*, 1999). Functional

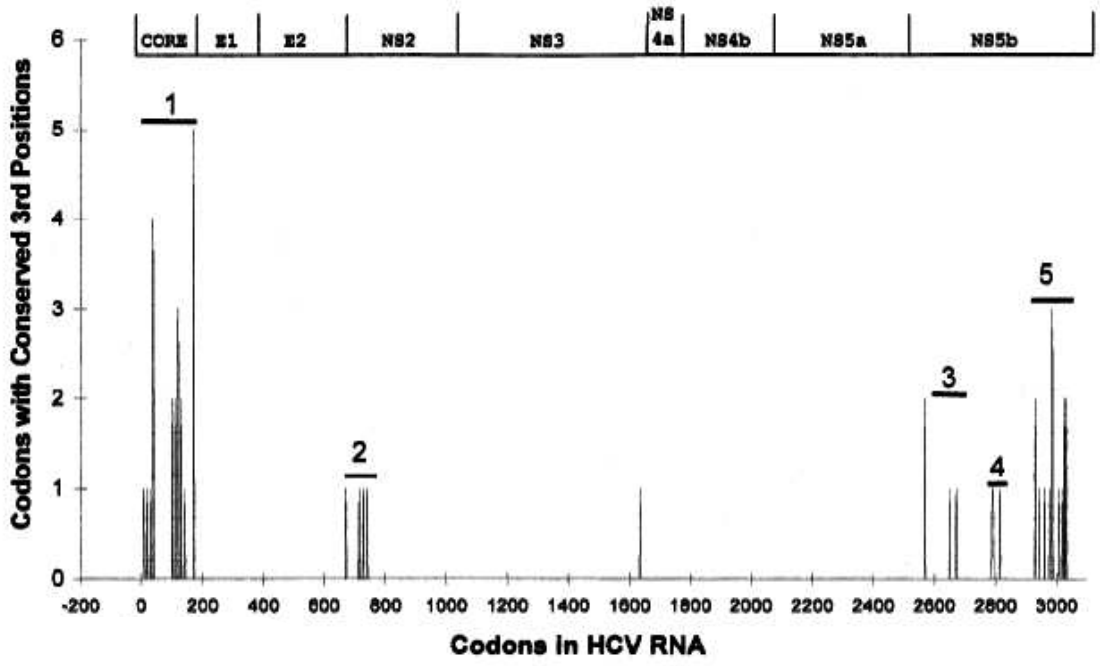


Figure 1.9: Clusters of synonymous codons in the main ORF of HCV. Codons with conserved third position nucleotides conserved in 8 highly divergent HCV sequences show a prominent cluster in the core-coding region, providing a rational basis for an alternative reading frame in this region (taken from Walewski *et al.*, 2001).

properties assigned to core protein have also been studied using the F protein including modulation of c-*myc* and p53 promoters, as well as effects on TNF-α-mediated apoptosis, however as yet none seem to be conclusively due to this alternative protein (Basu *et al.*, 2004).

1.5.5 Possible Pathogenic Roles of Core Protein

1.5.5.1 Effects on Apoptosis

Expression of HCV core has been shown to affect apoptosis. Apoptosis, or programmed cell death, is an orderly cellular process resulting in dell death via several morphological phases including cell shrinkage, nuclear condensation, blebbing of the plasma membrane and "laddering" of genomic DNA by digestion (McLauchlan, 2000). Induction of apoptosis can be caused by a number of stimuli including oxidative stress, heat shock, ionizing radiation, cytokines and virus infection. Induction of apoptosis by these stimuli normally occurs via cell surface molecules such as Fas or TNF receptors, resulting in a cascade of intracellular events including death domain clustering and activation of caspases, which are responsible for many of the morphological changes during apoptosis. Apoptosis is modulated by core protein via 3 receptor pathways, *Fas* receptor-mediated, TNFα receptor-mediated and lymphotoxin β receptor-mediated apoptosis.

Conflicting reports on the effect core protein has on *Fas*-mediated apoptosis have been published. The first report on the effect of core on this apoptotic pathway suggested that the interaction between anti-*Fas* antibody with the *Fas* receptor induced marked apoptosis in the presence of core expressed in HepG2 cells (Ruggieri *et al.*, 1997). In contrast, core protein was also shown to protect HepG2 cells from *Fas*-mediated apoptosis induced by the same anti-*Fas* antibody (Marusawa *et al.*, 1999). The methodologies used in both experiments were similar, however the use of clonal cell lines stably expressing core (Ruggieri *et al.*, 1997) as opposed to transient transfection of the full HCV open reading frame (Marusawa *et al.*, 1999), may explain the contradictory results.

As with the effect of core protein on *Fas*-mediated apoptosis, contradictory results have been reported on the effect core has on TNFα-mediated apoptosis. Transiently transfected core protein was initially shown to inhibit TNFα-induced apoptosis in MCF7 cells (Ray *et al.*, 1998a). In agreement with this, core protected HepG2 cells from TNFα-mediated

apoptosis, with suppression of apoptosis thought to be upstream of caspase-8 (Marusawa *et al.*, 1999). Core was also shown to activate NF-κB and activation was enhanced upon stimulation by either anti-*Fas* or anti-TNFα antibody, thus preventing apoptosis (Marusawa *et al.*, 1999). However, core protein was also shown to sensitise HepG2 and HeLa cells to TNFα-induced apoptosis (Zhu *et al.*, 1998). Residues 1-115 of core were identified as the binding region for TNFR1, while the death domain within the cytoplasmic tail of TNFR1 was shown to be required for interaction with core. TNFR1 has no catalytic activity therefore the death domain is thought to act as a signal transducer in the apoptopic pathway. Further to this, the effects core had on downstream protein interactions were analysed. Upon oligomerisation of TNFR1, TRADD is recruited which subsequently recruits FADD. In turn, FADD then interacts directly with downstream apoptotic proteases leading to apoptosis. Core was shown to bind FADD but not TRADD in core expressing 293 cells, while a dominant negative form of FADD blocked the core-induced sensitisation to TNFR1-mediated apoptosis. It is therefore suggested that core enhances TNFα-mediated apoptosis by recruiting FADD to TNFR1. Core, binding the death domain of TNFR1, blocks TRADD from binding TNFR1 (Zhu *et al.*, 2001). A recent report (Saito *et al.*, 2006) has contradicted this work by Zhu *et al.*, claiming that TNFα-induced apoptosis is inhibited by core protein and that core does not interact with TNFR1 or TRADD. This report suggests that core sustains levels of c-FLIP, which in turn inhibits the cleavage and activation of caspase-8, resulting in inhibition of apoptosis. As with *Fas*-mediated apoptosis, the contradictory reports may be due to different cell lines or mode of core expression.

Core protein can also enhance LT-βR-mediated apoptosis in a cell-type dependent manner. Enhancement was seen in HeLa cells expressing core, although this was not observed in HepG2 or Huh-7 cells also expressing core (Chen *et al.*, 1997).

Finally, a recent report suggests that core may induce ER stress, thus leading to apoptosis. Transient transfection of core in HepG2 or Huh-7 cells resulted in an increase in expression of glucose-regulated protein 78 (Grp78), an ER-resident chaperone, indicating ER stress. Similar results were also seen in replicon-harbouring cells and transgenic mice. Increase in Grp78 expression was not seen in cells transfected with HBV core. Levels of CHOP, an ER stress-induced cell death modulator (Rao *et al.*, 2004), are increased in the presence of core protein in cell culture and transgenic mice, suggesting that ER stress, induced by HCV core protein, may lead to apoptosis (Benali-Furet *et al.*, 2005).

1.5.5.2 Effects on Cell Transformation

Studies using primary rat embryo fibroblasts (REFs) have shown that HCV core, in combination with the oncogene H-*ras*, can convert cells to a transformed phenotype. Cells showed rapid proliferation, anchor-independent growth and tumour formation in athymic mice (Ray *et al.*, 1996). Although core, in combination with the oncogene could produce this effect, core alone was unable to do so. However, core alone was shown to transform established NIH3T3 cells, resulting in anchor-independent growth and tumourigenicity when injected into nude mice (Smirnova *et al.*, 2006, Ray *et al.*, 1996). Transformation of NIH3T3 cells by core was the result of core interacting with and activating (through phosphorylation), signal transducer and activator of transcription 3 (STAT3) (Yoshida *et al.*, 2002). Core, in combination with H-*ras*, can also prevent contact inhibition of growth and promote anchorage-independent growth of BALB/3T3 A31-1-1 cells by stimulating the activation of the Erk1/2-serum response element pathway, involved in signal transduction of growth stimuli (Tsuchihara *et al.*, 1999). In contrast, it has been reported that core is unable to transform REFs in the presence of H-*ras* (Chang *et al.*, 1998). This discrepancy may be due to the HCV isolates used in the two studies as Chang *et al.* used HCV-RH core while Ray *et al.* used HCV-1 core (although both are genotype 1a). HCV-1 core gene has been shown to produce a 16 kDa core species which shows predominant nuclear localisation (Lo *et al.*, 1995), therefore this species of core may be responsible for the differences reported by the two groups. In agreement with Ray *et al.* however, Chang *et al.* showed that the established REF line Rat-1 was readily transformed by core and showed anchorage-independent growth. Abnormal cellular proliferation is also seen due to the effects of core protein on the transcription factor LZIP. In the context of core protein, LZIP presents activities consistent with that of a tumour suppressor. LZIP is a nuclear CRE-activating factor whose transcriptional activity is repressed by core. Subcellular sequestration of LZIP by core results in inactivation of LZIP and subsequent abnormal cellular proliferation (Jin *et al.*, 2000).

1.5.5.3 Effects on Transcription and Regulatory Factors

A number of reports have indicated that core protein can modulate expression from cellular and viral promoters (McLauchlan, 2000). The first report of this kind showed that HCV core protein could suppress expression and replication of hepatitis B virus (HBV) in Huh-7 cells (Shih *et al.*, 1993). Suppression was evident between 6 and 9 days post-transfection and coincided with intracellular relocalisation of HCV core from the cytoplasm to the nucleus (Shih *et al.*, 1993). Other viruses may also be transcriptionally regulated by HCV

core. Core transactivates expression from the HIV-1 long terminal repeat (LTR) and also from the SV40 promoter, while suppressing expression from the rous sarcoma virus (RSV) LTR (Ray *et al.,* 1995). In terms of cellular promoters, core has been shown to transactivate the c-*myc* promoter while suppressing the c-*fos* and p53 tumour suppressor promoters (Ray *et al.,* 1995, Ray *et al.,* 1997). Contradictory effects of core on the promoter of a negative regulator of cell cycle progression (p21) have also been reported. Core was reported to suppress p21 promoter activity indicating that p21 regulation of cell growth was affected in the presence of core during liver regeneration, resulting in enhanced cell growth (Ray *et al.,* 1998b). On the other hand, enhancement of activity from the p21 promoter by core protein has also been shown (Lu *et al.,* 1999). An attempt was made to resolve this inconsistency by showing that core affected p21 promoter activity in different ways, depending on the cell line used (Kwun and Jang, 2003). While core transactivated p21 promoter activity in HepG2 and Hep3B cells, suppression of expression from the promoter was seen in NIH3T3 cells and primary hepatocytes from transgenic mice. Different cell lines were also used in the studies by Ray *et al.* (1998b) and Lu *et al.* (1999). While Ray *et al.* (1998b) used COS7 cells, Lu *et al.* (1999) used both HepG2 and Hep3B cells, results which were consistent with those of Kwun & Jang (2003). Finally it has been reported that core may counteract the antiviral effects of IFN by suppressing activity from the promoters of the IFN effector proteins MxA, PKR and 2'-5' oligoadenylate synthetase (2'-5' OAS) (de Lucas *et al.,* 2005).

1.5.5.4 Effects on Immune Presentation

As HCV establishes chronicity in the majority of cases, the virus must be able to avoid clearance by the immune system. Core protein may play a role in HCV immune evasion by inhibiting the immune response to infection. Cytotoxic T cells are primed against HCV as a result of interaction between antigen-presenting cells (APC), HCV antigen and the T cells themselves. APCs are a site of HCV replication (Lerat *et al.,* 1998) and have therefore been studied in the context of HCV proteins and stimulation of cytotoxic T cells. Expression of HCV proteins core, E1 and E2, or core protein alone using adenoviral vector systems have shown that dendritic cells expressing HCV proteins possess reduced stimulatory capacity for cytotoxic T cells and produced and released lower levels of T cell stimulatory cytokine IL-12 (Hiasa *et al.,* 1998, Sarobe *et al.,* 2002). Addition of exongenous IL-12 to dendritic cell/T cell cultures did not restore T-cell proliferation, indicating that lack of IL-12 is not the main cause of abnormal T cell priming (Sarobe *et*

al., 2002). Studies in mice using recombinant vaccinia virus showed that those expressing core protein had elevated virus titre 5 days post-infection compared to those expressing HCV non-structural proteins and, while those expressing non-structural proteins overcame infection, mice expressing core protein succumbed to lethal infection (Large *et al.*, 1999). Failure to clear vaccinia virus infection in the presence of HCV core protein led to the theory that core protein plays a role in inhibition of the cytotoxic T cell response. Analysis of cytotoxic T cell precursor indicated a 10-fold lower frequency in core expressing mice 5 days post-infection compared to mice expressing non-structural proteins. Interferon-γ production by immune splenocytes was also profoundly suppressed in the presence of core protein, with a reduction in antigen-stimulated IL-2 production also seen (Large *et al.*, 1999). Core protein has also been shown to modulate the immune response by interfering in the complement pathway (Kittlesen *et al.*, 2000). Binding of complement protein C1q to its receptor, gC1qR, blocks proliferation of cytotoxic T cells (Ghebrehiwet *et al.*, 1990). HCV core protein has been shown to bind gC1qR in both yeast-2-hybrid and GST pull-down assays and is believed to mimic C1q in blocking proliferation of cytotoxic T cells (Kittlesen *et al.*, 2000). Core protein is secreted from transfected cell lines expressing core (Sabile *et al.*, 1999) and circulating core protein is detectable in the plasma of HCV infected patients (Masalova *et al.*, 1998). Exposure to core protein inhibited T cell proliferation in a dose-dependent manner (Kittlesen *et al.*, 2000) and this was suggested to be due to inhibition of the ERK/MEK MAP kinase signalling pathway (Yao *et al.*, 2001). Inhibition of activation of the ERK/MER MAP kinase by C1q/gC1qR interaction inhibits transcription of early genes involved in T cell activation (such as IL-2) and leads to suppression of proliferation. Production of IL-2 is also inhibited in the presence of core protein, suggesting core may inhibit T cell proliferation by inhibiting the ERK/MEK MAP kinase signalling pathway (Yao *et al.*, 2001).

1.5.5.5 Effects on Lipid Metabolism

HCV infection has been suggested to have effects on lipid metabolism as shown by studies on steatosis in the liver of HCV infected patients (Bach *et al.*, 1992, Moriya *et al.*, 2001) and in transgenic mice harbouring HCV core protein or the entire HCV genome (Lerat *et al.*, 2002, Moriya *et al.*, 1997), as well as through an association between HCV core protein and apolipoprotein AII (Barba *et al.*, 1997). In comparison with patients with autoimmune chronic hepatitis, those with chronic hepatitis C presented more commonly with steatosis (72% vs. 19%) (Bach *et al.*, 1992). HCV core protein localises to the surface

of lipid droplets as shown in both chimpanzees and HepG2 cells stably expressing core protein (Barba *et al.*, 1997), as well as in transgenic mice (Moriya *et al.*, 1997), suggesting that core may regulate expression of cellular genes involved in lipid metabolism (Barba *et al.*, 1997). Transgenic mice, expressing HCV core from birth, presented with steatosis at 2 months and although mild at first, progressed in severity, coinciding with an increased frequency of large (in comparison to small) lipid droplets, suggesting a role for core in steatosis, possibly by binding enzymatic molecules or apolipoproteins involved in lipid metabolism (Moriya *et al.*, 1997). Interestingly, core was shown to colocalise with apolipoprotein II on lipid droplets within HepG2 cells (Barba *et al.*, 1997). Comparison of lipid levels and fatty acid composition of lipids in core-expressing transgenic mice, non-transgenic mice and non-transgenic obese mice indicated that triglyceride levels in the livers of transgenic mice and non-transgenic obese mice were significantly higher than in non-transgenic mice while the concentration of carbon 18 mono-unsaturated fatty acids (oleic and vaccenic acids) was increased only in transgenic mice, suggesting HCV core may affect a specific pathway in lipid metabolism, different to that affected by other causes of steatosis. Similar results on lipid composition were found in human livers (Moriya *et al.*, 2001). Core protein has also been shown to impair hepatic assembly and secretion of triglyceride-rich, very low-density lipoproteins (VLDL) (Perlemuter *et al.*, 2002) and a marked reduction in number of normal sized lipoprotein particles was seen in core expressing transgenic mice. Microsomal triglyceride transfer protein (MTP) and apolipoprotein B (apo B) are major regulators of VLDL assembly. Transgenic mice expressing core show significantly lower MTP activity compared to non-transgenic mice, suggesting that core protein may impair secretion of VLDL by decreasing MTP activity and thus VLDL assembly (Perlemuter *et al.*, 2002). Transgenic mice have also recently been used to show that the proteasome activator PA28γ is involved in core-induced steatosis. PA28γ specifically binds core protein in the nucleus, resulting in degradation of the viral protein in a PA28γ-dependent manner (Moriishi *et al.*, 2003). Vacuolation of mouse liver cells was seen in the presence of core and PA28γ but not in the absence of either core or PA28γ, suggesting that PA28γ is required for the induction of liver steatosis by HCV core (Moriishi *et al.*, 2007).

1.5.6 Host Cell Proteins Interacting with Core

A number of host cell proteins have been identified which interact with HCV core protein. These proteins were initially identified by yeast-2-hybrid screening of human cDNA

libraries using core sequences as bait. Once identified, the interactions were confirmed by biochemical analyses including GST-pull down assay and *in vivo* colocalisation by immunofluorescence. More recently, proteomic profiling using 2-dimensional electrophoresis and mass spectrometry has been used to identify proteins interacting with core (Kang *et al.*, 2005), and to analyse lipid droplet composition in core expressing cells (Sato *et al.*, 2006).

1.5.6.1 Lymphotoxin-β Receptor (LT-βR)

Core protein binds the cytoplasmic tail of LT-βR (Matsumoto *et al.*, 1997, Chen *et al.*, 1997), which is a member of the tumour necrosis factor receptor family. The exact function of LT-βR is unknown however it is thought to be involved in cytolytic and NF-κB activation in certain cell types. The region of core required for interaction with LT-βR includes residues 1-91 of domain I (Matsumoto *et al.*, 1997, Chen *et al.*, 1997). Core protein, expressed in HeLa cells, enhances the cytolytic effects of LT-βR ligand (lymphotoxin-α_1-β_2) while having no such effects in either Huh7 or HepG2 cells (Chen *et al.*, 1997).

1.5.6.2 Tumour Necrosis Factor Receptor 1 (TNFR1)

Core protein has also been shown to bind the prototype tumour necrosis factor receptor, TNFR1 (Zhu *et al.*, 1998). TNFR1 is the primary receptor mediating TNF induction and is involved in cell death signalling and NF-κB activation. GST-fusion protein pull-down assays were used to identify an interaction between core and TNFR1. This interaction requires residues 1-115 of core protein and the cytoplasmic tail region of TNFR1 (residues 345-407). In the presence of core, HepG2 and HeLa cells were more sensitive to TNF- or anti-TNFR1 antibody-induced cell death (Section 1.5.5.1) (Zhu *et al.*, 1998).

1.5.6.3 Heterogeneous Nuclear Ribonucleoprotein K (hnRNP K)

hnRNP K, a component of the hnRNP complex, is a transcriptional regulator with both RNA- and DNA-binding properties. Although predominantly found in the nucleus, hnRNP

K also shuttles to the cytoplasm and has been suggested to play a role in processing and transport of pre-mRNA. Core protein specifically interacts with hnRNP K as shown by yeast-2-hybrid, GST-fusion protein binding and colocalisation studies (Hsieh *et al.*, 1998). By yeast-2-hybrid analysis, residues 1-115 of HCV core domain I were required for the interaction. Core protein was also shown to partially reverse the suppressive effect of hnRNP K on the human thymidine kinase gene, possibly by binding proline-rich regions of hnRNP K which blocks the interaction of hnRNP K with other cellular factors (Hsieh *et al.*, 1998).

1.5.6.4 Apolipoprotein AII (apoAII)

In a study describing the cytoplasmic localisation of core protein, it was reported that core and apoAII colocalise on the surface of lipid droplets in HepG2 cells (Barba *et al.*, 1997). The region involved in the interaction was later identified as residues 160-173 of core. Truncation of this region leads to loss of interaction with apoAII and localisation of core to the nucleus (Sabile *et al.*, 1999). Association between apoAII and HCV core may be involved in the modulation of lipid metabolism by core protein, thus playing a role in HCV pathogenicity.

1.5.6.5 p53

Activation of the tumour suppressor p53 has been shown to be enhanced by HCV core protein (Lu *et al.*, 1999). Core was able to enhance the gene transactivation activity of exogenous p53 in p53-negative Hep3B cells as well as endogenous p53 in HepG2 cells. Enhancement of transactivation by p53 in the presence of core resulted in enhanced expression of the downstream p53 effector gene, the cdk inhibitor $p21^{waf1/Cip1/Sdi1}$. Increased expression of $p21^{Waf1/Cip1/Sdi1}$ can suppress the cell cycle and, in the presence of core protein, Hep3B cell growth was suppressed in a p53-dependent manner. Suppression of hepatocellular growth by core protein in a p53-dependent manner may have important implications in HCV pathogenesis. Direct interaction between core and p53 was identified by co-immunoprecipitation experiments. Binding p53 does not require residues downstream of amino acid 151 of core, while residues 366-380 of p53 are required for interaction (Lu *et al.*, 1999).

1.5.6.6 14-3-3ε protein

An interaction between core protein and the epsilon isoform of 14-3-3 protein (14-3-3ε) has been shown by yeast-2-hybrid analysis (Aoki *et al.*, 2000a). The 14-3-3 protein family associate with components of several signal transduction pathways such as the Raf-1 kinase cascade. Residues 49-97 of core protein are required for interaction, as are residues 165-234 of 14-3-3 protein. Interaction may occur in a phospho-serine dependent manner, as phosphorylation of serine-53 of core is essential for interaction with the cellular protein in HepG2 cells stably expressing core. Activation of Raf-1 kinase, a central component of the mitogen-activated protein (MAP) kinase pathway, requires binding of core protein to 14-3-3 protein. Interestingly, Ito *et al.* reported enhanced MAP kinase activation in HCC (Ito *et al.*, 1998b), suggesting core may play a role in the progression of HCV to HCC (Aoki *et al.*, 2000a).

1.5.6.7 $p21^{Waf1/Cip1/Sdi1}$ (p21)

Core protein may be involved in de-regulation of the cell cycle via interaction with the cell cycle regulator p21 (Wang *et al.*, 2000). *In vitro* pull-down studies identified an interaction between core and p21 with the binding site mapped to residues 24-52 of core protein and residues 139-164 of p21. As p21 is a regulator of the cell cycle, interaction with core protein may inhibit nuclear transport of newly synthesised p21 and its subsequent involvement in cell cycle regulation (Wang *et al.*, 2000).

1.5.6.8 Leucine Zipper Protein (LZIP)

The transcription factor LZIP has been shown to bind HCV core protein by yeast-2-hybrid analysis and co-immunoprecipitation studies (Jin *et al.*, 2000). Endogenous LZIP was shown to localise to the nucleus in HepG2 and HeLa cells, as was exogenously expressed LZIP. However, upon co-expression with HCV core protein, exogenously expressed LZIP was relocalised from the nucleus to the cytoplasm. Loss of LZIP function (by over-expression of a transcriptionally incompetent LZIP mutant) resulted in loss of contact inhibition in NIH3T3 cells, resulting in dense foci of cells. This dysregulation of cell growth was enhanced in the presence of core protein, suggesting that by sequestration of LZIP, core protein can prevent LZIP function and act as a co-factor in cell transformation (Jin *et al.*, 2000).

1.5.6.9 Complement Receptor gC1qR

Screening of a human lymphocyte-expression library identified the complement receptor gC1qR as a core-binding protein (Kittlesen *et al.*, 2000). The residues required for interaction include amino acids 26-124 of core and 188-259 of gC1qR. Binding of gC1qR to its natural ligand, complement protein C1q, specifically inhibits T-cell proliferation in a dose-dependent manner (Ghebrehiwet *et al.*, 1990). Naked core protein has been shown to circulate in the plasma of HCV-infected patients (Kanto *et al.*, 1994) and be secreted from transfected cell lines (Sabile *et al.*, 1999). Kittlesen *et al.* showed that core protein could specifically inhibit T-cell proliferation in a dose-dependent manner and that inhibition could be blocked by either anti-gC1qR or anti-core antibody (Kittlesen *et al.*, 2000). Inhibition of T-cell proliferation by circulating, naked core protein may be a mechanism by which the virus evades the immune system and establishes persistence.

1.5.6.10 p73

As well as interacting with p53 (section 1.5.6.5) (Lu *et al.*, 1999), core protein has also been shown to bind p73, another member of the p53 superfamily (Alisi *et al.*, 2003). p73 has been reported to transactivate endogenous targets of p53 such as the $p21^{Waf1/Cip1/Sdi1}$ promoter (Jost *et al.*, 1997) and core protein can modulate the transcriptional activity of p73 on the p21 promoter (Alisi *et al.*, 2003). An interaction between core and p73 was identified by co-immunoprecipitation studies in HepG2 cells while deletion studies identified residues 321-353 of p73 as being required for the interaction (Alisi *et al.*, 2003). Core protein is able to inhibit p73 dependent cell growth arrest in HepG2 cells, suggesting an involvement of the core-p73 interaction in the pathogenesis of HCV (Alisi *et al.*, 2003).

1.5.6.11 Sp110b

During a study on the molecular mechanisms behind core modulation of all-*trans*-retinoic acid (ATRA)-induced cell death, Sp110b was identified as a core-interacting protein (Watashi *et al.*, 2003). Residues 21-80 of core and 389-453 of Sp110b are required for this interaction. Within the nucleus, Sp110b acts as a transcriptional corepressor of the retinoic acid response element (RARE), preventing the enhancement of downstream proapoptotic

gene expression (Watashi *et al.*, 2003). Core protein sequesters Sp110b from the nucleus to the cytoplasmic surface of the ER, thus releasing the suppressive function of Sp110b and activating RARα-mediated transcription, leading to ATRA-induced cell death (Watashi *et al.*, 2003).

1.5.6.12 DEAD-box RNA Helicase (DDX3/CAP-Rf/DBX)

Finally, core protein has been shown to bind a DEAD-box RNA helicase termed DBX/DDX3/CAP-Rf (Mamiya and Worman, 1999, Owsianka and Patel, 1999, You *et al.*, 1999). The name DDX3 has been approved by the HUGO/GDB Nomenclature Committee and will therefore be used in the remainder of this thesis. The interaction between core and DDX3 will be discussed in detail in section 1.6.4.

The following section describes the properties and functions of DEAD-box RNA helicases as a family, before reviewing the current literature on DDX3.

1.6 DEAD-box RNA Helicases

1.6.1 General Features

Proteins capable of catalysing the separation of RNA-RNA, RNA-DNA or RNA-protein complexes in an energy-dependent manner are termed RNA helicases. They are ubiquitous proteins, found in all cellular organisms and in many viral genomes (Linder and Daugeron, 2000), and play vital roles in all processes involving RNA such as transcription, splicing, translation, ribosomal biogenesis, RNA transport and RNA turnover (de la Cruz *et al.*, 1999). Despite being termed helicases, *in vitro* helicase activity has only been shown for a few proteins including cellular proteins eIF4A (Rozen *et al.*, 1990), p68 (Hirling *et al.*, 1989), *X. laevis* An3 (Gururajan and Weeks, 1997) and *Drosophila* Vasa (Liang *et al.*, 1994), and viral proteins NPH-II of vaccinia virus (Shuman, 1992) and NS3 of HCV and BVDV (Kim *et al.*, 1995, Jin and Peterson, 1995, Warrener and Collett, 1995). The requirement of co-factors by some helicases for activity (e.g. eIF4A, (Rozen *et al.*, 1990)) may explain why many putative helicases lack demonstrable activity. RNA helicases are classified into 3 superfamilies and 2 families (SF1-SF5), based on characteristics of

conserved motifs in their amino acid sequence (Gorbalenya and Koonin, 1993). DEAD-box RNA helicases belong to SF2 (Caruthers and McKay, 2002) and contain 9 conserved motifs required for ATPase and helicase function (Tanner *et al.*, 2003).

1.6.2 DEAD-box Helicase Motifs

All DEAD-box helicases contain a cluster of 9 conserved motifs spanning a central ~400-residue domain (Fig. 1.10) (Caruthers and McKay, 2002, Tanner *et al.*, 2003). The N- and C-termini however, are highly variable in both sequence and length and are thought to be involved in substrate interaction as well as subcellular localisation (Cordin *et al.*, 2006). The N-terminal most motif of the central domain is termed the Q motif and consists of a 9 amino acid sequence containing an invariant glutamine as well as a conserved aromatic group (usually phenylalanine) 17 residues further upstream (Tanner *et al.*, 2003). Site-directed mutagenesis of either the conserved phenylalanine or conserved glutamine in the essential yeast proteins eIF4A or Ded1 is lethal. Mutants show significantly reduced ATPase activity and RNA binding activity, indicating that the Q motif is involved in control of ATP binding and hydrolysis as well as RNA substrate affinity (Cordin *et al.*, 2004, Tanner *et al.*, 2003). Although highly conserved throughout other RNA and DNA helicase families, the glutamine residue is not essential outwith the DEAD/DEAH subfamilies (Tanner *et al.*, 2003). Motif I, also known as the Walker A motif (Walker *et al.*, 1982), is essential for ATPase and helicase activities. Mutation of a conserved lysine to asparagine within this motif in eIF4A results in vastly reduced ATP binding (Rozen *et al.*, 1989), while mutation of a conserved alanine to valine inhibits ATP hydrolysis and RNA helicase activity, without adverse effects on ATP binding (Blum *et al.*, 1992). Motif II, also known as the Walker B motif (Walker *et al.*, 1982), gives rise to the name DEAD-box and is involved in ATP-binding and/or ATP hydrolysis (Walker *et al.*, 1982, Pause and Sonenberg, 1992). Motif III is thought to link ATPase and helicase activities and mutations in this motif inhibit the helicase activity of eIF4A while not significantly interfering with ATP binding/hydrolysis (Pause and Sonenberg, 1992). Motif IV is the least-well studied DEAD-box protein motif, encompassing between 3 and 8 residues, with no definite function assigned as yet (Cordin *et al.*, 2006). Motif V is proposed to be an RNA binding motif (Caruthers *et al.*, 2000) and motif VI required for RNA binding and ATP hydrolysis (Pause *et al.*, 1993).

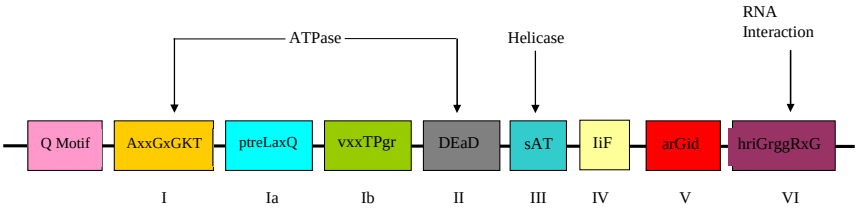


Figure 1.10: Conserved motifs of the DEAD-box family of RNA helicases. The conserved central region contains 9 motifs flanked by diverse N- and C-termini of varying length, thought to be involved in substrate specificity, subcellular localisation and interactions with cellular co-factors (adapted from de la Cruz *et al.*, 1999).

1.6.3 Functional Classification of RNA Helicases

RNA helicases are ubiquitous cellular proteins involved in all aspects of RNA metabolism, including transcription, pre-mRNA splicing, ribosome biogenesis, RNA export, translation and RNA decay (de la Cruz *et al.*, 1999). Below, examples are given of RNA helicases involved in each of these processes.

1.6.3.1 Transcription

Saccaromyces cerevisiae protein POP2, a component of the multi-subunit transcriptional regulator complex Ccr4p, has been shown to physically interact with the putative RNA helicase DHH1 (Hata *et al.*, 1998). Overexpression of DHH1 suppressed phenotypes associated with POP2 mutants, suggesting DHH1 may be part of the Ccr4p transcription complex, while the DEAD-box RNA helicase RHII/Gu has been suggested to be involved in c-jun mediated transcription activation (Westermarck *et al.*, 2002). The N-terminal transcription activation region of c-jun interacts with RHII/Gu and a dominant negative form of RHII/Gu interferes with c-jun mediated transcription (Westermarck *et al.*, 2002).

1.6.3.2 Pre-mRNA Splicing

Before transcribed molecules leave the nucleus, pre-mRNA undergoes a series of processes including 5’ capping, 3’ modification and intron excision. Intron excision, or splicing, is carried out by the spliceosome, a ribonucleoprotein (RNP) composing of a large number of proteins and small nuclear RNAs. The spliceosome is an energy-dependent machine and RNA helicases are thought to be the driving force behind the extensive structural rearrangements that take place during splicing (Staley and Guthrie, 1998). At least 8 RNA helicases are believed to be involved in yeast pre-mRNA splicing including the DEAD-box RNA helicases Prp5p and Prp28p as well as the DexH-box RNA helicase Brr2p (Staley and Guthrie, 1998).

1.6.3.3 Ribosome Biogenesis

Similar to the spliceosome, ribosome biogenesis involves a large complex of proteins as well as rRNAs. In yeast this number is approximately 80 proteins and 4 ribosomal (r) RNAs (de la Cruz *et al.*, 1999). A number of putative RNA helicases, essential for yeast viability, have been suggested to be involved in ribosome biogenesis. It is possible that these RNA helicases may be required to establish and/or dissociate small nucleolar RNAs (snoRNA)-pre-RNA base pairs, facilitate the activities of endo- and exo-nucleases or recruit, rearrange or dissociate *trans*-acting factors during processing (de la Cruz *et al.*, 1999).

1.6.3.4 RNA Export

Upon processing, mRNA (in the form of RNPs) is transported from the nucleus to the cytoplasm via the nuclear pore complex. It is plausible that RNA helicases are required at this stage to alter the conformation and/or composition of these RNPs and allow transit through the nuclear pore complex or to dissociate mRNA from protein once in the cytoplasm. The RNA helicase Dbp5p/Rat8p, which accumulates around the nuclear envelope, has been reported to be involved in mRNA export. Poly(A)$^+$ RNA was shown to accumulate in the nucleus in the absence of functional Dbp5p/Rat8p (Tseng *et al.*, 1998, Snay-Hodge *et al.*, 1998). More recently, the DEAD-box helicase DDX3 has been shown to be required for nuclear export of incompletely spliced HIV RNA transcripts via the CRM1 export pathway (Yedavalli *et al.*, 2004) (See section 1.6.4).

1.6.3.5 Translation

Translation of eukaryotic mRNA involves recruitment of the 40S ribosome subunit to the 5’ end and involves initiation factors (eIFs). Upon recruitment, the 40S subunit scans the mRNA for the correct initiation codon before recruitment of the 60S ribosome subunit and initiation of translation. eIF4A is a DEAD-box RNA helicase that is, along with other eIFs, required for 40S ribosome binding. eIF4A has ATP-dependent helicase activity (Rozen *et al.*, 1990), suggesting its function may be in unwinding secondary structures in the 5’UTR of mRNA. *Saccaromyces cerevisiae* RNA helicase Ded1p has also been reported to be

involved in translation. Conditional Ded1p mutants showed severely impaired translation with evidence suggesting initiation of translation was affected, indicating a similar role to that of eIF4A (Chuang *et al.*, 1997).

1.6.3.6 RNA Decay

Degradation of defective RNA molecules occurs rapidly in *Saccaromyces cerevisiae* by both 5'→3' and 3'→5' exonucleolytic processing. The RNA helicase Ski2p is a component of the 3'→5' exosome complex (Anderson and Parker, 1998), indicating involvement in RNA degradation. In an alternative degradation pathway, eukaryotic mRNA containing premature stop codons is subjected to decay via the nonsense mediated decay (NMD) pathway. Identification of molecules containing premature stop codons requires a surveillance complex, a component of which is the RNA helicase Upf1p (Mitchell and Tollervey, 2003), highlighting another RNA decay associated RNA helicase.

1.6.4 DDX3

DDX3 is a ubiquitous cellular protein belonging to the DEAD-box family of RNA helicases, possessing ATPase and helicase activities (Yedavalli *et al.*, 2004). Known homologues include mouse PL10 (Leroy *et al.*, 1989), mDEAD3 (Gee and Conboy, 1994), *Xenopus* An3 (Gururajan *et al.*, 1991) and yeast Ded1 (Jamieson and Beggs, 1991). The exact cellular function of DDX3 has yet to be identified however it has been suggested to be involved in splicing (Deckert *et al.*, 2006, Zhou *et al.*, 2002), translation initiation (Mamiya and Worman, 1999), cell cycle regulation (Chang *et al.*, 2006, Chao *et al.*, 2006), nucleo-cytoplasmic RNA shuttling (Yedavalli *et al.*, 2004) and RNA transport (Kanai *et al.*, 2004). An accumulation of data (Scott, 2002, Owsianka and Patel, 1999, Mamiya and Worman, 1999), suggest that DDX3 is primarily a cytoplasmic protein although some forms are detected in the nucleus. An involvement in splicing has been indicated by its presence in highly purified and functional human spliceosomes and in spliceosomal B complexes which undergo catalytic activation leading to catalysis of pre-mRNA splicing (Zhou *et al.*, 2002, Deckert *et al.*, 2006). DDX3 also possesses an C-terminal arginine / serine rich RS-domain consisting of 7 serine-arginine or arginine-serine dipeptides, similar to that of other splicing factors (Owsianka and Patel, 1999). Related to splicing, DDX3 has

also been found in affinity-purified, spliced messenger-ribonucleoproteins (mRNPs) (Merz *et al.*, 2006). DDX3 was present in mRNPs after treatment with heparin, suggesting a stable association. This association was also dependent on splicing, as, under the same physiological conditions, DDX3 did not associate with intron-less, m^7G-capped mRNAs (Merz *et al.*, 2006). An association with kinesin RNA-transport granules has also been reported, however knockdown of DDX3 by RNAi did not affect RNA transport (Kanai *et al.*, 2004). DDX3 has also been shown to be involved in cell cycle regulation and may have a role in tumourigenesis. DDX3 was overexpressed in liver cancer cell lines showing anchorage independent growth (AIG) (Huang *et al.*, 2004). Overexpression of DDX3 in AIG-negative Tong cells led to a 60-80-fold increase in transformed colonies in AIG assays. In contrast, 2 recent reports have indicated DDX3 may be down-regulated in liver tumour cells and may affect cell cycle progression via regulation of $p21^{waf}$ gene expression. Firstly, an expression profile of DDX3 in liver tumour tissue and adjacent non-tumour parts indicated a decrease in DDX3 expression in 59% of cases in tumour compared to non-tumour tissue (Chang *et al.*, 2006). DDX3 knockdown in NIH3T3 cells resulted in an accelerated proliferation rate. Interestingly, cyclin D_1, a key regulator in early-mid G_1 phase, was expressed at higher levels (and at earlier time points) while the cyclin-dependent kinase (cdk) inhibitor $p21^{waf}$ was expressed at lower levels at all time points (Chang *et al.*, 2006). Similarly, DDX3 was shown to exert an inhibitory effect on cell growth in Huh-7 and NIH3T3 cells (Chao *et al.*, 2006). Overexpression of DDX3 inhibited colony formation and slowed growth rates. Overexpression also led to a 2-4-fold upregulation in $p21^{waf}$-promoter driven luciferase expression and endogenous $p21^{waf}$ expression, which required the ATPase activity of DDX3 but not the helicase activity (Chao *et al.*, 2006). Similar to the report by Chang *et al.*, DDX3 expression was lower in liver tumour specimens compared to non-tumour specimens (Chao *et al.*, 2006). These two reports provide firm evidence for a role for DDX3 in cell cycle progression and tumourigenicity via regulation of $p21^{waf}$ gene expression.

DDX3 also functions in the life cycle of human immunodeficiency virus (HIV)-1 (Yedavalli *et al.*, 2004). HIV-1 gene expression is regulated post-transcriptionally by HIV-1 Rev-mediated export of Rev response element (RRE)-containing un-spliced/partially spliced viral RNAs from the nucleus to the cytoplasm (Cullen, 2003). In doing so, Rev interacts with chromosome maintenance region 1 (CRM1) (Bogerd *et al.*, 1998), which is involved in nucleo-cytoplasmic shuttling of proteins, small nuclear RNAs and rRNAs (Fornerod *et al.*, 1997). DDX3 was identified as an HIV-1 Tat-induced cellular protein, with further analysis linking it to the Rev-RRE/CRM1 export pathway (Yedavalli *et al.*, 2004). This report showed that DDX3 possesses ATPase-dependent RNA unwinding

properties and that over-expression of DDX3 resulted in an increase in Rev-dependent expression of an RRE reporter plasmid while knockdown of DDX3 by antisense DDX3 resulted in reduced expression of un-spliced RNA encoded p24 and p55 Gag proteins. Co-immunoprecipitation studies showed interaction between DDX3 and Rev as well as DDX3 and CRM1, while inhibition of CRM1 export by leptomycin B resulted in a build up of DDX3 in the nucleus. As the ATPase-dependent helicase activity of DDX3 is critical for Rev-RRE/CRM1 mediated HIV RNA export, it is possible that DDX3 is required for unwinding of viral RNA/protein complexes to allow passage through the nuclear pore (Yedavalli *et al.*, 2004).

The interaction between HCV core and DDX3 was discovered using yeast-2-hybrid screening and confirmed by both *in vitro* binding studies and immunofluorescence analysis. The interaction domains were mapped by deletion analysis to be within the N-terminal 59 residues of core protein (You *et al.*, 1999, Owsianka and Patel, 1999) although interaction between this deletion mutant and DDX3 was weaker than binding using core residues 1-114, indicating that residues 59-114 may also contribute to the interaction (Owsianka and Patel, 1999). The core-binding domain of DDX3 was mapped to the C-terminal 409-622 residues (Owsianka and Patel, 1999, You *et al.*, 1999, Mamiya and Worman, 1999). The minimal region of DDX3 required for interaction (residues 553-622) includes almost all of a 50 amino acid domain rich in arginines and serines (residues 582-632) which resembles the RS domains of splicing factors such as ASF/SF2 and SC-35. Although both core and DDX3 are expected to bind RNA, this was not a requirement for the interaction (Owsianka and Patel, 1999). In HeLa cells, DDX3 has a diffuse cytoplasmic distribution, although nuclear staining was seen with some anti-DDX3 antibodies (Mamiya and Worman, 1999, Owsianka and Patel, 1999, You *et al.*, 1999). In cells expressing either core alone, or together with E1 and E2, the distribution of DDX3 is altered from a diffuse cytoplasmic localisation to discrete cytoplasmic foci on lipid droplets where it colocalises with HCV core protein. Nuclear DDX3 was not re-distributed in the presence of core protein (Owsianka and Patel, 1999).

Although the interaction between HCV core and DDX3 is well established, the functional relevance of the interaction has still to be confirmed. DDX3 has been suggested to be involved in translation and that interaction with core protein may inhibit translation of capped mRNA (Mamiya and Worman, 1999). Mouse PL10 and DDX3 were able to rescue the lethality of cells with chromosomal deletion of the DDX3 yeast homologue Ded1, suggesting DDX3 can replace the function of Ded1. Expression of full-length core protein severely inhibited growth of DDX3/PL10 complemented (but not Ded1 complemented)

Ded1 deletion yeast, consistent with the fact that core binds DDX3 and PL10 but not Ded1. Core protein also inhibited *in vitro* translation of luciferase from capped but not un-capped RNA, suggesting it may inhibit translation of capped mRNA, possibly by inhibiting DDX3 function (Mamiya and Worman, 1999). It has also been suggested that DDX3 is involved in gene expression, however, in contrast to the findings of Mamiya and Worman (1999), it was reported that gene expression was enhanced in the presence of HCV core protein (You *et al.*, 1999). Exogenous DDX3 enhanced luciferase reporter expression 1.5-3-fold in Huh7 cells, while co-transfection with HCV core protein increased luciferase reporter expression 5.5-34-fold. Core protein also enhanced DDX3 ATPase activity 4-5-fold, suggesting that core may modulate the activity of DDX3, thus affecting the function of DDX3 in gene expression (You *et al.*, 1999).

As yet, the exact function of DDX3 and its role in the life cycle of HCV is not known. It is possible that core protein recruits DDX3 from its normal cellular function for use in the viral life cycle, for example during replication or packaging of progeny RNA. Alternatively, core may be sequestering DDX3 in order to prevent it from carrying out its normal cellular function, possibly to reduce protein synthesis or affect cell cycle regulation.

1.7 Aims

The aim of this project is to identify critical residues of HCV core protein required for interaction with cellular DDX3. Mutation of these critical residues and analysis of their effects in the infectious JFH-1 cell culture system will allow study of the role of DDX3 in HCV replication, translation and production of progeny virus. It is also hoped that mutational abrogation of core-DDX3 interaction in HCV-infected cells may provide clues and future avenues for research on the possible role of this interaction in disrupting critical cellular processes which may lead to some of the pathologies associated with HCV infection.

2. Materials and Methods

2.1 Materials

2.1.1 Chemical and Additional Reagent Suppliers

Most analytical grade chemicals and reagents were supplied by Sigma Aldrich Ltd or BDH Laboratory Supplies. The remainder are listed below:

Chemical / Reagent	**Supplier**
30% Acrylamide / bis solution 37.5:1	Bio-Rad laboratories
Absolute ethanol	Bamford Laboratories, UK
Agarose	Melford
Ampicillin (Penbritin)	Beecham Research
Ammonium persulphate (APS)	Bio-Rad Laboratories
Chloroform	Prolabo
Citifluor™ AF1 (Glycerol:PBS)	UKC Chemical Laboratories
Coomassie Brilliant Blue	BioRad
Coverslips (13 mm diameter)	VWR International
Developer and fixer	Kodak X-OMAT
DNA miniprep kit	Qiagen
dNTPs	Amersham Biosciences
Dried skimmed milk	Marvel
Ethanol	Fisher Scientific
Electroporation cuvettes (1mm)	Apollo
Hybond ECL Nitrocellulose Membrane	Amersham Biosciences
Immunolon II ELISA plates	Dynal, UK
IPTG	Invitrogen
Isopropanol	Prolabo
Lambda DNA	Promega
Luminol	Fluka
Methanol	Prolabo
Phosphoimager screens	BioRad
Photographic film (S-film)	Kodak X-OMAT
Pipette tips (Rnase free)	Molecular Bio Products
QIAquick gel purification kit	Qiagen
QIAquick nucleotide purification kit	Qiagen
Rainbow protein markers	Amersham Biosciences
TEMED	Bio-Rad Laboratories
TMB substrate	Zymed Laboratories Inc.
Tris	Roche
TRIzol	Invitrogen
Tween-20	Bio-Rad Laboratories
Whatman 3mm filter paper	Whatman

2.1.2 Enzyme Suppliers

Restriction endonuclease enzymes and buffers were supplied by New England Biolabs (NEB) and Promega. Other enzymes and their suppliers are shown below:

Enzyme	Supplier
Calf intestinal phosphatase	New England Biolabs
Ribonuclease A	Sigma
T4 DNA ligase	Invitrogen
Taq DNA polymerase	Roche
KOD Hotstart DNA polymerase	Novagen
Expand High Fidelity PCR System	Roche
Mung Bean nuclease	New England Biolabs
Proteinase K	New England Biolabs
M-MLV RT (including First-strand buffer, DTT, RNase-OUT)	Invitrogen

2.1.3 Immunological Reagent Suppliers

Monoclonal antibodies (mAbs) and polyclonal antisera (pAbs) used in this study are shown below:

2.1.3.1 Primary antibodies

Antibody	Name	Type	Raised in	Source
HCV core	R308	Polyclonal antiserum	Rabbit	Hope & McLauchlan, (2000)
HCV E2	AP33	Monoclonal antibody	Mouse	Clayton *et al.*, (2002)
HCV NS5a	NS5a	Polyclonal antiserum	Sheep	MacDanoald *et al.*, (2003)
DDX3	AO196	Monoclonal antibody	Mouse	A.H. Patel, unpublished
DDX3	R648	Polyclonal antiserum	Rabbit	A.H. Patel, unpublished
ADRP	ADRP 4	Polyclonal antiserum	Sheep	Target-Adams *et al.*, (2003)
GFP	GFP	Polyclonal antiserum	Rabbit	Abcam

2.1.3.2 Secondary antibodies

Antibody	Source
FITC-conjugated donkey anti-rabbit IgG	Jackson ImmunoResearch Laboratories Inc.
TRITC-conjugated donkey anti-sheep IgG	Jackson ImmunoResearch Laboratories Inc.
Cy5-conjugated donkey anti-mouse IgG	Jackson ImmunoResearch Laboratories Inc.
FITC-conjugated donkey anti-sheep IgG	Molecular Probes
Protein A-HRP (Whole molecule)	Sigma

2.1.4 Cells

Laboratory stocks of human hepatoma cell line Huh-7 (Nakabayashi *et al.*, 1982) were used in this study.

2.1.5 Cell Culture Supplier

Phosphate buffered saline (PBS), versene, trypsin, and L-broth were all made in-house by the media department using standard recipes, while Dulbecco's Modified Eagle's medium (DMEM), Foetal calf serum (FCS), Penicillin, Streptomycin, Glutamine, Non-essential amino acids (NEAA) and OptiMEM were supplied by GIBCO-BRL^{TM} Life Technologies, Invitrogen.

2.1.6 Plasmid constructs

Plasmid constructs used in this study are shown below.

Name	Vector	Details	Source
pGFP-SpAb	pKK223-3	Domain B of Staphylococcal protein $A^{194-271}$ fused to the 3'end of GFP.	Aoki *et al.*, (2002)
pGFP-$Core_{1-59}$	pKK223-3	HCV (H77c strain) core residues 1-59 fused to the 3' end of GFP.	D.A. Dalrymple, unpublished
pGST-DDX3C	pGEX-2T	C-terminal 253 amino acids (409-662) of DDX3 fused to the 3'end of GST.	Owsianka & Patel, (1999)
pC-E1-E2	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2.	A.H. Patel, unpublished
pC-E1-E2 mut25	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutations R9K, V34G, Q57R	D.A. Dalrymple, unpublished
pC-E1-E2 mut36	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutations V34D, R40W, T52S	D.A. Dalrymple, unpublished
pC-E1-E2 mut90	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutation I30N	D.A. Dalrymple, unpublished
pC-E1-E2 mut99	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutations V22D, V34D	D.A. Dalrymple, unpublished
pC-E1-E2 mut110	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutations I30N, R43S	D.A. Dalrymple, unpublished
pC-E1-E2 mut111	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutations K6E,	D.A. Dalrymple, unpublished

		Y35N	
pC-E1-E2 mut115	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutations K10D, K12E, F24S	D.A. Dalrymple, unpublished
pC-E1-E2 mut125	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutations N16I, Q29R, Y35N, Q57R	D.A. Dalrymple, unpublished
pC-E1-E2 mut126	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutations Q8H, G26D, I30N, L36W	D.A. Dalrymple, unpublished
pC-E1-E2 mut25b	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutation V34G	D.A. Dalrymple, unpublished
pC-E1-E2 mut36b	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutation V34D	D.A. Dalrymple, unpublished
pC-E1-E2 mut99b	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutation V34D	D.A. Dalrymple, unpublished
pC-E1-E2 mut110b	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutation I30N	D.A. Dalrymple, unpublished
pC-E1-E2 mut111b	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutation Y35N	D.A. Dalrymple, unpublished
pC-E1-E2 mut125b	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutations Q29R, Y35N	D.A. Dalrymple, unpublished
pC-E1-E2 mut126b	pcDNA3.1 (+)Zeo	HCV (H77c strain) sequence encoding core, E1 and E2. Core contains mutations G26D, I30N, L36W	D.A. Dalrymple, unpublished
pC-E1-E2 F24A	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2. Core contains mutation F24A	D.A. Dalrymple, unpublished
pC-E1-E2 P25A	pcDNA3.1	HCV (JFH-1 strain) sequence encoding core,	D.A. Dalrymple,

	(+)Zeo	E1 and E2. Core contains mutation P25A	unpublished
pC-E1-E2 G26A	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2. Core contains mutation G26A	D.A. Dalrymple, unpublished
pC-E1-E2 G27A	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2. Core contains mutation G27A	D.A. Dalrymple, unpublished
pC-E1-E2 G28A	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2. Core contains mutation G28A	D.A. Dalrymple, unpublished
pC-E1-E2 Q29A	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2. Core contains mutation Q29A	D.A. Dalrymple, unpublished
pC-E1-E2 I30A	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2. Core contains mutation I30A	D.A. Dalrymple, unpublished
pC-E1-E2 V31A	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2. Core contains mutation V31A	D.A. Dalrymple, unpublished
pC-E1-E2 G32A	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2. Core contains mutation G32A	D.A. Dalrymple, unpublished
pC-E1-E2 G33A	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2. Core contains mutation G33A	D.A. Dalrymple, unpublished
pC-E1-E2 V34A	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2. Core contains mutation V34A	D.A. Dalrymple, unpublished
pC-E1-E2 Y35A	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2. Core contains mutation Y35A	D.A. Dalrymple, unpublished
pC-E1-E2 L36A	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2. Core contains mutation L36A	D.A. Dalrymple, unpublished
pC-E1-E2 JFH-1	pcDNA3.1 (+)Zeo	HCV (JFH-1 strain) sequence encoding core, E1 and E2.	D.A. Dalrymple, unpublished
pJFH-1	pUC	Full length JFH-1 cDNA downstream of the T7 RNA polymerase promoter.	Wakita *et al.*, (2005)

pJFH-1 GND	pUC	Full length JFH-1 cDNA carrying a mutation in the NS5B GDD motif, downstream of the T7 RNA polymerase promoter.	Wakita *et al.*, (2005)
pJFH-1 (1-2837)	pGEM T Easy	HCV (JFH-1 strain) nucleotides 1-2837	D.A. Dalrymple, unpublished
pJFH-1 F24A	pUC	Full length JFH-1 cDNA carrying a mutation in core (F24A), downstream of the T7 RNA polymerase promoter.	Wakita *et al.*, (2005)
pJFH-1 G27A	pUC	Full length JFH-1 cDNA carrying a mutation in core (G27A), downstream of the T7 RNA polymerase promoter.	Wakita *et al.*, (2005)
pJFH-1 I30A	pUC	Full length JFH-1 cDNA carrying a mutation in core (I30A), downstream of the T7 RNA polymerase promoter.	Wakita *et al.*, (2005)
pJFH-1 G33A	pUC	Full length JFH-1 cDNA carrying a mutation in core (G33A), downstream of the T7 RNA polymerase promoter.	Wakita *et al.*, (2005)
pJFH-1 V34A	pUC	Full length JFH-1 cDNA carrying a mutation in core (V34A), downstream of the T7 RNA polymerase promoter.	Wakita *et al.*, (2005)
pJFH-1 Y35A	pUC	Full length JFH-1 cDNA carrying a mutation in core (Y35A), downstream of the T7 RNA polymerase promoter.	Wakita *et al.*, (2005)

2.1.7 Bacterial Strains

Plasmids were manipulated and grown in the *Escherichia coli* (*E.coli*) strain JM109 (Stratagene), (genotype *e14-(McrA-) recA1 endA1 gyrA96 thi-1 hsdR17*(rK- mK+) *supE44 relA1 Δ(lac-proAB)* [F' *traD36 proAB lacI^{q}ZΔM15]).*

2.1.8 Solutions

Standard solutions used in this study are shown below:

Solutions	**Contents**
Alkaline lysis buffer for plasmid purification	0.2 M NaOH , 1 % SDS
Neutralisation buffer for plasmid purification	3 M potassium acetate 11.5 % glacial acetic acid
Cell lysis buffer	20 mM Tris pH 7.5 , 150 mM NaCl , 1 mM EDTA , 1 % NP40
Coomassie stain	0.2 % Coomassie brilliant blue dye R200, 50 % methanol, 7 % acetic acid
Destain	5 % (v/v) methanol, 7 % (v/v) acetic acid (in H_20)
DNA loading dye	30 % glycerol, 0.25 % bromophenol blue
Electrochemiluminescence I	1 % luminol, 0.44 % coumaric acid, 100 mM Tris-HCl pH 8.5
Electrochemiluminescence II	30 % H_2O_2 , 100 mM Tris-HCl pH 8.5
LB broth agar	L-broth, 15 % (w/v) agar
Luria-Bertani (LB) broth	1 % NaCl, 1 % Bactopeptone, 0.5 % yeast extract
PBS	PBSA, 68 mM $CaCl_2 2H_2O$, 4 mM $MgCl_2 6H_2O$
PBSA	170 mM NaCl, 34 mM KCl, 10 mM Na_2HPO_4, 18 mM KH_2PO_4, 25 mM Tris-HCl pH 7.2
PBST	PBSA, 0.05 % Tween-20
Protease inhibitors	20 mM AEBSF, 100 mM Benzamidine, 50 mM EDTA, 100 μg/ml Apronitin, 100 μg/ml Leupeptine
SDS-PAGE resolving buffer	0.5 M Tris-HCl pH 8.9, 0.4 % SDS
SDS-PAGE running buffer	40 mM Tris, 185 mM Glycine, 0.1 % SDS
SDS-PAGE sample loading buffer	100 mM Tris-HCl pH 6.9, 2 % SDS, 10 % glycerol, 5 % β mercaptoethanol, 1 μg/ml bromophenol blue
Stacking gel buffer	0.5 M Tris-HCl pH 6.9, 0.4 % SDS
Trypsin solution	0.25 % Difco trypsin dissolved in PBS(A), 0.002 % phenol red

Versene	0.6 mM EDTA in PBS(A), 0.002 % phenol red
10x TBE	8.9 M Tris-borate, 8.9 M boric acid, 0.02 M EDTA (pH 8.0)

2.2 Methods

2.2.1 Tissue Culture

2.2.1.1 Serial Passage of Cells

Huh-7 cells were propagated at 37°C in Dulbeccos Modified Eagle's Medium (DMEM) containing 10 % FCS, 1 % non-essential amino acids (NEAA) and 100 units / ml penicillin / streptomycin. Passage of cells was carried out when cells reached 90 % confluency using trypsin (1:100). Cells were seeded in new flasks using the appropriate media and incubated in a humidified CO_2 incubator at 37°C.

2.2.1.2 Long Term Storage of Cells

Aliquoted cells were stored in DMEM containing 25 % FCS and 10 % DMSO. Aliquots were left overnight at -70°C before being transferred to -180°C for long-term storage.

2.2.1.3 Transfection of Cells with DNA

DNA was transfected into cells using Genejuice transfection reagent (Novagen), according to the manufacturers instructions.

2.2.2 Preparation, Manipulation and Analysis of DNA

2.2.2.1 Extraction of Plasmid DNA by Alkaline Lysis

A single bacterial colony was inoculated in 5 ml LB broth containing ampicillin (final concentration 100 μg/ml). The culture was grown in a shaker (200 rpm) at 37°C for 16 hrs. 200 μl of culture was added to 200 μl of alkaline lysis buffer and mixed gently. Neutralisation buffer (200 μl) was then added and the solution was again mixed gently. Samples were then centrifuged at 18,000 g for 2 minutes and the supernatant decanted into 500 μl isopropanol. DNA was pelleted at 18,000 g for 1 minute and the supernatant was removed. The pellet was resuspended in 30 μl H_2O (+ ribonuclease A, final concentration 16 μg/ml).

2.2.2.2 Oligonucleotide Synthesis

Oligonucleotides were ordered from Sigma-Genosys.

2.2.2.3 Quantitation of DNA

DNA (2 μl) was added to 48 μl dH_2O and the optical density (O.D.) measured using a BioPhotometer (Eppendorf).

2.2.2.4 Restriction Enzyme Digestion of DNA

All restriction enzyme digests of plasmid DNA were carried out at 37°C for at least 1 hr unless otherwise specified by the manufacturer. Ten units of each enzyme per μg DNA was used along with enzyme buffer in a total volume of 50 μl. Calf intestinal phosphatase (CIP) enzyme (10 units / μg DNA) was added to vector digestions to remove 5' phosphates from the vector DNA, preventing vector self-ligation.

2.2.2.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to resolve DNA fragments produced by restriction enzyme digestion or PCR. Slab gels containing 1 % agarose were prepared in 0.5 % TBE containing ethidium bromide (1 μg/ml). DNA samples were mixed with 0.1 volumes of 10x agarose gel loading buffer before being loaded into the wells of the gel. Gels were run at 100 V in 0.5x TBE buffer. On completion of electrophoresis, DNA was visualised using either short-wave UV light or long-wave UV light for preparative gels. A BioRad Gel Doc 2000 imager using Quantity One software was used for analysis.

2.2.2.6 Bacteriophage Lambda DNA Markers

Digestion of bacteriophage lambda DNA (40 μg) with *Eco*RI and *Hind*III and the appropriate buffer was carried out at 37°C for 2 hrs in a total volume of 400 μl. The reaction was then mixed with 1.4 ml dH_2O and 200 μl 10x DNA loading dye.

2.2.2.7 DNA Purification from Agarose Gels

DNA fragments were excised from agarose gels under long-wave UV light using a sterile scalpel. DNA was recovered from the gel using the Qiagen gel extraction kit, following the manufacturers instructions.

2.2.2.8 Ligation of DNA Fragments

Using the appropriate ligation buffer, DNA fragments were mixed with T4 DNA ligase and incubated overnight at 16°C. Following ligation, DNA was precipitated by adding 0.1 volumes 3 M sodium acetate (pH 5.1) and 400 μl absolute ethanol and incubated at -20°C for 2 hrs. Ligated DNA was pelleted at 18,000 g for 15 mins, washed with 200 μl 70% ethanol, air dried and resuspended in 5 μl dH_2O.

2.2.2.9 Production of Electrocompetent Cells

LB-broth (400 ml) was inoculated with 5 ml of fresh overnight *E. coli* culture and grown at 37°C to an OD_{600} of 0.5-0.8. The flask was chilled on ice for 30 mins and centrifuged at 4,000 g for 5 mins at 4°C. Supernatant was removed and the pellet resuspended in 500 ml ice-cold dH_2O. Cells were re-centrifuged as before and resuspended in 250 ml ice-cold dH_2O. Again cells were centrifuged as before and resuspended in 10 ml ice-cold dH_2O (10% glycerol). Finally, cells were centrifuged as before and resuspended in 1 ml ice cold dH_2O (10% glycerol) before being aliquoted into 70 µl aliquotes and stored for up to 6 months at -70°C.

2.2.2.10 Transformation of Electrocompetent *E.coli* Cells

Ligated DNA was ethanol-precipitated and the pellet resuspended in 5 µl dH_2O, 2 µl of which was added to 70 µl electrocompetent *E.Coli* in a pre-chilled 1 mm cuvette and electroporated (1.8 kV, 25 µF, 200 Ω) using a BioRad GenePulser Xcell. The *E.coli* were then resuspended in 1 ml L-broth and incubated at 37°C for 1 hr before being plated on LB-agar plates (+ ampicillin, final concentration 100 µg/ml). Plates were then incubated overnight at 37°C.

2.2.2.11 Storage of bacterial stocks

Bacterial cultures were mixed with glycerol to a final concentration of 15 % and stored at -70°C.

2.2.2.12 Sequencing

Nucleotide sequencing was carried out in-house, using an ABI 377 DNA sequencer. Sequences were analysed using Chromas (ABI) and Vector NTI (Invitrogen) software.

2.2.2.13 PCR Amplification of DNA

PCR (Mullis *et al.*, 1986) was carried out using a GeneAmp PCR machine (Applied Biosystems). Briefly, cDNA or plasmid DNA was amplified using relevant primers (see appendix) in the presence of dNTPs, DNA polymerase and appropriate buffer according to the manufacturer's instructions. Cycle conditions were as follows -

Denaturation	94ºC	3 min	
Denaturation	94ºC	45 sec	35 cycles
Annealing	66ºC	1 min	
Elongation	72ºC	3 min	
Termination	72ºC	10 min	

2.2.2.14 Site-Directed Mutagenesis

Site-directed mutagenesis was carried out using Quikchange (Stratagene) according to the manufacturer's instructions.

2.2.3 RNA Electroporation

2.2.3.1 Plasmid Linearisation for *In Vitro* Transcription

Plasmid pJFH-1 (or pJFH-1 GND) was linearised by *Xba I* digestion and treated with Mung Bean nuclease to digest overhangs (30°C for 30 mins) before adding proteinase K (final concentration 100 μg/ml) and SDS (final concentration 0.5 %) to clean template of proteins and incubating at 50°C for 30 mins. Neutral phenol-chloroform (100 μl) was added and the sample centrifuged (18,000 g for 2 mins). The aqueous (top) layer was placed in a fresh centrifuge tube and 0.1 volumes 5 M NH_4OAc added along with 3 volumes 100% ethanol. The sample was stored at -20°C for 30 mins before being centrifuged at 18,000 g for 15 mins and the pellet resuspended in 30 μl dH_2O.

2.2.3.2 *In Vitro* Transcription

In Vitro transcription was carried out using a T7 Megascript kit (Ambion) following the manufacturer's instructions.

2.2.3.3 Cell Electroporation

RNA (5 μg) was electroporated into $1x10^7$ cells using a BioRad GenePulser Xcell (250 V, 950 μF), following the manufacturers instructions. Cells were then diluted in the appropriate media and plated out.

2.2.3.4 Preparation of Total RNA

Culture dishes (35 mm) containing electroporated cells were washed with PBSA then lysed using 750 μl TRIzol LS. Chloroform (200 μl) was added and the sample shaken vigorously for 15 seconds before being incubated at RT for 15 mins. Samples were then centrifuged at 18,000 g for 15 mins and the aqueous (upper) phase added to 500 μl isopropanol. This was incubated at RT for 10 mins before being centrifuged at 18,000 g for 15 mins. After decanting, the pellet was washed in 75% EtOH, mixed by vortexing, and centrifuged at 18,000 g for 10 mins. The pellet was then dried at RT and resuspended in 16 μl of "The RNA Storage Solution" (Ambion). RNA was stored at -70°C.

2.2.3.5 First-Strand cDNA Synthesis

The following reagents were added to a nuclease-free centrifuge tube – 1 μl gene specific primer (2 pmole / μl), 5 μl RNA, 1 μl 10 mM dNTPs and made up to 12 μl with sterile dH_2O. The mixture was heated to 65°C for 5 mins and quickly chilled on ice (1 min). Upon brief centrifugation, the following were added – 4 μl 5x First-strand buffer, 2 μl 0.1 M DTT, 1 μl Rnase-OUT. The contents were mixed and incubated at 37°C for 2 mins. Moloney Murine Leukemia virus reverse transcriptase (M-MLV RT, 200 units) was then

added and mixed gently before incubation at 37°C for 50 mins. The reaction was inactivated at 70°C for 15 mins. PCR was then carried out as in section 2.2.2.13.

2.2.3.6 Real-Time PCR

Total RNA (1 µl) was reverse transcribed using random hexamers and reverse transcription kit (both Applied Biosystems) according to the manufacturers instructions (10 min at 25ºC, 60 min at 37ºC, 5 min at 95ºC then cooled to 4ºC) with the reaction composition as follows:

10 x Taqman buffer	4 µl
25 mM $MgCl_2$	8.8 µl
10 mM dNTPs	8 µl
Random hexamers	2 µl
Rnase inhibitor	0.8 µl
Multiscribe RT	1 µl
H_2O	14.4 µl
RNA	1 µl

Resultant cDNA was amplified using both HCV-specific (see appendix) and GAPDH-specific primers (Applied Biosystems) in the presence of FAM™ (HCV-specific) and VIC® (GAPDH-specific) labelled probes, using Applied Biosystem's Fast Universal Mastermix. Real-Time PCR was carried out using Applied Biosystems 7500 Fast Real-Time PCR System using Fast Universal PCR conditions (95ºC for 20 seconds followed by 40 cycles of 95ºC for 3 seconds and 60ºC for 30 seconds) and data analysed using Applied Biosystems software (SDS version 1.3.1), according to the manufacturers instructions.

2.2.4 Protein Analysis

2.2.4.1 Crude Protein Expression in Bacteria

A single bacterial colony was inoculated in 5 ml L-broth (+ ampicillin, final concentration 100 µg/ml) and incubated at 37°C overnight. The next day 1.5 ml of L-broth was inoculated with overnight culture (1:50). The culture was grown at 37°C for 2.5 hrs before IPTG was added to a final concentration of 0.3 mM. Cultures were incubated for a further

16 hrs at 37°C before being spun (4,000 g for 2 mins) and the pellet resuspended in 250 µl PBS. Samples were freeze-thawed, sonicated and centrifuged (18,000 g for 15 mins) and the supernatant (soluble protein) was then subjected to SDS-PAGE or used in ELISA.

2.2.4.2 Purification of GST-tagged Proteins

L-broth (400 ml) (+ ampicillin, final concentration 100 µg/ml) was inoculated with overnight bacterial culture (1:50) and incubated at 37°C for 3 hrs. IPTG was then added (final concentration 0.1 mM) and the culture was incubated for a further 4 hrs at 37°C. The culture was then centrifuged (4,000 g for 5 mins) and the pellet resuspended in 20 ml PBSA (+ protease inhibitors 1:100). Samples were freeze-thawed twice then sonicated before 10 % Triton-100 was added (final concentration 1 %). This was then incubated at RT for 30 mins before being centrifuged (18,000 g for 10 mins). One millilitre of slurry (equilibrated in PBS) of 50 % glutathione-agarose beads was incubated with the supernatant at RT for 2 hrs before being centrifuged (100 g for 1 min). Supernatant was stored for analysis and the pellet was washed three times in PBS with supernatant being collected for analysis after each wash. Bound protein was then eluted using PBS containing 15 mM reduced glutathione. A fraction of each sample was run on a polyacrylamide gel followed by coomassie staining. Clean samples containing the desired protein were then assayed for protein concentration and used in ELISA.

2.2.4.3 Denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sample loading buffer was added to protein samples (1:1 v/v) before being denatured by boiling. Resolving gels (10 %) were used to analyse proteins [resolving gel buffer (1.5 ml), dH_20 (2.5 ml), 30% Acrylamide / bis solution 37.5:1 (2 ml), 25 % w/v APS (20 µl) and temed (15 µl)]. Gels were poured and levelled using 1 ml dH_20. When set, gels were overlayed with 4 % stacking gel [stacking gel buffer (1 ml), dH_20 (2.5 ml), 30% Acrylamide / bis solution 37.5:1 (0.532 ml), 25 % APS (20 µl) and temed (15 µl)] before placing a comb in the overlay. Gels were placed in one times running buffer and samples loaded into wells. Gels were run for 3 hrs at 80 V or until the loading dye ran off the bottom of the gel. Samples were run using a Mini Protean II gel kit (BioRad) according to

the manufacturer's instructions. Gels were then either western blotted or stained with coomassie stain.

2.2.4.4 Western Immuno-blotting of Polyacrylamide Gels

Proteins separated on polyacrylamide gels were transferred to Hybond^{TM}-ECL^{TM} nitrocellulose membranes essentially as described (Towbin *et al.*, 1979) using a BioRad transblot SD blotting device. Transfer was carried out at 20 V for 20 mins and membranes were incubated in PBST containing 5 % milk powder to block non-specific binding of antibody. Membranes were washed three times in PBST at RT and probed with the appropriate antibody (diluted in PBSA) for 2 hrs at RT. The membrane was again washed three times with PBST and incubated with the appropriate secondary antibody conjugated to HRP for 2 hrs at RT. Finally the membrane was washed three times in PBST and bound antibody was detected using Enhanced Chemiluminescence Reagents I and II (ECL I & II) (Amersham Pharmacia Biotech) in equal ratio. Bands were visualised by autoradiography using Kodak X-OMAT film and a Konica SRX-101-A film processor.

2.2.4.5 Coomassie Staining

Proteins separated on a polyacrylamide gel were stained with Coomassie Brilliant Blue for 30 mins at RT then washed with coomassie destain at RT until background staining was removed. Protein bands were viewed using a lightbox.

2.2.4.6 Coomassie Protein Assay Reagent

Purified protein (20 µl) / BSA standard (range 0.125 - 1.5 mg/ml) was added to 980 µl of coomassie reagent (Pierce) and absorbance measured at 595 nm using a Genios plate reader (Tecan). A standard curve was then prepared by plotting each BSA standard against its concentration in µg/µl.

2.2.4.7 Enzyme-Linked Immunosorbent Assay

Immulon II flat-bottom plates were coated with protein (in PBSA) and incubated at RT overnight. Wells were blocked with PBST (2% milk powder) for 2 hrs at RT and washed three times with PBST before addition of ligand (in PBSA) and incubation for 2 hrs at RT. Plates were washed three times with PBST and primary antibody added for 1 hr at RT before being washed three times with PBST again. HRP conjugated secondary antibody was then added (1 hr RT) and plates again washed three times with PBST. TMB (100 µl) was added for 10 mins at RT (in dark) before the reaction was stopped using 100 µl 0.5 M H_2SO_4. Absorbance was then measured at 450 nm using a Genios plate reader (Tecan).

2.2.4.8 Indirect Immunofluorescence

Cells (grown on 13 mm coverslips) were washed three times with PBS and fixed in methanol at -20°C for 30 mins. Coverslips were then washed three times with PBSA and incubated with primary antibody (diluted in PBST) for 1 hr at RT. Coverslips were again washed three times with PBST and incubated with the relevant secondary antibody for 1 hr at RT. Finally, coverslips were washed in PBS twice and dH_2O once before being mounted downwards on microscope slides using citifluor. Cells were viewed under UV light using LSM510 software and a ZEISS confocal microscope.

2.2.4.9 Fluorescence Assay

Crude cell lysate was serially diluted in PBSA and added to a black 96-well plate. Fluorescence was measured at 485 nm using a Genios plate reader (Tecan).

3. Identification of HCV core residues critical for the interaction with DDX3

3.1 Introduction

HCV core protein associates with lipid droplets in the cytoplasm of cells (Moradpour *et al.*, 1996). Using Yeast-2-hybrid screening, an interaction between HCV core protein and the cellular DEAD-box RNA helicase DDX3 has been reported by 3 independent groups (Mamiya and Worman, 1999, Owsianka and Patel, 1999, You *et al.*, 1999). Comparative proteomic analysis of lipid droplet proteins in core-expressing and non-expressing hepatoma cell lines also indicated that DDX3 associates with lipid droplets only in the presence of HCV core protein (Sato *et al.*, 2006). In order to understand the significance of core-DDX3 interaction in terms of virus replication and pathogenesis, the HCV life cycle must be studied in a system where the interaction between core and DDX3 is inhibited. One approach would be to knockdown DDX3 expression by siRNA. However, it has been reported that knockdown of mouse DDX3 in NIH-3T3 cells leads to premature entry into S-phase (Chang *et al.*, 2006). Assuming the same is true in hepatocytes, this may offset the virus life cycle. Another approach is to mutate residues of core, which are critical for the interaction with DDX3, thus disrupting core-DDX3 interaction. This approach would ensure the normal cellular role of DDX3 was not affected.

Previously, it has been reported that amino acids 1-59 of core protein are involved in the interaction with DDX3 (Owsianka and Patel, 1999). In order to discover critical residues within this region, a library of $core_{1\text{-}59}$ (HCV core residues 1-59) mutants was constructed by error-prone PCR. The mutated sequences were cloned downstream of Green Fluorescent Protein (GFP) and analysed by GFP-display (Aoki *et al.*, 2000b) to confirm the presence of mutations. GFP-display is a gel electrophoresis technique for detecting single amino acid mutations in a polypeptide. GFP is stable in the presence of protein denaturing solutions such as 8 M urea and 1% sodium dodecyl sulphate (SDS) and therefore does not lose its fluorescence in SDS-PAGE (Ward, 1998). This allows GFP to act as a chemical label attached to a polyprotein. It is known that the migration of a protein in a urea-containing SDS-PAGE gel can vary due to the introduction of even a single amino acid substitution mutation. This is believed to be due to the interplay between urea, dodecyl ions and their strength of binding to different amino acids (Aoki *et al.*, 2000b, Aoki *et al.*, 2002). If the protein to be mutated is fused in-frame to GFP then the fusion proteins carrying mutations can be readily identified in urea/SDS-PAGE gel simply by exposing the gel under UV light and examining the protein migration profiles. Here, the intention was to rapidly screen the library to identify clones carrying amino acid substitutions in core and transfer mutated sequences into a full-length core background in a

mammalian expression vector for further analysis. Unfortunately, the GFP-display assay was not reproducible in my hands and failed to generate meaningful results. Therefore, a high throughput assay was designed with a view to rapidly screen large numbers of substitution mutants of HCV $core_{1-59}$. This assay was performed in an ELISA format in which a soluble GST-DDX3 fusion protein, previously used to delineate the domain of DDX3 interacting with core (Owsianka and Patel, 1999), was bound to the ELISA plate. The N-terminal 59 amino acids of core protein (containing single or multiple amino acid substitutions generated by error-prone PCR) was cloned downstream of green fluorescent protein (GFP) in a bacterial expression vector (pKK223-3). The mutants were analysed for their ability to bind DDX3. Bound mutants were detected using a rabbit polyclonal anti-GFP antiserum in order to rule out false negatives occurring due to core antibody recognition sites being mutated during error-prone PCR. Mutants unable to bind DDX3 were then transferred into an expression vector (pcDNA 3.1(+)zeo) containing core, E1 and E2 sequence (derived from HCV strain H77c). Mutant core protein was then studied in a transient transfection system and abolishment of the interaction with cellular DDX3 confirmed by immunofluorescence in transfected cells. Upon nucleotide sequencing of core mutants unable to bind DDX3, systematic reversal of mutations to wild type lead to the identification of a 13 amino acid region containing essential residues. Sequential alanine substitutions were then introduced across this region in wild type pC-E1-E2 to identify individual residues of core protein required for the interaction between core and DDX3.

3.2 Results

3.2.1 Cloning of Core$_{1-59}$ into GFP Expressing pKK223-3 Vector

Bacterial expression vector pKK223-3 (Amersham Pharmacia Biotech, Sweden), containing the B domain of *Staphylococcus aureus* protein A (SpAb) fused to GFP, was kindly donated by Takashi Aoki (Department of Biochemistry, Health Sciences University of Hokkaido, Japan) (Aoki *et al.*, 2002). The construct was digested with *Hind*III / *Pst*I, and the SpAb fragment removed and replaced by a *Hind*III / *Pst*I digested PCR product of nucleotides 1-177, encoding amino acids 1-59, of HCV core (strain H77c). This was generated by PCR using template pCS2 (an expression plasmid containing core, E1 and E2 sequence derived from H77c) and primers AP233 (AAACTGCAGCACGATAATACC**ATG**GGC, *Pst*I site underlined, core initiation codon in bold) and AP234 (CCCAAGCTTCTATTAGGGGATAGGCTGACGTCTACC, *Hind*III site underlinded) (Fig. 3.1). Correct sequence of the PCR product was confirmed by nucleotide sequence analysis. Crude bacterial lysates containing GFP-Core$_{1-59}$ fusions (GFP fused to amino acids 1-59 of HCV core) were produced (see methods), and the presence of GFP-Core$_{1-59}$ confirmed by exposure of the lysates to UV light to detect fluorescence.

3.2.2 Construction of Mutant GFP-Core$_{1-59}$ Library

To construct a library of mutant core$_{1-59}$ proteins, error-prone PCR (EP-PCR) was used. This method is ideal for introducing random mutations into a defined segment of DNA. The low fidelity of *Taq* DNA polymerase means this enzyme can misincorporate nucleotides with a frequency as high as 0.02% per position (Eckert and Kunkel, 1991). The fidelity of *Taq* can be further decreased by differing the relative dNTP concentrations, using a high Mg^{2+} concentration and including Mn^{2+} in the reaction (Leung *et al.*, 1989). EP-PCR can have various outcomes including no nucleotide substitutions in the product, silent substitutions (a nucleotide substitution occurs which does not alter the amino acid), or amino acid substitutions (a nucleotide substitution occurs which either alters the amino acid or introduces a premature stop codon). Of these 4 possible outcomes from EP-PCR, the PCR products of interest in this project are those in which a nucleotide substitution results in an amino acid change. Using primers AP233 and AP234 and pCS2 as a template

A

*Pst*I Start
AP233 – 5’ AAA CTG CAG CAC GAT AAT ACC ATG GGC 3’

*Hind*III Stop Stop
AP234 – 5’ CCC AAG CTT *CTA TTA* GGG GAT AGG CTG ACG TCT ACC 3’

B

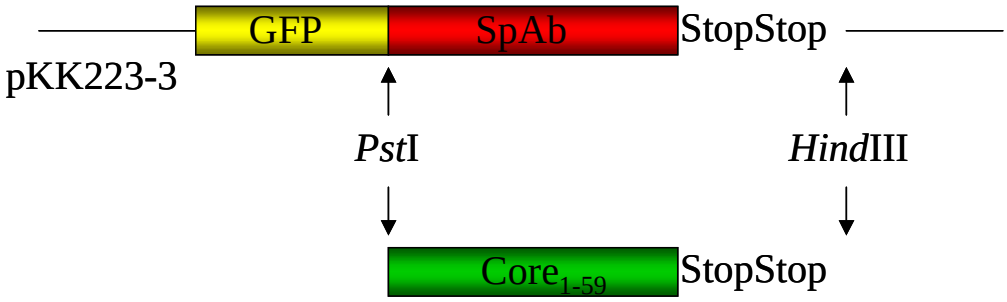


Figure 3.1: Construction of pGFP-core$_{1\text{-}59}$. Vector pKK223-3 containing GFP sequence upstream of the B domain of *Staphylococcus aureus* protein A (SpAb) (Aoki et al., 2002), was digested with *Pst*I and *Hind*III to release and replace SpAb with PCR generated nucleotides 1-177 of HCV genotype 1a H77c strain (encoding amino acids 1-59 of core sequence), followed by two stop codons. A) AP233 and AP234 primer sequences. Restriction sites *Pst*I and *Hind*III are underlined, core protein start codon shown in bold and stop codons in italics. B) Schematic diagram of cloning strategy.

as before (section 3.2.1), EP-PCR was performed to generate mutant core$_{1-59}$ molecules. These products were then inserted into the cloning vector pGEM-T Easy for sequencing. Nucleotide sequence analysis of a subset of these mutants showed that between 1 and 3 nucleotide mutations occurred per PCR molecule, translating to 0-3 amino acid changes per core$_{1-59}$ molecule, some of which were stop codons (Fig. 3.2). These molecules were then released from pGEM-T Easy by digestion with *Hind*III and *Pst*I and sub-cloned into pGFP-SpAb (after removal of the SpAb fragment).

3.2.3 GFP-Display Assay

Constructs pGFP-SpAb and pGFP-SpAb (N214D) were a kind gift from T. Aoki. Fusion proteins expressed from these constructs in bacteria have previously been shown to migrate at different rates on urea / SDS-PAGE gels (Aoki *et al.*, 2002). Inconsistent results meant we were unsuccessful in our attempts to reproduce these results. Constructs were expressed in bacteria and crude bacterial lysate fractionated on a 12% polyacrylamide gel containing 0.2% SDS and 6 M urea. Fluorescent proteins were visualised by exposing the gel under UV light. Results were inconsistent, with differences in migration seen on some occasions but not on others, although the reason for this is unclear (Fig. 3.3). Despite this, we attempted to detect differences in migration between our wild type and mutant GFP-core$_{1-59}$ fusion proteins. Crude bacterial cell lysates were separated on a 12% polyacrylamide gel, containing 0.2% SDS and 6 M urea. Unfortunately, differences in migration patterns were not detected with mutant proteins (Fig. 3.4). The reason for this was unknown although it is possible that certain amino acid changes have more effect on migration compared to others, depending on their isoelectric point. Multiple amino acid changes may also cancel out the differences in isoelectric points of individual mutations.

3.2.4 Core – DDX3 ELISA Design

As the GFP-display assay was not consistently reproducible it was decided to design a rapid screening assay that could make use of the pre-made GFP-core$_{1-59}$ mutant library to identify mutants of interest. This assay was in ELISA format, in which a soluble GST-DDX3C fusion protein (GST fused to residues 409-662 of DDX3), previously used to delineate the domain of DDX3 interacting with core (Owsianka and Patel, 1999) was

MUTANT	NUCLEOTIDE CHANGES	AMINO ACID CHANGES
1	170(A-T)	Q57L
2	26(G-A)	R9K
	101(T-G)	V34G
	170(A-G)	Q57R
3	46(A-T)	N16Y
4	40(A-G)	N14D
	98(G-T)	G33V
	110(T-A)	L37Stop
5	34(A-T)	K12Stop
6	16(A-G)	K6E
	23(A-T)	Q8L
7	37(C-T)	R13C
	41(A-C)	N14T
8	107(T-A)	L36Stop
9	102(T-A)	No Change
10	65(T-G)	V22G
	109(T-A)	L37M
	130(T-A)	L44M

Figure 3.2: Identification of mutations in an initial subset of $core_{1-59}$ mutants. Error-prone PCR was shown to introduce between 1 and 3 nucleotide changes per PCR molecule. These mutations translated to 0 to 3 amino acid changes per molecule or into premature stop codons.

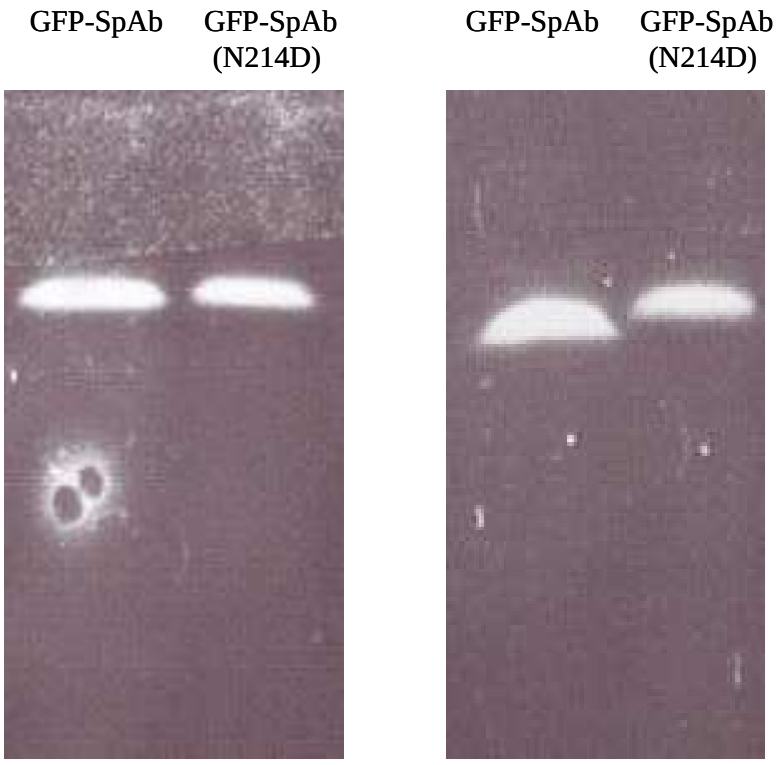


Figure 3.3: Detection of GFP-fused SpAb in bacterial cell lysates. Crude bacterial lysates containing wild type or mutant GFP-SpAb were fractionated on a 12% polyacrylamide gel containing 0.2% SDS and 6 M urea on 2 separate experiments. GFP-fused proteins were detected by viewing gel under UV light. The two gels show that the results were not reproducible in my hands.

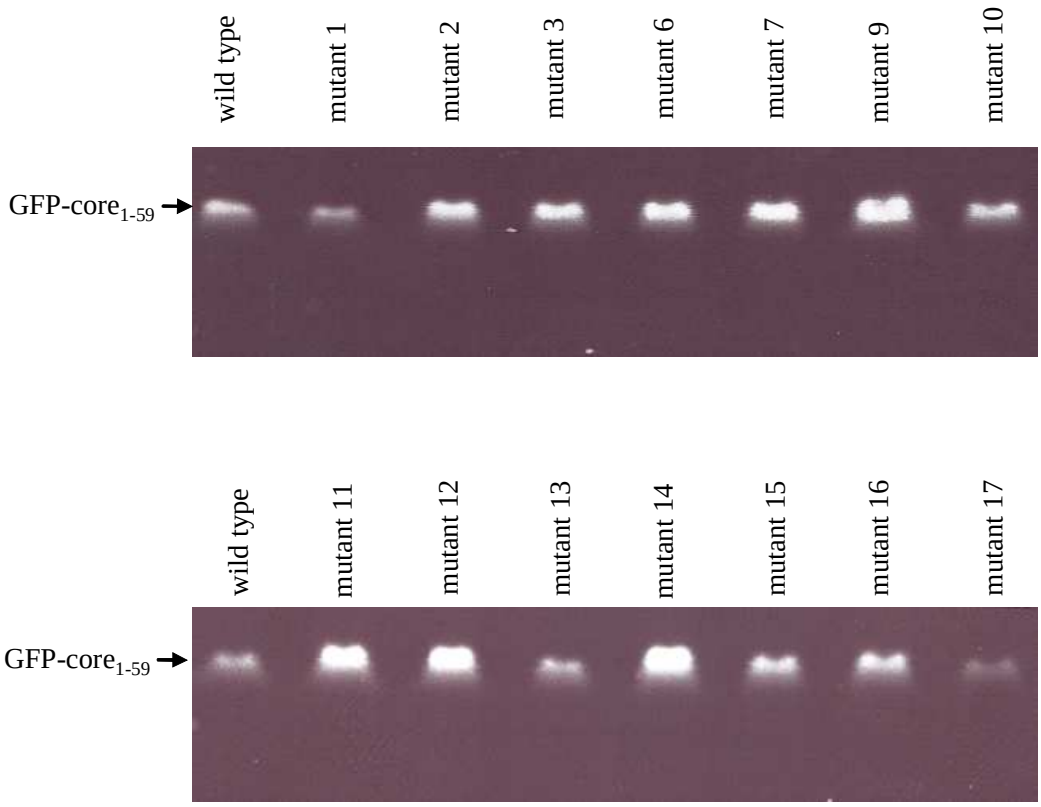


Figure 3.4: Detection of GFP-fused core$_{1-59}$. Crude bacterial lysates containing wild type or mutant GFP- core$_{1-59}$ were separated on a 12% polyacrylamide gel containing 0.2% SDS and 6 M urea. GFP-fused proteins were detected by viewing gel under UV light.

bound to the wells of an ELISA plate. Crude bacterial cell lysate containing GFP-core$_{1-59}$ mutants, after normalising for GFP fluorescence, was then incubated with the GST-DDX3. Bound GFP-core$_{1-59}$ was detected with rabbit polyclonal anti-GFP antiserum followed by protein A-HRP. HRP enzymatic activity was then measured upon addition of TMB substrate (see methods).

3.2.5 Purification of GST-DDX3 fusion protein

GST-DDX3C-expressing *E. coli* were lysed by freeze-thaw and sonication and incubated with glutathione-agarose beads. Unbound protein was washed off and bound GST-DDX3C eluted from the beads using reduced glutathione. Most GST-DDX3C remained bound to the beads but sufficient protein was eluted for use in ELISA. As shown in Fig. 3.5, GST-DDX3C fusion protein of expected molecular weight was expressed at least partially in a soluble form and could be affinity-purified using glutathione agarose. By Coomassie protein assay it was calculated that 0.3 μg/μl purified soluble GST-DDX3C was expressed.

3.2.6 Construction of GFP as a Negative Control

To use as a negative control for our ELISA, GFP alone was expressed in *E. coli*. Using primers quickchange 1 (GATGGACTATACAAACTGTAGCACGATAATACCATG) and quickchange 2 (CATGGTATTATCGTGCTACAGTTTGTATAGTTCATC), site-directed mutagenesis was carried out on pGFP-Core$_{1-59}$ in order to introduce a stop codon (CAG → TAG) in the *Pst*I site between the GFP and core$_{1-59}$ sequences (red nucleotides indicate site-directed substitution; see Fig. 3.1). The substitution was confirmed by nucleotide sequence analysis.

3.2.7 Recognition of Bacterially Expressed Proteins by Antisera

The ELISA assay designed required bacterially expressed GST-DDX3C to be bound to wells of an ELISA plate followed by incubation with crude bacterial lysate containing GFP or GFP-Core$_{1-59}$ fusion proteins. GFP protein binding GST-DDX3C would then be detected

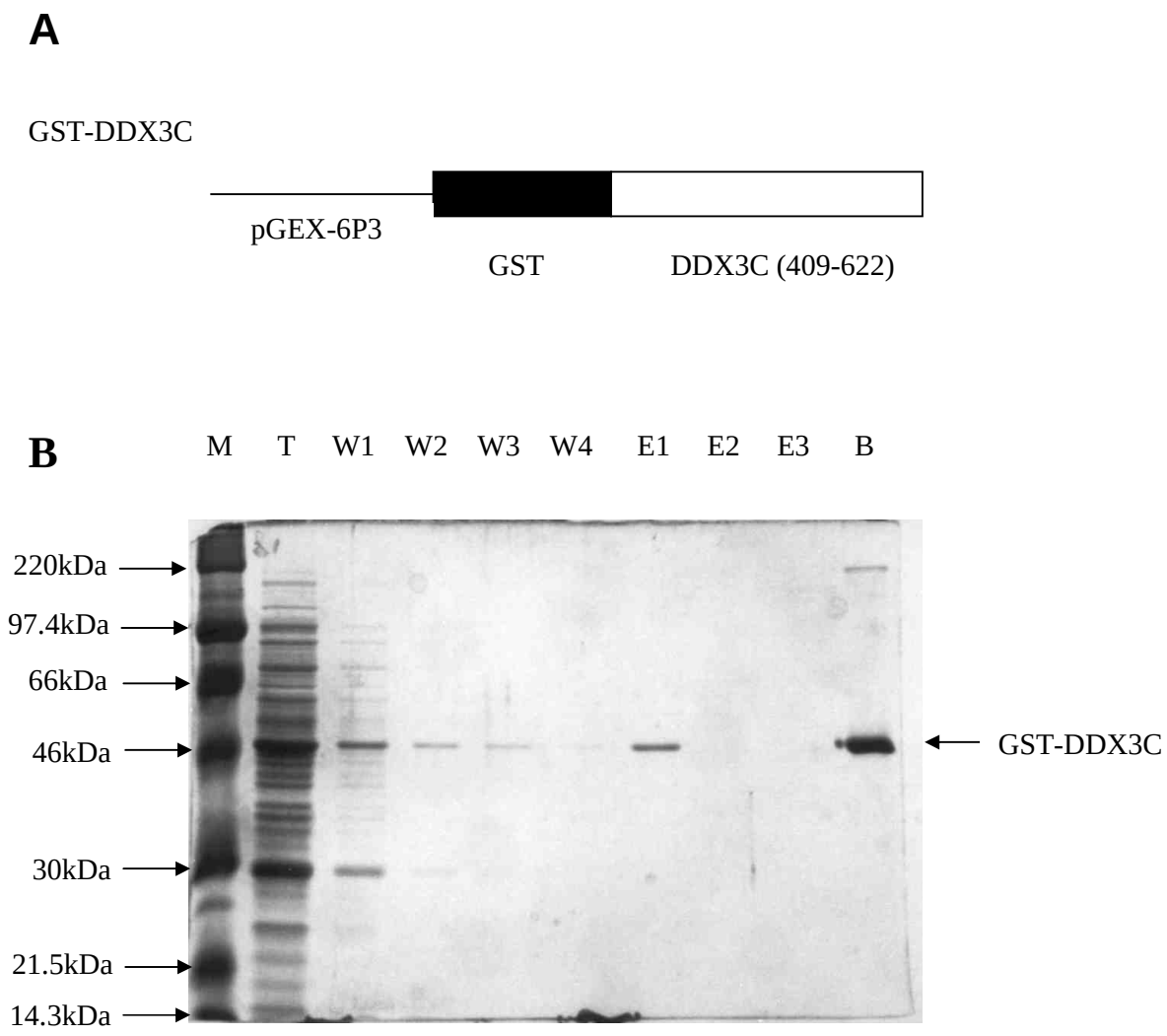


Figure 3.5: Purification of GST-DDX3C. (A) Schematic diagram of GST-DDX3C in the pGEX-6P-3 vector (Pharmacia) used for ELISA. The GST-coding sequence is not drawn to scale. (B) Coomassie brilliant blue-stained SDS-PAGE (10%) of amino acids 409-622 of DDX3 (fused to GST). *E. coli* expressing GST-DDX3 were lysed by freeze-thaw and sonication before addition of glutathione-agarose beads to bind GST-DDX3C. Samples were then washed in PBS and GST-DDX3C eluted from glutathione-agarose beads by addition of reduced glutathione. Samples harvested from each stage were analysed by SDS-PAGE and Coomassie brilliant blue staining. (M- Rainbow markers, T- Total cell lysate, W- Wash, E- Elution, B- Beads).

by a combination of rabbit polyclonal anti-GFP antiserum followed by protein A-HRP. TMB substrate is then added and the enzymatic reaction stopped using H_2SO_4 after an appropriate time. HRP enzymatic activity (indicative of GFP-core$_{1-59}$ binding to GST-DDX3) would then be measured by optical density. A number of validation experiments were initially carried out in order to test the specificity of the ELISA. Antibodies were tested for their affinity and specificity to bacterially expressed fusion proteins bound to Immulon II ELISA plates. Either GST-DDX3C or crude bacterial lysate containing GFP or GFP-core$_{1-59}$ was bound to Immulon II plates overnight and probed with either rabbit polyclonal anti-GFP, anti-core (R308) or anti-DDX3 (R648) antisera followed by protein A-HRP and then incubated with TMB substrate. Rabbit polyclonal antisera against both GFP and HCV core (R308) recognised GFP-core$_{1-59}$, indicating that the fusion of GFP and core$_{1-59}$ did not affect antibody recognition sites. Anti-GFP, and not anti-core, was able to detect the negative control GFP, while only rabbit polyclonal anti-DDX3 antiserum (R648) recognised GST-DDX3C (Fig. 3.6).

3.2.8 Optimization of Protein Concentration for ELISA

Using crude bacterial lysate as a source of GFP-Core$_{1-59}$ meant it was not possible to quantify the amount of ligand (GFP or GFP-core$_{1-59}$) present in each sample by standard Coomassie protein assay since other bacterial proteins would affect quantification. To ensure equal amounts of each ligand were used in our assay we normalised our lysates based on GFP fluorescence. Varying dilutions of crude bacterial lysate were analysed by optical density and fluorescence plots calculated (Fig. 3.7). In order to find optimal concentrations of both DDX3C and ligand for ELISA, titrations were carried out on Immulon II plates. Concentration of GST-DDX3C (coated on plate) was serially diluted 3-fold from left to right across the plate while GFP-Core$_{1-59}$ concentration remained the same for each well (20,000 Relative Fluorescence Units, RFU). Bound protein was detected using rabbit polyclonal anti-GFP antiserum (1:5,000) followed by protein A-HRP (1:10,000). ELISA plates were then incubated with TMB substrate, the reaction stopped with H_2SO_4 after an appropriate time, and absorbance levels read by optical density. Results showed that 0.67 μg of GST-DDX3C/well was optimal for the ELISA (Fig. 3.8a).

In order to find the optimal concentration of GFP/GFP-core$_{1-59}$, a similar experiment was carried out in which crude bacterial lysates were titrated across GST-DDX3C coated ELISA plates (GST-DDX3C was used at its previously optimized concentration of 0.67

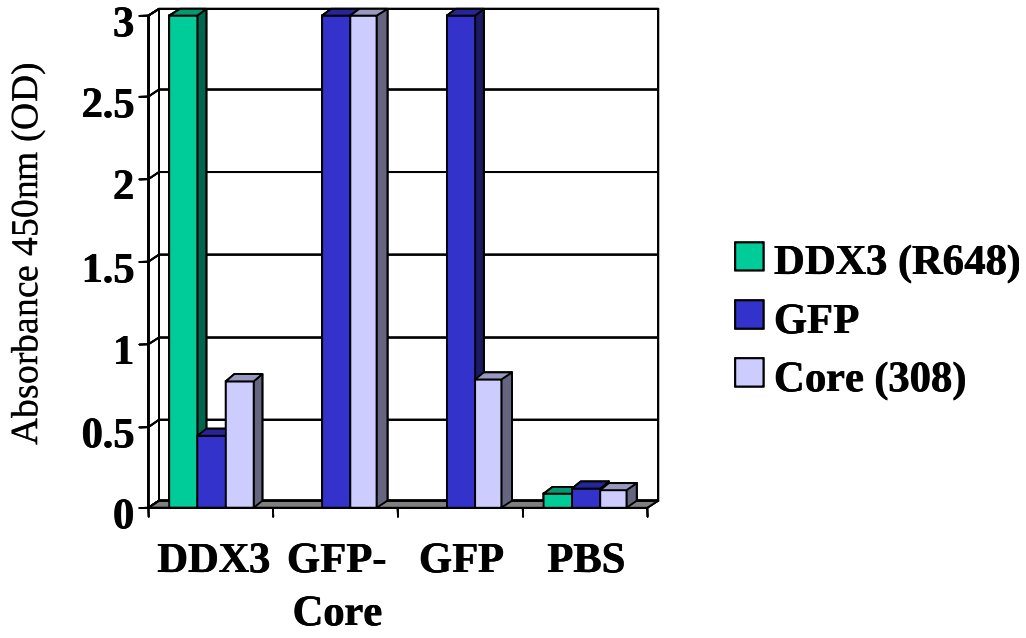


Figure 3.6: Antibody recognition of bacterially expressed fusion proteins. Proteins to be used in the ELISA were analysed for their ability to be recognised by various antibodies. GST-DDX3C or crude bacterial lysate containing either GFP or GFP-core$_{1\text{-}59}$ was bound to Immulon II plates and incubated with anti-DDX3 R648, anti-GFP or anti-core R308 antiserum, followed by protein A-HRP. TMB was added and HRP enzymatic activity measured by optical density at 450 nm. Polyclonal anti-DDX3 antibody detected DDX3C while polyclonal anti-GFP and polyclonal anti-core antibodies did not. Anti-GFP antibody efficiently detected both GFP and GFP-core$_{1\text{-}59}$ while only anti-GFP and not anti-core antibody detected GFP.

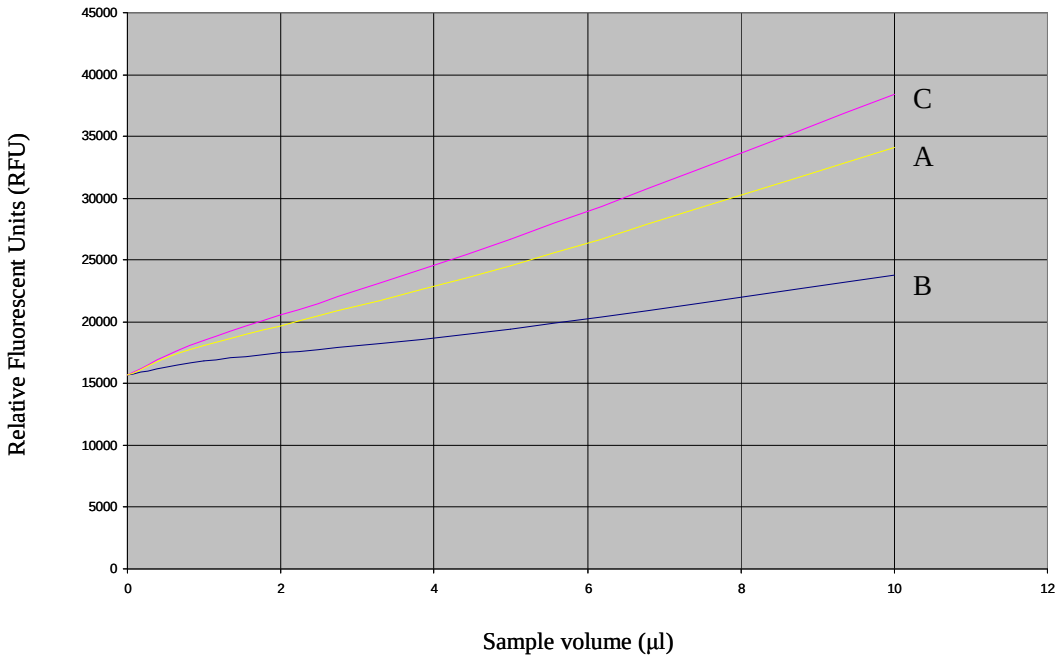


Figure 3.7: Relative fluorescence intensity of GFP-core$_{1-59}$ mutants. Increasing concentrations (1, 5 or 10 µl in 100 µl PBS) of crude bacterial lysates containing either wild type, mutant 1 or mutant 2 GFP- core$_{1-59}$ protein were analysed for GFP fluorescence levels by optical density. Relative fluorescence units were used to normalise lysates before use in ELISA. All samples were normalised to a fluorescence of 20,000 RFU. A) GFP- core$_{1-59}$, B) GFP- core$_{1-59}$ mutant 1, C) GFP-core$_{1-59}$ mutant 2.

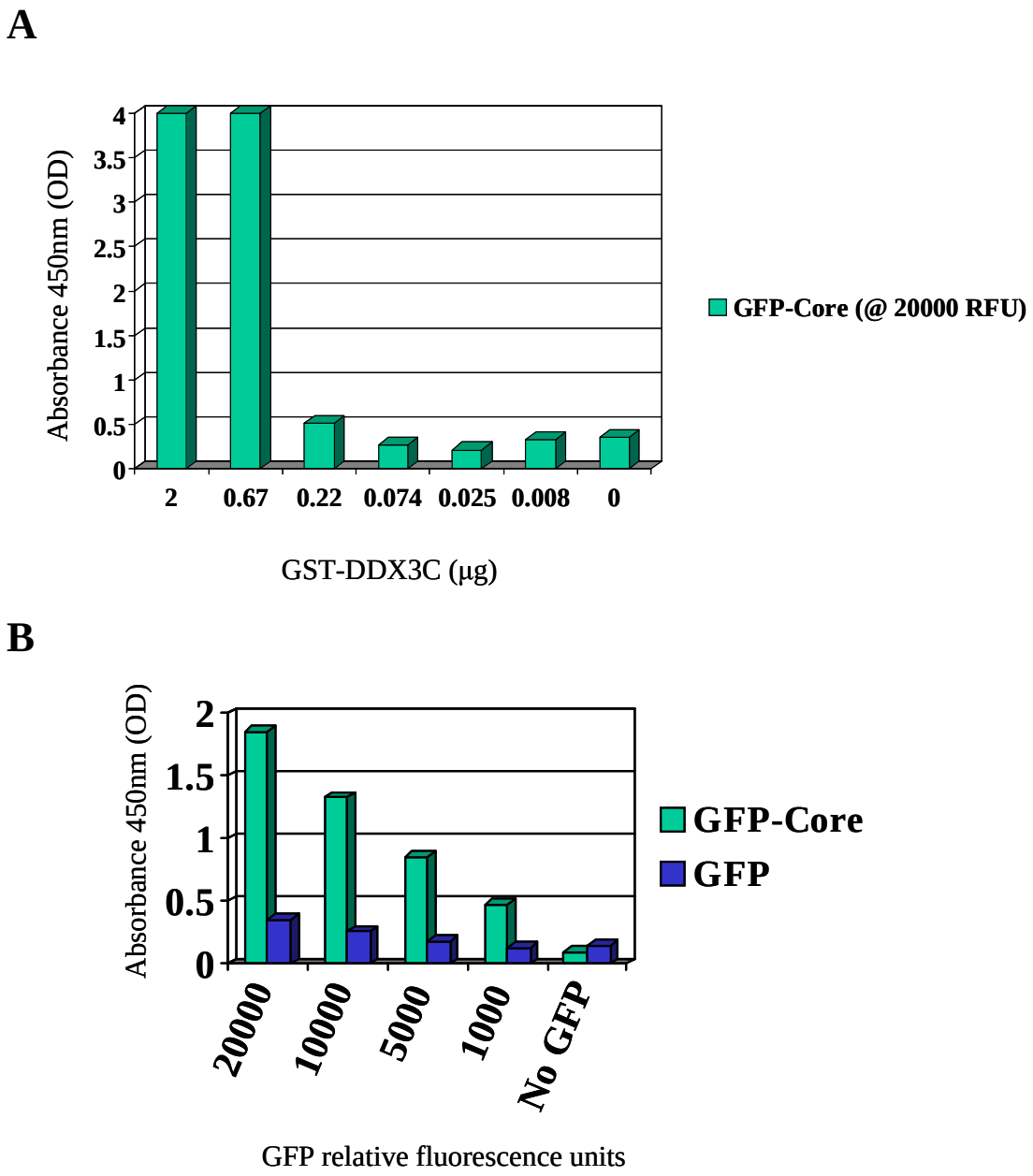


Figure 3.8: Optimization of GST-DDX3C/GFP-core$_{1-59}$ levels for ELISA. A) Wells of an Immulon II plate were coated with a series of 3-fold diluted GST-DDX3C from left to right across the plate. Crude bacterial lysate containing GFP- core$_{1-59}$ was incubated with the bound GST-DDX3C at a constant RFU of 20,000. Upon incubation firstly with anti-GFP antibody, then protein A-HRP followed by TMB substrate, optical density was calculated (see methods). Results suggest that 0.67 µg of GST-DDX3C was optimal for the assay. B) Wells of an Immulon II plate were coated with dilutions of crude bacterial lysate containing either GFP or GFP- core$_{1-59}$ (normalised for GFP content). Optimized levels of GST-DDX3 (0.67 µg/well, Fig. 3.8A) were incubated with bound crude lysate. Upon incubation firstly with anti-GFP antibody, then protein A-HRP followed by TMB substrate, optical density was calculated. Results showed that relative fluorescence units of 20,000 gave optimal contrast between GFP and GFP- core$_{1-59}$.

μg/well). Protein bound to GST-DDX3C was detected using rabbit polyclonal anti-GFP antiserum followed by protein A-HRP. ELISA were carried out as described above and absorbance levels read by optical density as before. From this titration it was confirmed that the concentration of GFP-core$_{1-59}$ used in our previous GST-DDX3C titration (fluorescence = 20,000 RFU) gave optimal contrast between GFP-core$_{1-59}$ and GFP (Fig. 3.8b).

3.2.9 Identification of GFP-core$_{1-59}$ Mutants Unable to Bind DDX3C

Of 130 mutants tested by ELISA, 9 were found to have less than 50% absorbance levels compared to that of wild-type GFP-Core$_{1-59}$, indicating a reduced affinity for GST-DDX3C, whereas other mutants bound GST-DDX3C efficiently (Fig. 3.9). In order to confirm these results, all 9 mutants were individually subcloned into the HCV strain H77c encoded core, E1 and E2 background in a mammalian expression vector pcDNA3.1 (+)Zeo (Fig. 3.10). This was achieved using a triple fragment cloning strategy. Briefly, to overcome restriction site problems, pC-E1-E2 was digested with *Pst*I and *Cla*I to provide the vector backbone and 3' end of core-E1-E2 insert. Similarly, pC-E1-E2 was digested with *Xho*I and *Cla*I, producing a 195 bp insert, which, together with the vector backbone fragment, was ligated to mutant core$_{1-59}$ sequence (digested with *Pst*I and *Xho*I). These mutant pC-E1-E2 constructs were then analysed in a transient transfection system.

3.2.10 Identification of Core Residues Required For Interaction With Cellular DDX3

Wild type or mutant pC-E1-E2 was transfected into 95% confluent naïve Huh7 cells on coverslips using GeneJuice Transfection Reagent (Novagen). Two days post-transfection, cells were fixed in methanol, permeablised with PBS-T and probed for core and E2 by immunofluorescence to confirm that all transfections had been efficient and that viral proteins were being expressed in transfected cells (Fig. 3.11). E2 was distributed throughout the cytoplasm while core was seen in typical punctate cytoplasmic spots. Initial experiments to identify core mutants unable to interact with DDX3 relied on crude bacterial lysates containing core residues 1-59 fused to GFP and affinity purified DDX3 C-terminus fused to GST. To investigate the interaction between full-length HCV core

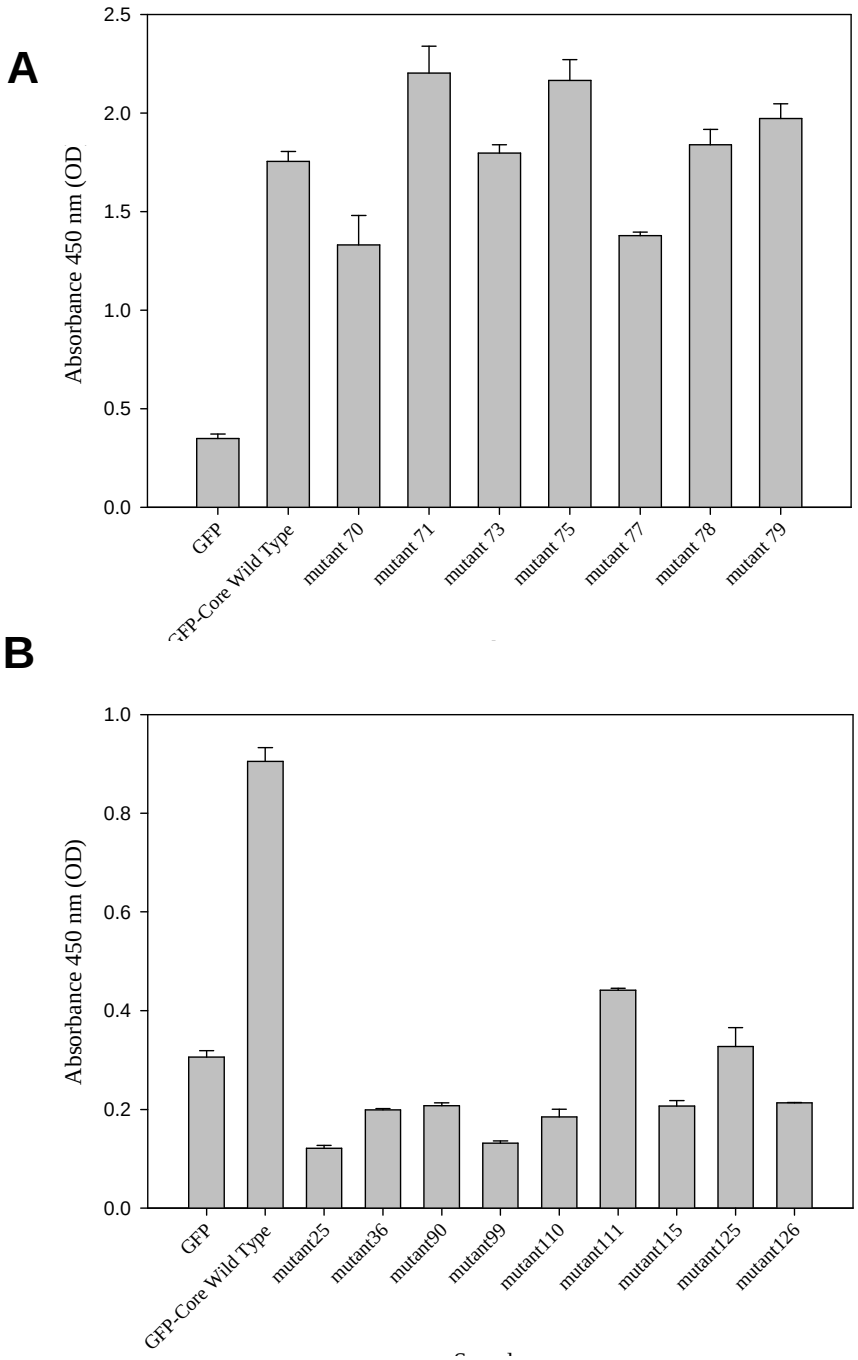


Figure 3.9: Reactivity of GFP- $core_{1-59}$ mutants to GST-DDX3C. Crude bacterial extracts expressing GFP- $core_{1-59}$ mutants (normalised to 20,000 RFU) were added to wells of Immulon II plates coated with 0.67 μg GST-DDX3C. Bound protein was detected using anti-GFP antiserum followed by protein A- HRP. Each mutant was tested in triplicate and error bars shown. A) Mutants binding GST-DDX3C. B) Mutants unable to bind GST-DDX3C. Sample names represent mutant sample number.

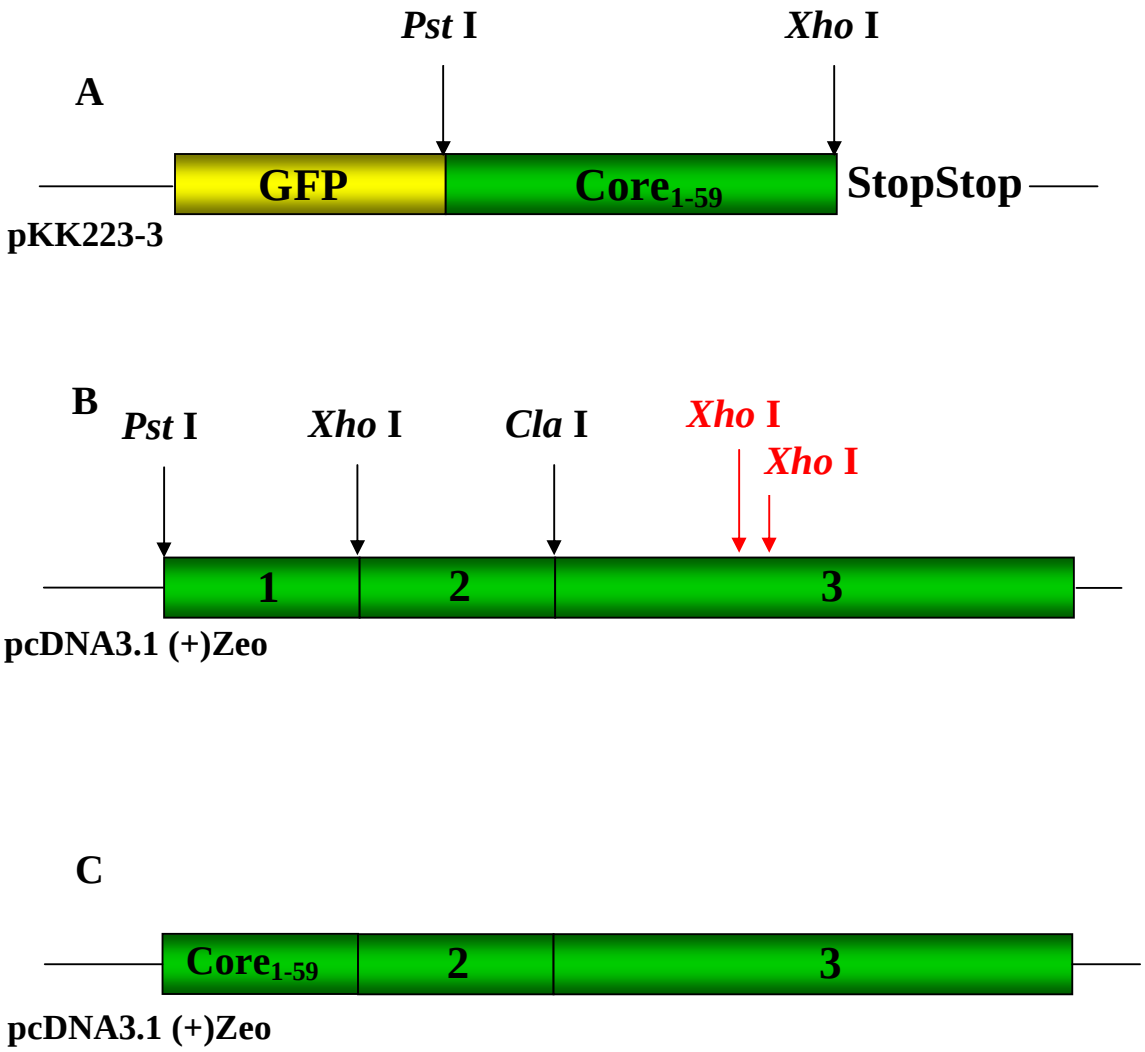
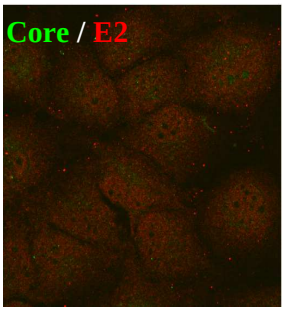
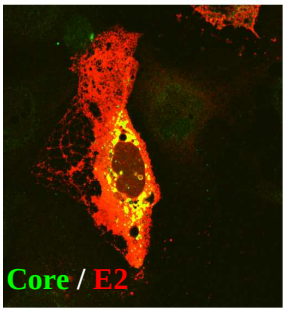


Figure 3.10: Cloning strategy for insertion of mutations into core-E1-E2 expressing vector. Single fragment cloning of mutant core$_{1-59}$ was not possible due to two other *Xho*I sites (red) in pcDNA3.1 (+)Zeo CE1E2. A) Mutant core sequence was digested from pKK223-3 GFP- core$_{1-59}$ using restriction enzymes *Pst*I and *Xho*I. B) pcDNA3.1 (+)Zeo containing core, E1 and E2 sequence was digested with *Xho*I and *Cla*I to produce fragment 2, and, in a separate reaction, digested with *Pst*I and *Cla*I to produce fragment 3 (including the vector backbone). C) Digested fragments 2, 3 and mutant core$_{1-59}$ were then ligated together to produce mutant core-E1-E2 in the pcDNA3.1 (+)Zeo background.

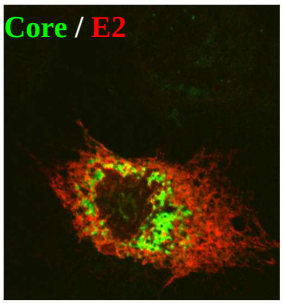
Mock



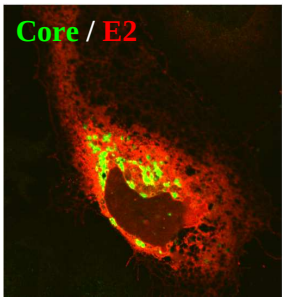
Wild type



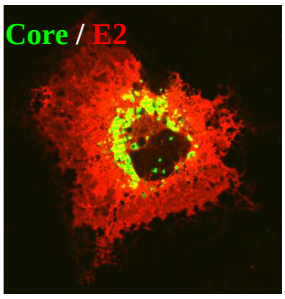
Mutant 25



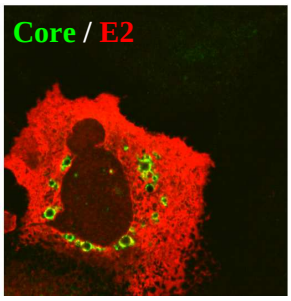
Mutant 36



Mutant 90



Mutant 99



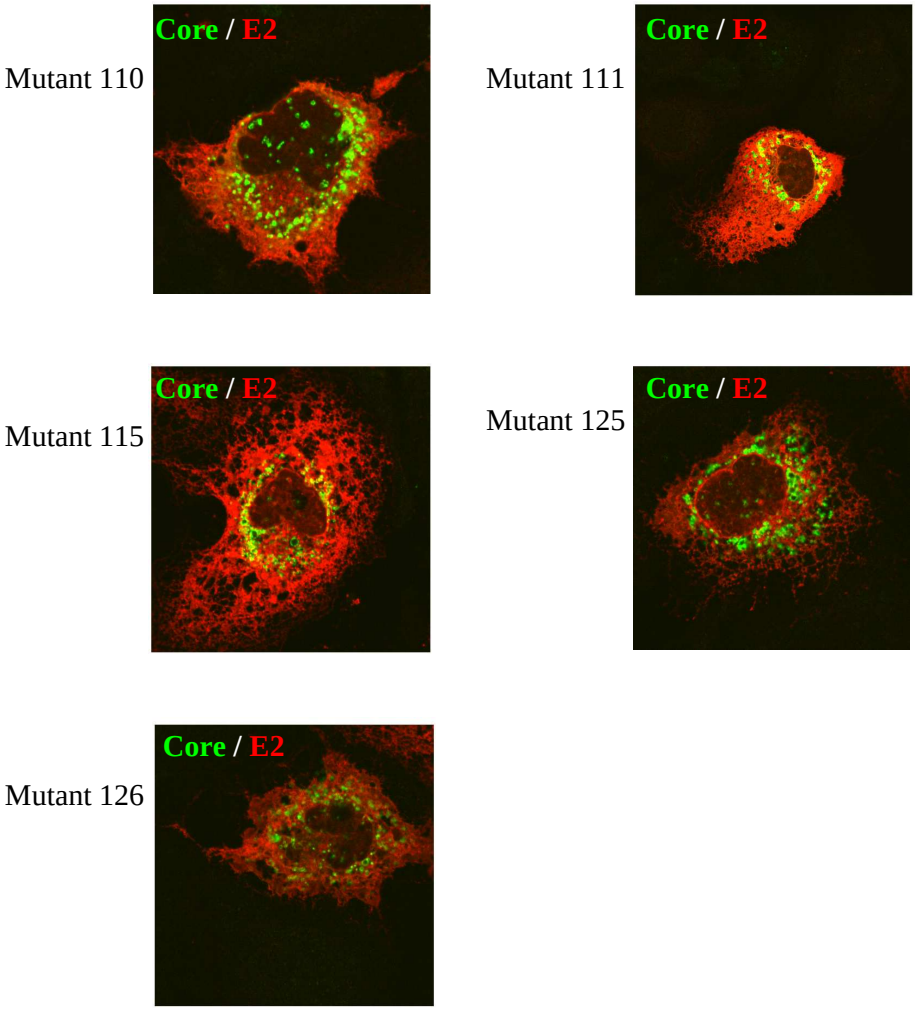
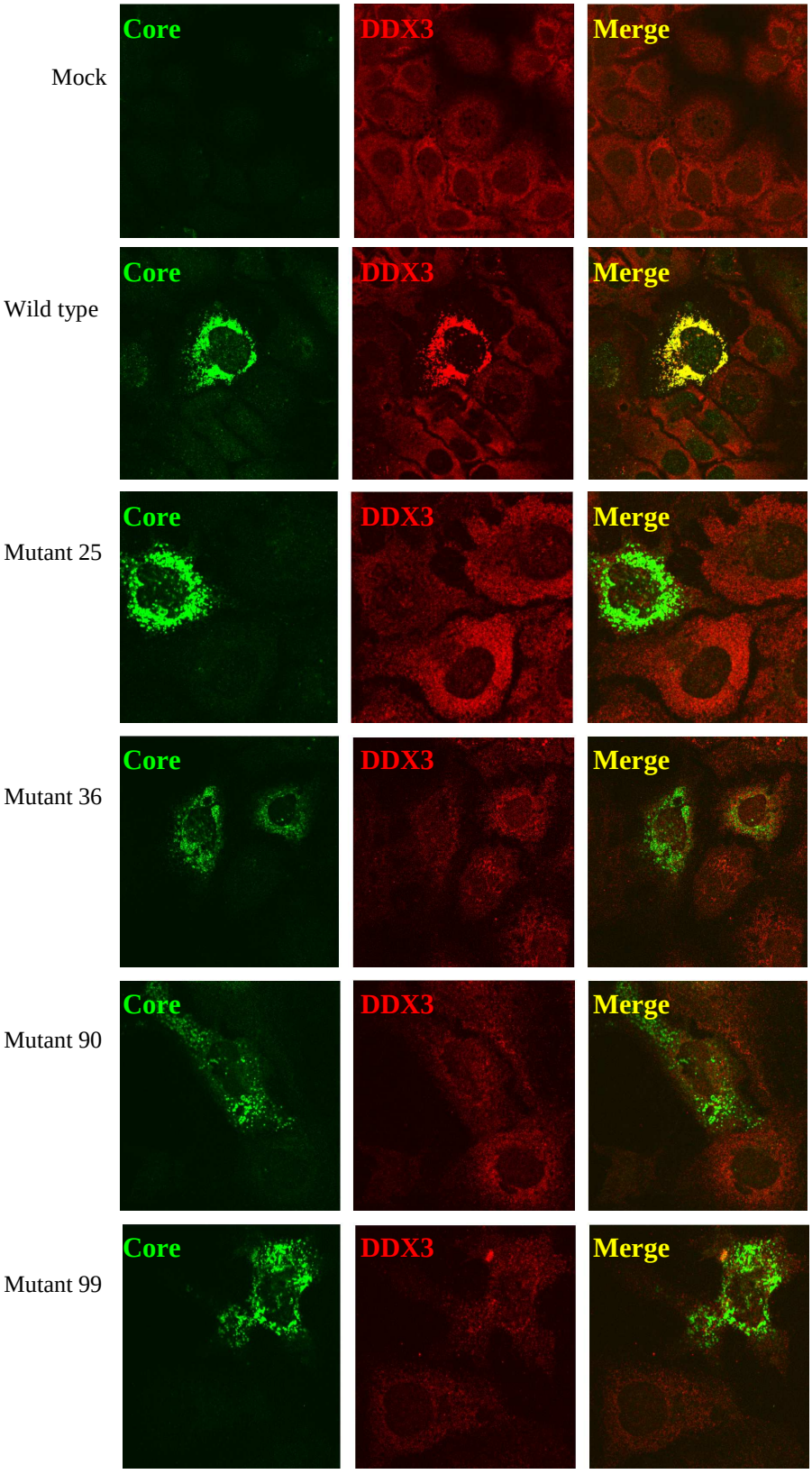


Figure 3.11: Expression and localisation of transiently transfected HCV structural proteins core and E2. Mutant core sequences of interest were cloned into core-E1-E2 expression plasmid pC-E1-E2 and transiently transfected into Huh7 cells (see methods). Forty-eight hrs post-transfection, cells were fixed in methanol, permeablised with PBS-T and probed with rabbit polyclonal core antiserum (R308) and mouse monoclonal anti-E2 antibody (AP33), followed by anti-rabbit FITC and anti-mouse Cy5, respectively.

mutants and cellular DDX3, cells transfected with mutant pC-E1-E2 were fixed in methanol 48 hrs post-transfection, permeablised with PBS-T and analysed by immunofluorescence for core and DDX3. The results obtained were in accordance with the data from our initial ELISA screen in that all 9 mutant core proteins failed to interact with DDX3 in the way wild-type core did (Fig. 3.12). Instead, DDX3 was seen to have a typical diffuse cytoplasmic localisation similar to that in non-transfected cells. Nucleotide sequence analysis was carried out on these 9 mutants and each was found to have between 1 and 4 amino acid substitutions (Fig 3.13). Of interest, mutant 90 had only one amino acid substitution (I30N) indicating this residue must have a negative effect on the interaction of core with DDX3. It was also noted that all 9 mutants had at least one amino acid substitution in the region spanning amino acids 24-36, indicating that this region may harbour amino acids that are critical for the core-DDX3 interaction. To test this hypothesis, site-directed mutagenesis was carried out to revert any mutation out-with this 13 amino acid region back to wild type. As previously stated, site-directed mutagenesis requires PCR amplification of the total plasmid, introducing the possibility of random PCR errors throughout the synthesised product. To overcome this possibility, site-directed mutagenesis was carried out on the original pGFP-core$_{1-59}$ mutant constructs. Primers were designed which would individually substitute each “out-lying” mutation for wild type. Each “out-lying” mutation was systematically reverted to wild type by site-directed mutagenesis and substitutions confirmed by nucleotide sequence analysis. These new mutant core$_{1-59}$ fragments were then sub-cloned into pC-E1-E2 as previously described (section 3.2.9).

These new mutants (designated “mutant Xb”) were transiently transfected into Huh7 cells as before. Two days post-transfection, cells were fixed in methanol and permeablised with PBS-T. To investigate the interaction between these mutants and cellular DDX3, cells were probed by immunofluorescence for core and DDX3. As with the original 9 mutants unable to interact with DDX3, none of these new core mutants interacted with DDX3 (Fig. 3.14), indicating that this 13 amino acid region of core is involved in the interaction between core and DDX3. As expected, all transfected cells expressed HCV viral protein E2 (data not shown).

To determine which residues in this 13 amino acid region were essential for the interaction between core and DDX3, alanine-scanning mutagenesis across amino acids 24-36 was performed, in which each amino acid in this region was sequentially mutated to alanine. Alanine was chosen as the replacement residue as it is the most common amino acid in proteins (Klapper, 1977). This non-polar, hydrophobic amino acid (Fig. 3.15) is the second smallest of all amino acids (behind glycine) and does not impose any unwanted steric


Mock
Core
DDX3
Merge
Wild type
Core
DDX3
Merge
Mutant 25
Core
DDX3
Merge
Mutant 36
Core
DDX3
Merge
Mutant 90
Core
DDX3
Merge
Mutant 99
Core
DDX3
Merge

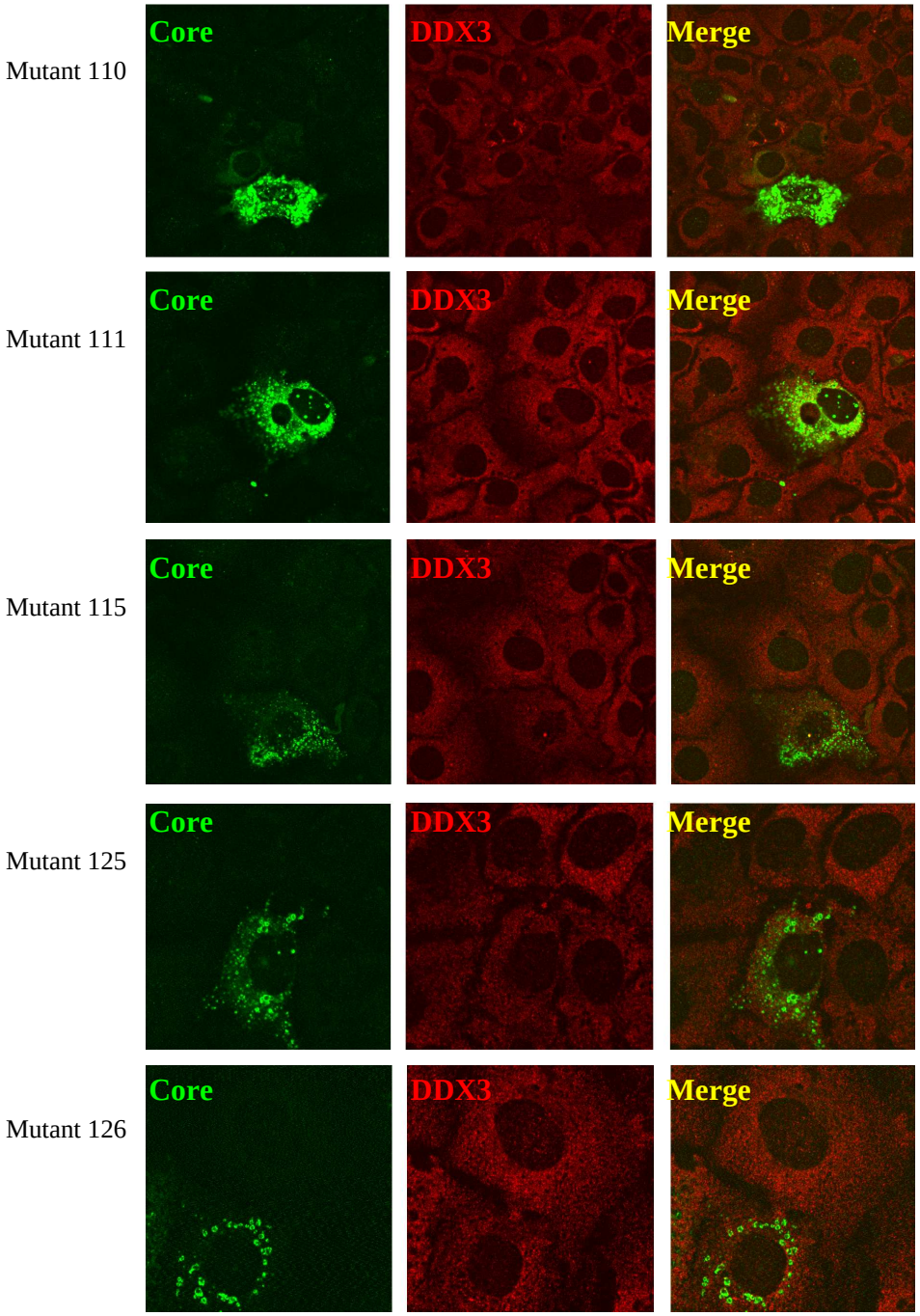


Figure 3.12: Analysis of the interaction between mutant core proteins and DDX3. Mutant core sequences of interest cloned into core-E1-E2 expression plasmid pC-E1-E2 were transiently transfected into Huh7 cells (see methods). Forty-eight hrs post-transfection, cells were fixed in methanol, permeablised with PBS-T and probed with rabbit polyclonal core antiserum (R308) and mouse monoclonal anti-DDX3 (AO196), followed by anti-rabbit FITC and anti-mouse Cy5, respectively.

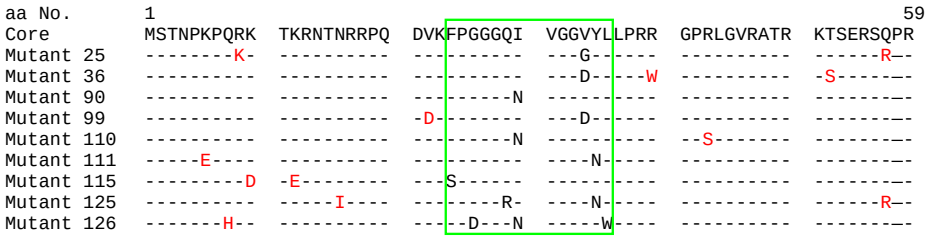
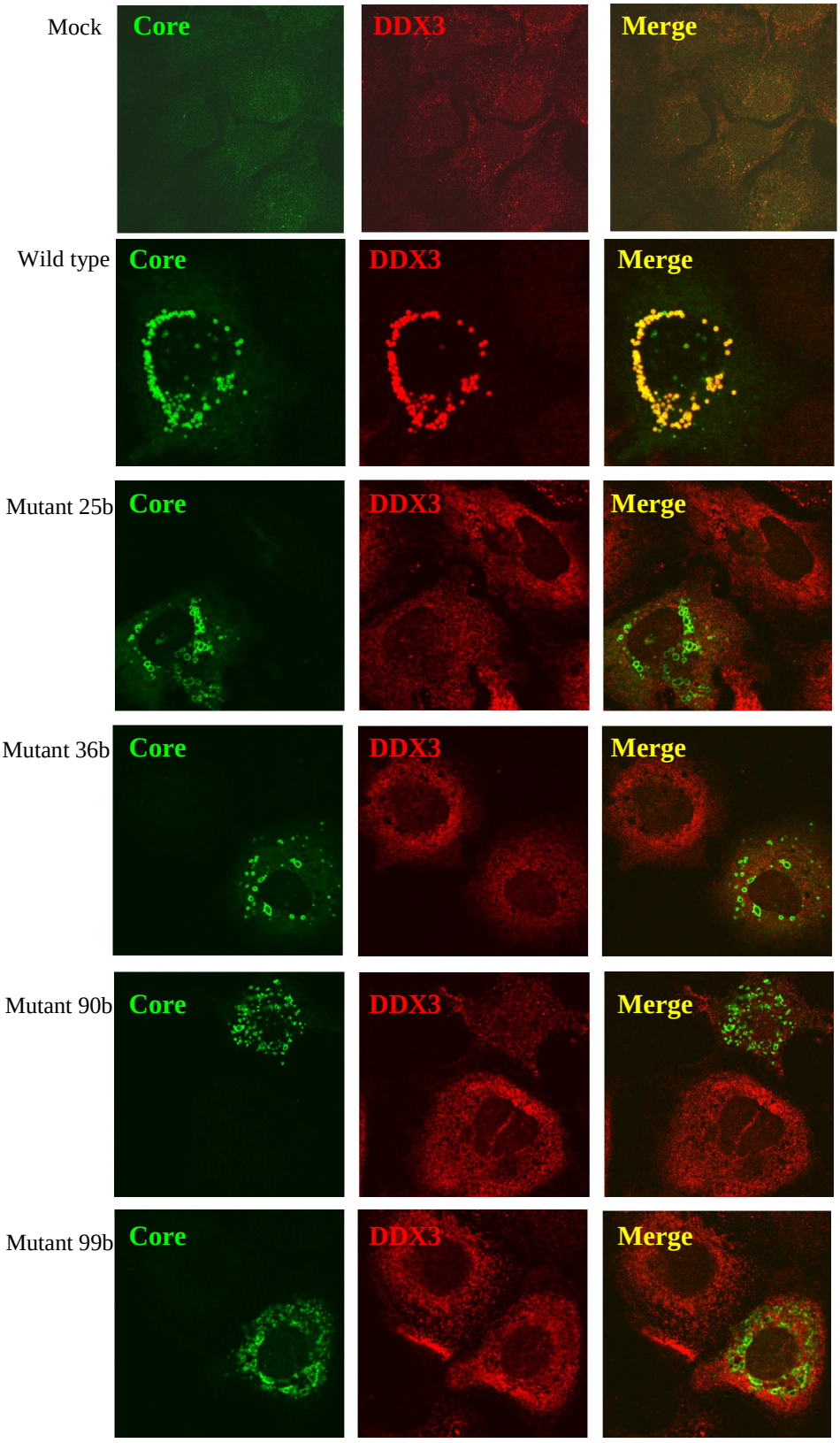


Figure 3.13: Nucleotide sequence analysis of core mutants unable to interact with cellular DDX3. The 9 core mutants unable to bind DDX3 both in ELISA and transient transfection assay were analysed by nucleotide sequencing. Amino acid substitutions found in these 9 mutants are shown (red). The green box denotes a region in which all 9 HCV core mutants unable to bind DDX3 had at least one mutation. In order to confirm if the substitutions within this central region were responsible for abolishing the core-DDX3 interaction, site-directed mutagenesis was used to revert out-lying substitutions (red) back to wild type. These new core mutants (designated “mutant Xb”), were then analysed for core-DDX3 interaction by immunofluorescence in the transient transfection assay as described in section 3.2.9.


Mock
Core
DDX3
Merge
Wild type
Core
DDX3
Merge
Mutant 25b
Core
DDX3
Merge
Mutant 36b
Core
DDX3
Merge
Mutant 90b
Core
DDX3
Merge
Mutant 99b
Core
DDX3
Merge

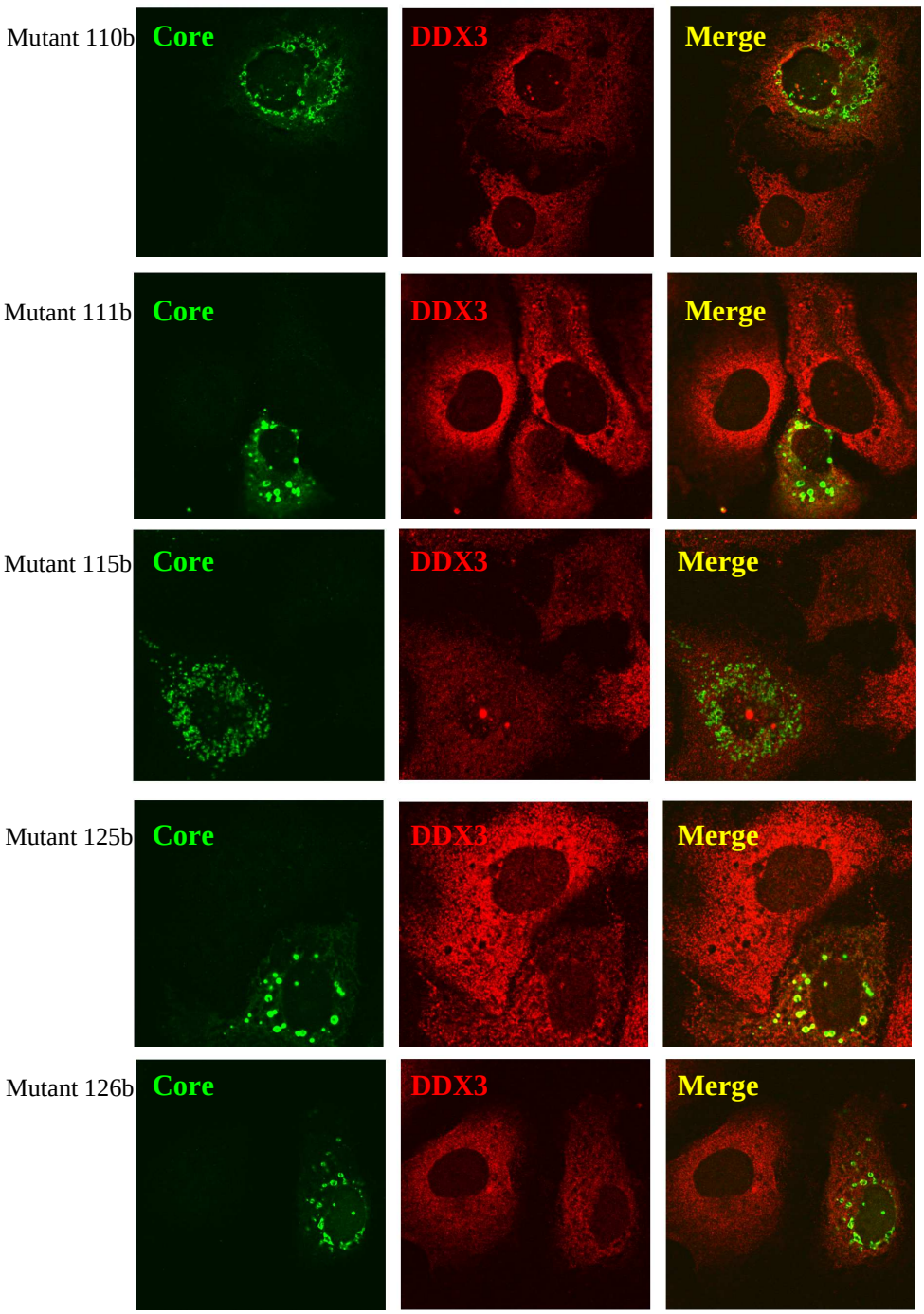


Figure 3.14: Analysis of the interaction between mutant core proteins and DDX3. Mutant core proteins constructed by site-directed mutagenesis were transiently transfected into Huh7 cells. Forty-eight hrs post-transfection, cells were fixed in methanol, permeablised with PBS-T and probed with rabbit polyclonal core antiserum (R308) and mouse monoclonal anti-DDX3 antibody (AO196), followed by anti-rabbit FITC and anti-mouse Cy5, respectively.

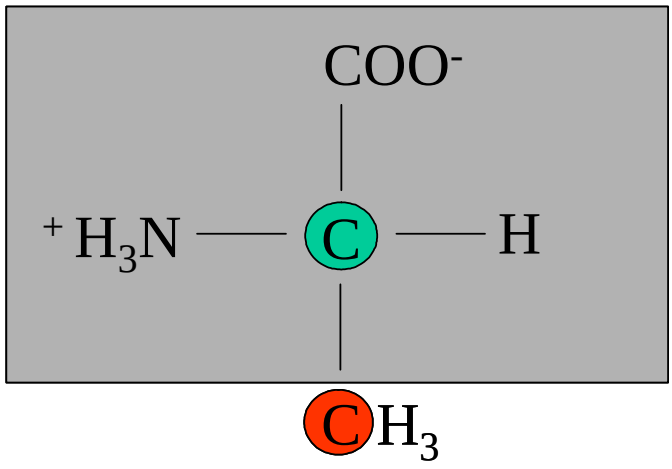
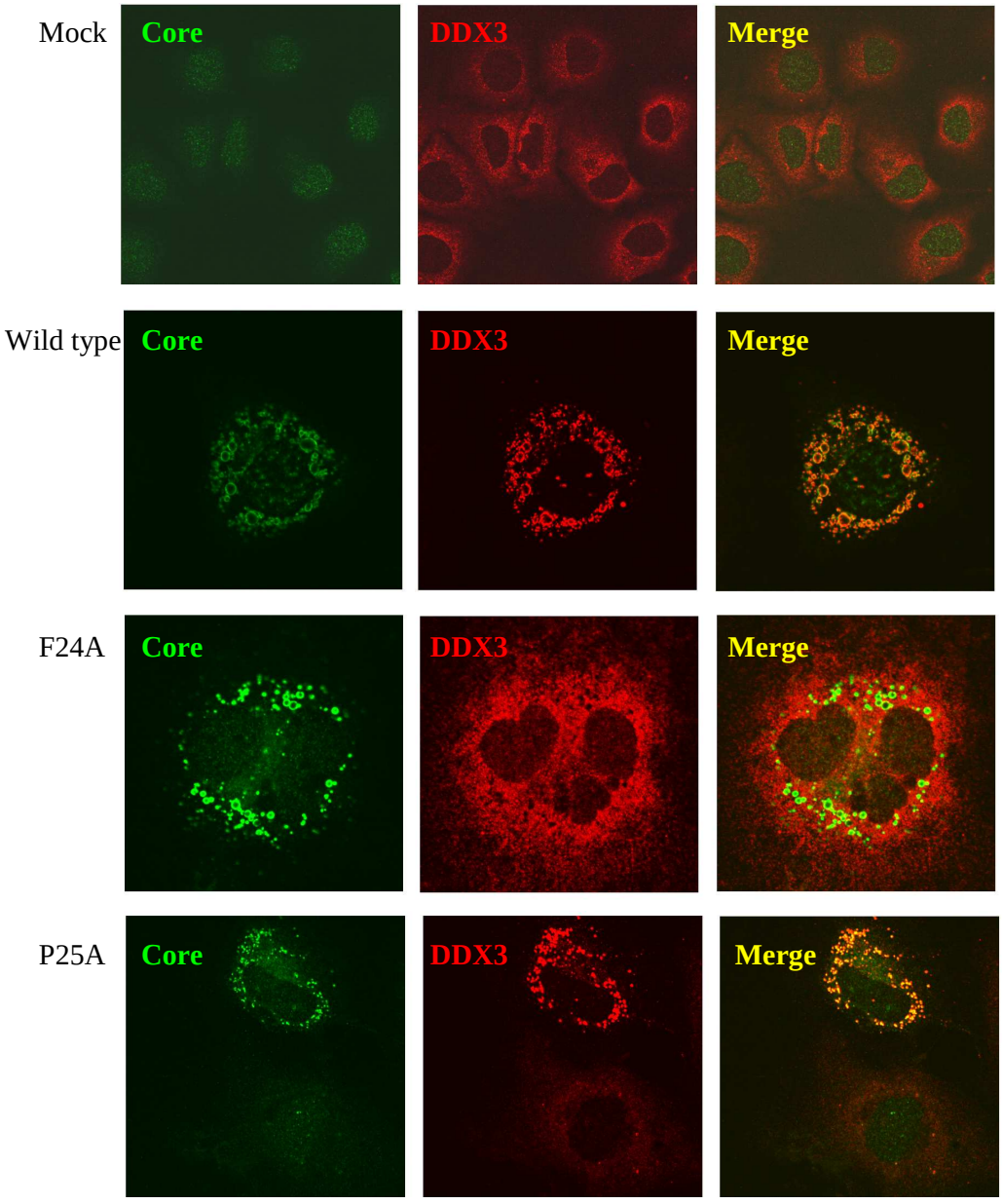
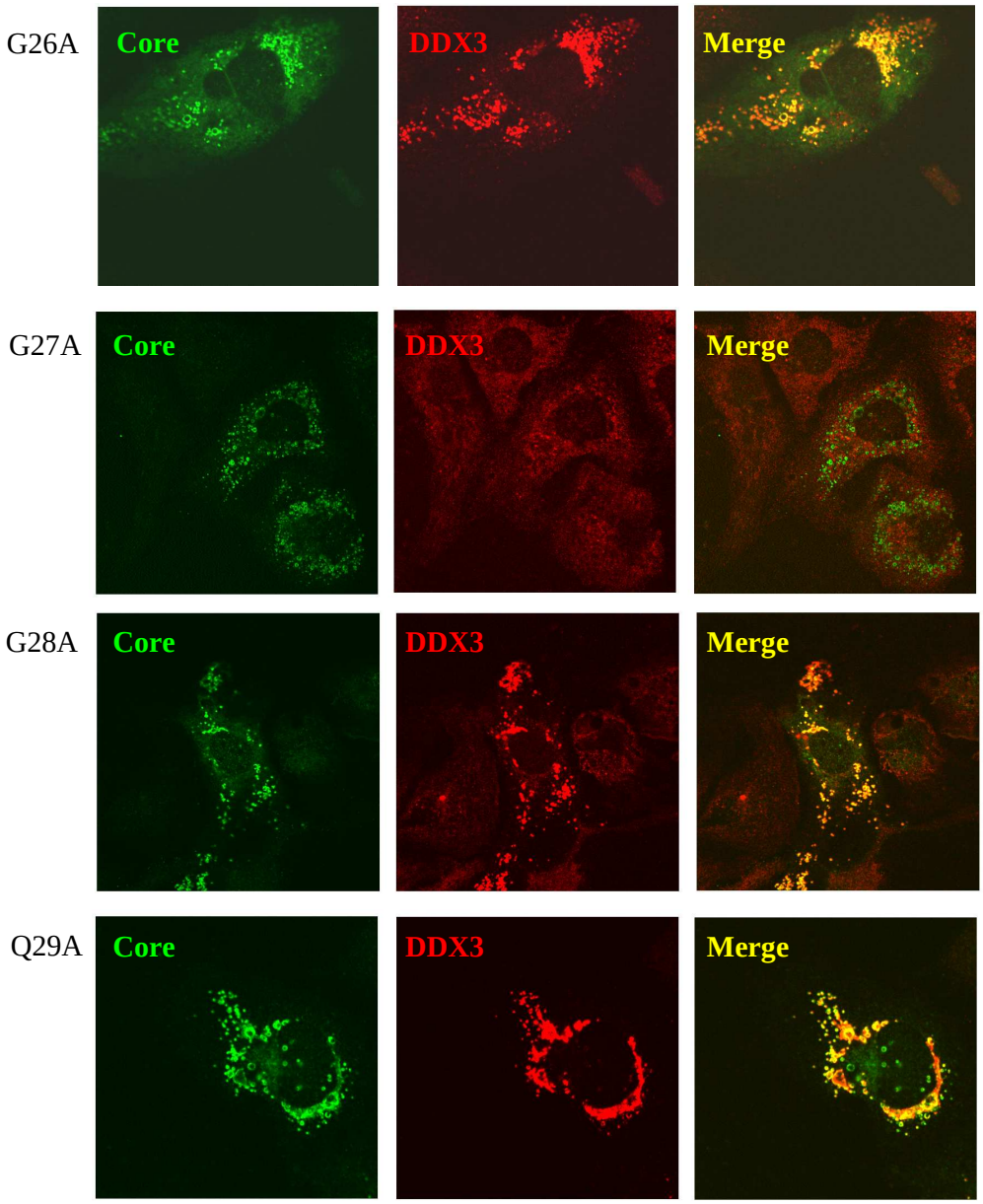
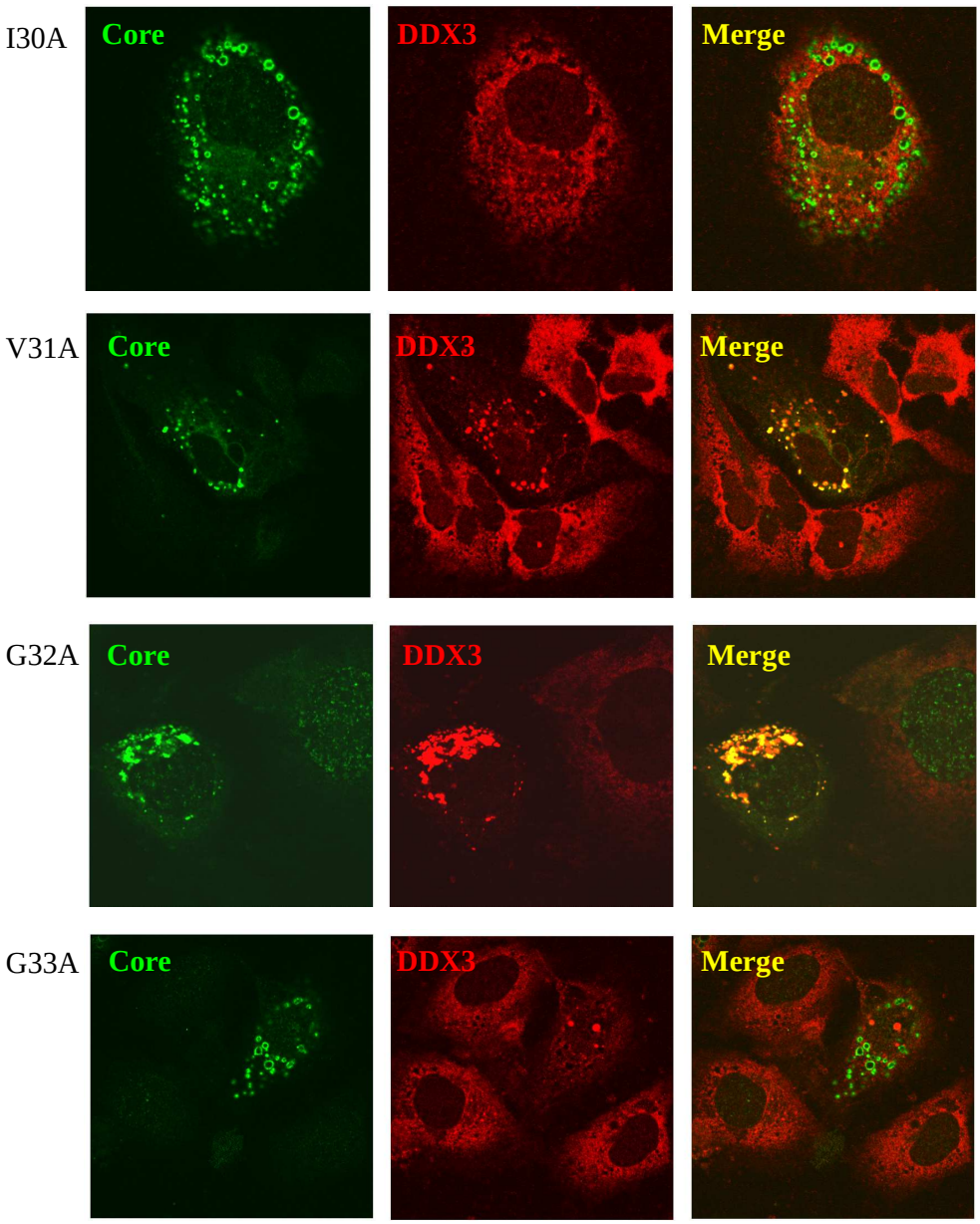


Figure 3.15: Structure of alanine. The shaded area shows the basic amino acid backbone common to all amino acids (amino group, carboxyl group, hydrogen atom and a-carbon (green)) while the side chain specific to alanine (CH_3) is attached to the a-carbon by the ß-carbon (red).

effects, unlike those with bulky side chains such as tryptophan. The elimination of side chains beyond the β-carbon (as in alanine) allows a more informative analysis of the importance of specific side chains, thus reducing the functional comparisons required between mutant and wild-type protein. For these reasons, alanine was chosen as the replacement residue for mutagenesis. To overcome the possibility of unwanted mutations (caused by PCR), site-directed mutagenesis was carried out on pGFP-core$_{1-59}$ (wild type) as before. Primers were designed to introduce specific nucleotide substitutions into the wild type core construct. These nucleotide substitutions resulted in amino acid substitutions from wild type sequence to alanine. This proceduce was carried out for each of the 13 HCV core residues between 24 and 36 and mutagenesis confirmed by nucleotide sequence analysis. As before, these alanine mutant sequences were subcloned into pC-E1-E2 and transiently transfected into Huh7 cells. Two days post-transfection, cells were fixed in methanol, permeablised with PBS-T and probed for core and DDX3. Immunofluorescence analysis of these transfected cells showed interesting results. All alanine mutants showed typical punctate cytoplasmic core staining similar to wild type core protein, however, while some mutants (P25A, G26A, G28A, Q29A, V31A, G32A and L36A) showed distinct colocalisation between core and DDX3 (similar to that seen with wild type HCV core), other mutants (F24A, G27A, I30A, G33A, V34A and Y35A) showed no interaction with DDX3 at all (Fig. 3.16). In cells transfected with these core mutants, DDX3 had a diffuse cytoplasmic localisation, similar to that seen in non-transfected cells. In total, 6 residues within this 13-residue region were shown to be critical for interaction with cellular DDX3 (residues 24, 27, 30, 33, 34 and 35). When substituted for alanine, these residues led to the inhibition of interaction between HCV core and cellular DDX3.


Mock
Core
DDX3
Merge
Wild type
Core
DDX3
Merge
F24A
Core
DDX3
Merge
P25A
Core
DDX3
Merge


G26A
Core
DDX3
Merge
G27A
Core
DDX3
Merge
G28A
Core
DDX3
Merge
Q29A
Core
DDX3
Merge


I30A
Core
DDX3
Merge
V31A
Core
DDX3
Merge
G32A
Core
DDX3
Merge
G33A
Core
DDX3
Merge

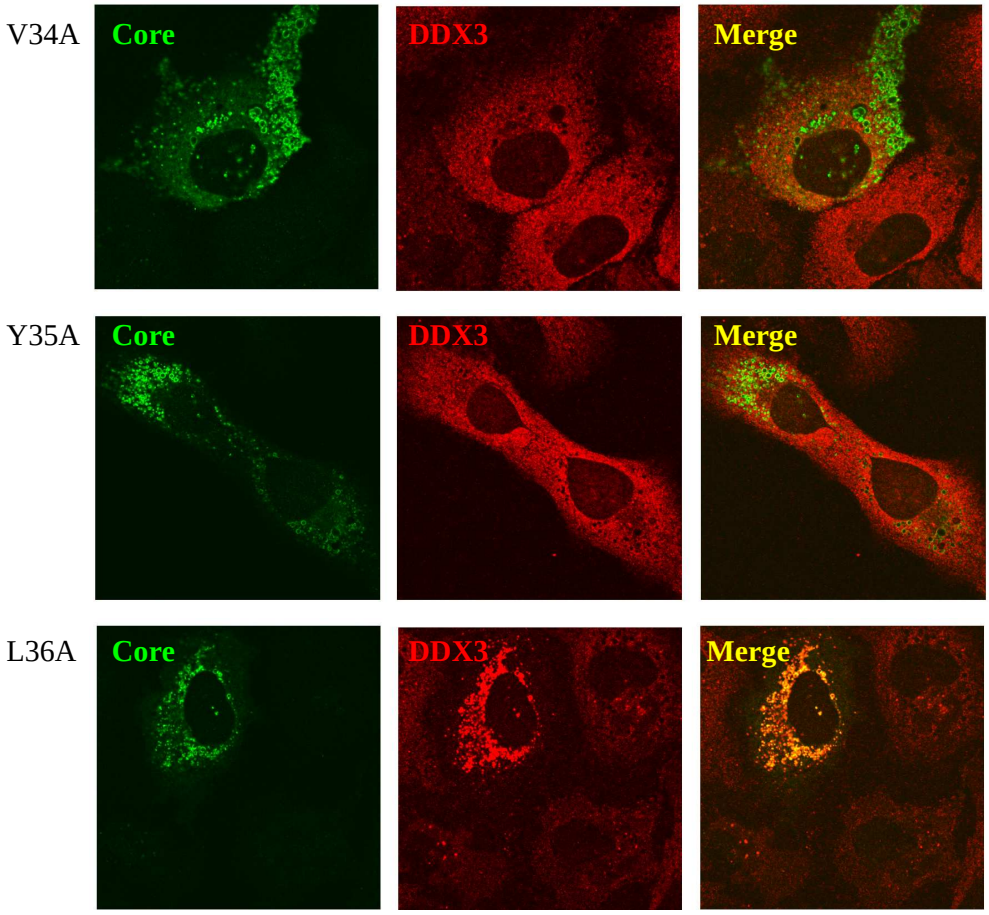


Figure 3.16: Analysis of alanine-substitute mutant core-DDX3 interaction. Using site-directed mutagenesis to introduce nucleotide changes, residues 24-36 of wild type HCV core were sequentially substituted for alanine (within pC-E1-E2). Alanine mutants were then transiently transfected into Huh7 cells. Forty-eight hrs post-transfection, cells were fixed in methanol, permeablised with PBS-T and probed with rabbit polyclonal anti-core antiserum (R308) and mouse monoclonal anti-DDX3 antibody (AO196), followed by anti-rabbit FITC and anti-mouse Cy5, respectively.

3.3 Discussion

The aim of this chapter was to identify individual residues of core protein required for the interaction between core and DDX3. The discovery of such residues would enable further studies on virus replication in which DDX3 did not interact with core protein, allowing direct comparisons of replication kinetics with and without DDX3 interaction. If interaction is essential for viral replication in cell culture, mutant viruses will not replicate. However, it is also possible that the interaction is required for some other process in the virus life cycle such as translation (DDX3 has been shown to rescue yeast lacking the translation factor and DDX3 homologue Ded1p (Mamiya and Worman, 1999)), packaging of viral RNA (the helicase activity of DDX3 may be required for packaging viral RNA into particles) or release of viral RNA in newly infected cells. As well as having an effect on the virus life cycle, it is also possibile that the interaction between core and DDX3 plays a role in the pathogenesis of the virus, doing so by inhibiting some cellular process.

In order to identify critical residues of core required for this interaction it was necessary to generate and test a large number of core mutants. To identify these residues, EP-PCR was used to produce a library of $core_{1-59}$ mutants, each carrying 0-4 amino acid substitutions. Since EP-PCR products carrying premature stop codons or having no amino acid substitutions were of little interest, a method for identifying mutants with amino acid substitutions was required. GFP-display is a technique used for identifying single amino acid substitutions in proteins (Aoki *et al.*, 2000b). Proteins fused to GFP are expressed in bacteria and crude lysates separated by SDS-PAGE in the presence of urea. GFP is stable in the presence of urea (Ward, 1998) and therefore can act as a chemical label attached to the fused protein. The interplay between urea, SDS ions and the strength of binding between the ions and specific amino acids is believed to determine the mobility of the polyprotein through the gel (Aoki *et al.*, 2000b, Aoki *et al.*, 2002). Single amino acid substitutions can therefore alter the mobility of the polyprotein. The $core_{1-59}$ mutants produced by EP-PCR were cloned downstream of GFP in a bacterial expression vector (pKK223-3) and expressed in *E. coli*. Crude bacterial lysates containing GFP-$core_{1-59}$ mutants were separated by SDS-PAGE containing 6 M urea however differences in mobility were not seen between mutants. Using constructs donated by T. Aoki (University of Hokkaido, Japan) -shown to have different mobility patterns under these conditions despite only one amino acid substitution (SpAb and SpAb N214D, (Aoki *et al.*, 2002))- inconsistent mobility patterns were seen. This may simply be due to experimental error as accurate urea concentration is critical for this technique (Aoki *et al.*, 2000b).

To overcome this problem, and still make use of the GFP-core$_{1-59}$ library, an ELISA based assay was designed allowing rapid screening of the mutant library. GST-tagged DDX3C (DDX3 amino acids 409-622) was coated on ELISA plates and incubated with either wild-type core (fused to GFP) or error-prone PCR produced mutant core (also fused to GFP). Bound protein was detected with rabbit polyclonal anti-GFP antiserum followed by protein A-HRP. This assay allowed us to quickly identify possible core mutants with low affinity for DDX3. Although mutants with premature stop codons may also have no affinity for GST-DDX3C, these were easily identified by nucleotide sequence analysis. In total, 9 mutant core proteins were found to have low binding affinity for DDX3 while having no premature stop codons. Upon sub-cloning of these mutant core$_{1-59}$ sequences into a mammalian expression vector (pcDNA 3.1(+)zeo) containing core, E1 and E2 sequence, transient transfection of naïve Huh-7 cells followed by immunofluorescence of transfected cells (probing for core and DDX3), confirmed none of the core mutants were able to interact with cellular DDX3. DDX3 remained diffusely localised throughout the cytoplasm as in non-transfected cells while core showed typical cytoplasmic staining similar to that of wild type core.

Nucleotide sequence analysis showed that a variety of mutations were present in the 9 non-binding mutants. Interestingly, mutant 90 had only one amino acid substitution (I30N), indicating this residue was essential for core-DDX3 interaction. Concentrating on this region of core, it was noted that all mutants had at least one amino acid substitution within a 13-residue area surrounding amino acid 30. To confirm if the substitutions located within this 13-residue region were responsible for abolishing the interaction between core and DDX3, site-directed mutagenesis was used to revert “out-lying” mutations back to wild type. These new core mutants (containing only the mutations found between amino acids 24-36) were transiently transfected into Huh7 cells as before and core-DDX3 interaction analysed by immunofluorescence. Again, none of the 9 mutants were able to interact with DDX3, indicating that the mutations within this 13-residue region of core were responsible for abolishing the interaction with DDX3. In order to identify which of these 13 residues were involved in the interaction between core and DDX3, alanine-scanning mutagenesis was carried out across this region. Each of the 13 residues between amino acids 24 and 36 of core were substituted individually for alanine and transfection studies carried out as before. While 7 of the 13 substitutions had no adverse effect on the interaction between core and DDX3, 6 alanine substitutions abolished the interaction completely. Substituting any one of residues 24, 27, 30, 33, 34 and 35 for alanine was enough to abolish the colocalisation between core and DDX3.

Residues 19-41 of core protein, written on a classical 3.6 amino acids per turn α-helix (the proposed structure of a polypeptide produced by the ribosome before native folding (Lim, 1978)) show the proximity of these 6 essential amino acids to each other (Fig. 3.17). Residues 24, 27, 30, 34 and 35 all reside on the same side of the α-helix, however in contrast, residue 33 appears on the opposite side of the structure. The close proximity of these residues in this α-helix model would suggest that this is indeed the binding site for DDX3. In order to identify any major structural alterations these mutations may have on core, the putative structure of mutant core protein was compared to that of the wild type NMR structures proposed by Ladaviere *et al.* (unpublished data, Protein Data Bank I.D. 1CWX). Ladaviere *et al.* described four different structures using residues 2-45 of core, fortunately however the region of interest in the present study (including a proposed helical structure between residues 30 and 39) was identical in each model. Using the molecular modelling program AMBER 99 (Ponder and Case, 2003) to find the minimum energetic position (minimisation) of each mutant structure, the 6 core mutations were individually inserted into the proposed core structure and compared to that of the wild type model (Fig. 3.18). Put simply, if adjacent amino acids can be imagined jostling for position next to each other, then the minimum energetic position is the most naturally comfortable position for each amino acid in that environment. With the exception of residue 33 which, when mutated to alanine caused a slight “kink” in the helix structure, none of the mutations had any significant effect on the predicted secondary structure of core within this region and all retained the predicted helix (D. Gatherer, personal communication), suggesting that mutation of these residues inhibits interaction with DDX3 not by altering the overall structure of core but instead by alterations to the interacting surface domain required by DDX3. These alterations to the interacting surface domain are caused by the presence of a different side chain when the amino acids are replaced with alanine. Figure 3.19 shows the properties and side chain of each amino acid involved in the interaction as well as that of alanine. Although each amino acid is similar in charge and polarity to alanine (with the exception of tyrosine which is non-polar compared to alanine which is polar), amino acids isoleucine, phenylalanine, tyrosine and valine have large side chains whereas alanine has only a small side chain, suggesting these large side chains may play a role in binding DDX3. It should be noted however that glycine, which is essential at residues 27 and 33 of core (for interaction with DDX3), has a small side chain similar to alanine.

Analysis of 52 core protein amino acid sequences from different genotypes showed that HCV core has a highly conserved amino acid sequence across all genotypes with 85.3% - 100% conservation observed (Bukh *et al.*, 1994). Here, the amino acid sequence of the first 59 residues of core protein were analysed from 112 isolates from all genotypes. This

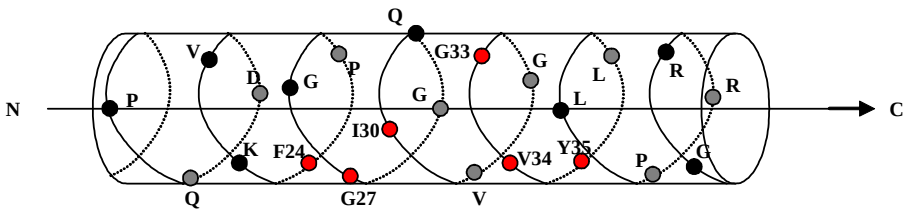


Figure 3.17: Relative positions of HCV core residues required for interaction with DDX3. HCV core residues 19-41 are written on a classical a–helix (3.6 amino acids per turn). Red residues indicate those required for interaction between core and DDX3. Black and grey residues distinguish amino acids on front and rear of 3D helix.

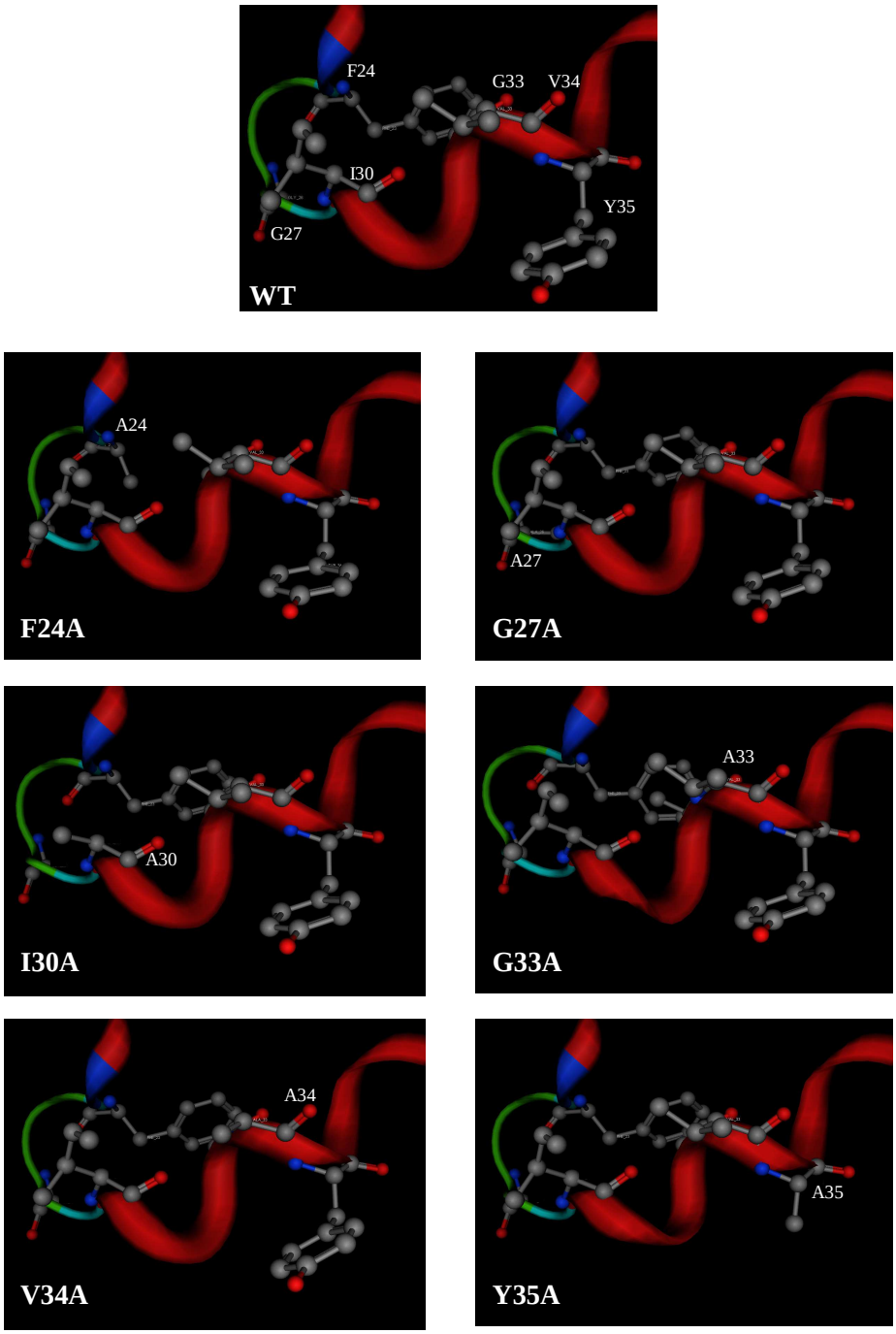


Figure 3.18 : Analysis of predicted structural changes in core between residues 21 and 39 due to alanine mutations. Using an NMR model of core proposed by Ladaviere *et al.* (Protein Data Bank I.D. 1CWZ), amino acids 24, 27, 30. 33, 34 and 35 were individually mutated to alanine and mutant structures minimised by AMBER99 to find the minimum energetic position of each residue.

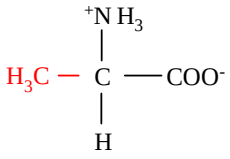
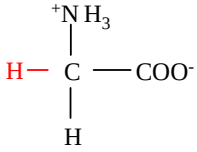
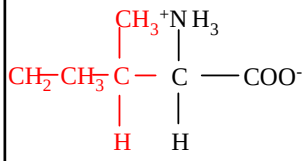
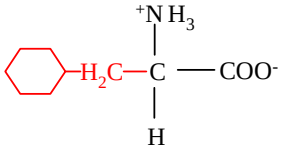
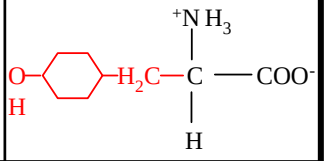
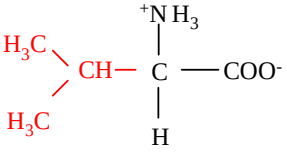
Amino acid	Side chain polarity	Side chain acidity or basicity	Structure
Alanine	Non-polar	Neutral	
Glycine	Non-polar	Neutral	
Isoleucine	Non-polar	Neutral	
Phenylalanine	Non-polar	Neutral	
Tyrosine	Polar	Neutral	
Valine	Non-polar	Neutral	

Figure 3.19: Properties of the amino acids essential for interaction with DDX3 and comparison with alanine. Structures in red indicate amino acid side chains.

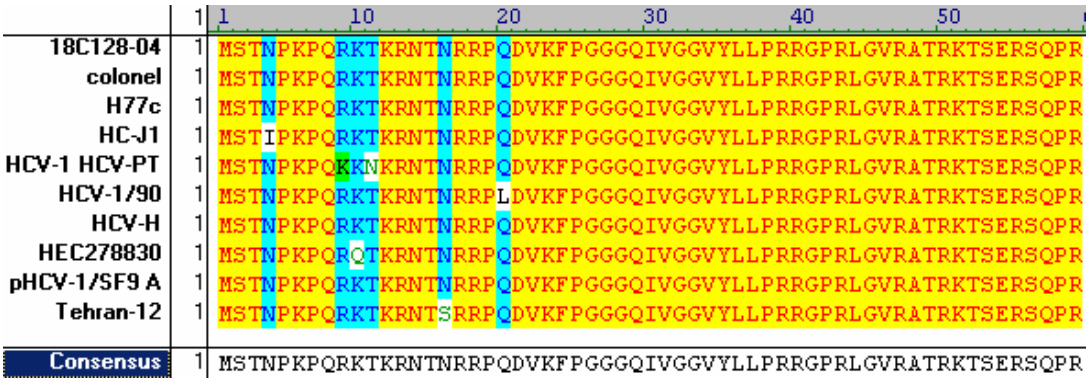
region was found to be highly conserved, with intra-genotypic identity ranging from 79.7% (genotype 3b) to 98.3% (genotype 2c) (Fig. 3.20). Interestingly, of 104 isolates analysed covering all genotypes (excluding those from genotype 3b), the 6 residues shown to be required for the interaction between core and DDX3 were 100% conserved (Fig. 3.21). Analysis of the 8 genotype 3b isolates showed some interesting results. Four of the isolates had 100% conservation of the essential amino acids 24, 27, 30, 33, 34 & 35 while the other 4 had a glycine to glutamic acid substitution at residue 33. As well as this substitution at residue 33, these 4 isolates also share common substitutions at residues 26 (Gly – Arg), 43 (Arg – Gln) and 48 (Ala – Glu) (Fig. 3.21), however the infectivity of these isolates is unknown.

The highly conserved nature of these 6 residues indicates that the interaction between core protein and cellular DDX3 may play some essential role in the virus life cycle. In the following chapter, these identified residues of core are introduced into the newly described infectious JFH-1 system in order to study the effects the abolition of this interaction has on virus infectivity, replication and particle release.

Genotype	Identity
1a	89.8%
1b	86.4%
1c	94.9%
2a	89.8%
2b	96.6%
2c	98.3%
3a	89.8%
3b	79.7%
4	94.9%
5	96.6%
6	89.8%

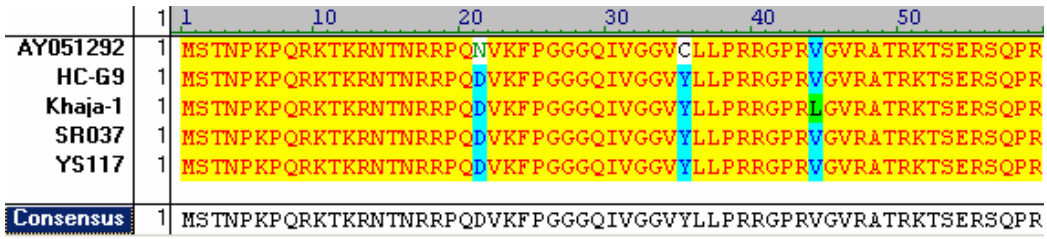
Figure 3.20: Amino acid identity within HCV genotypes. Amino Acids 1-59 of HCV core protein isolates were analysed for identity with other isolates from the same genotype. Analysis was carried out using Vector NTI (Invitrogen).

Genotype 1a

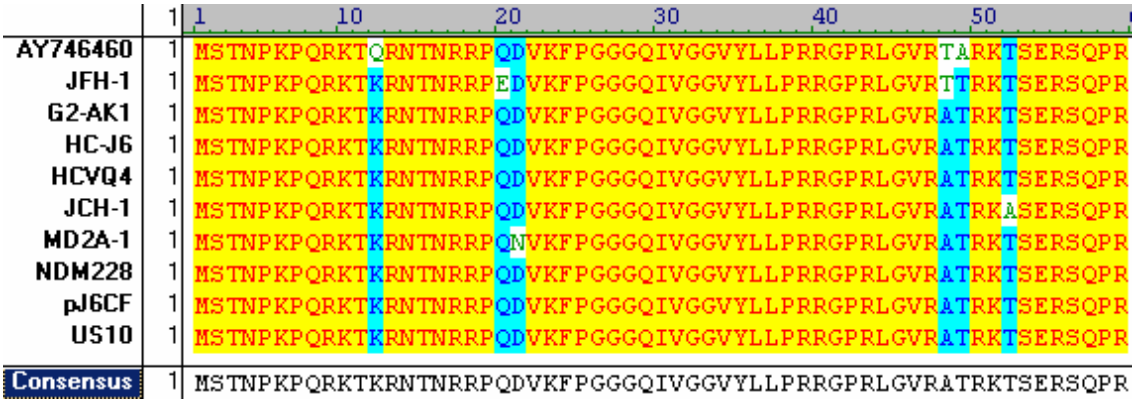


Genotype 1b

Genotype 1c

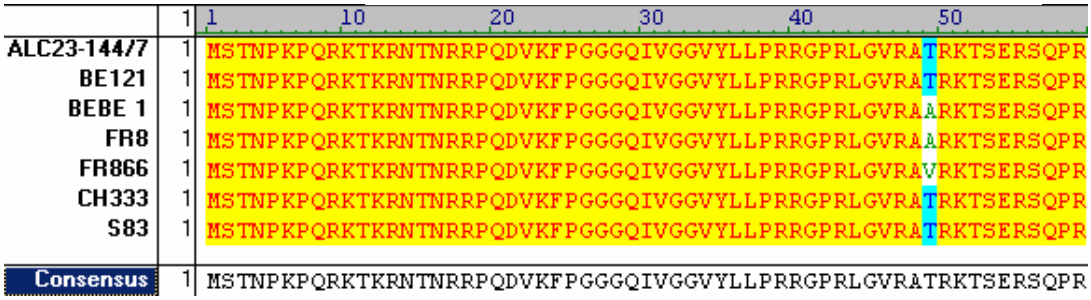


Genotype 2a

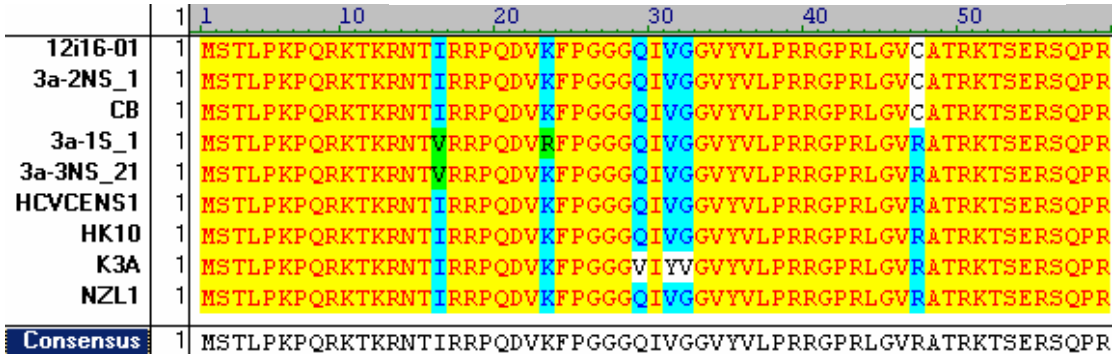


Genotype 2b

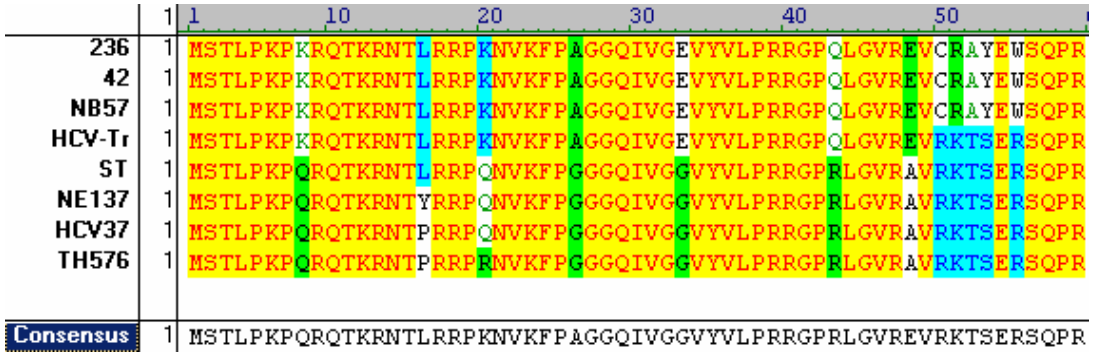
Genotype 2c



Genotype 3a



Genotype 3b



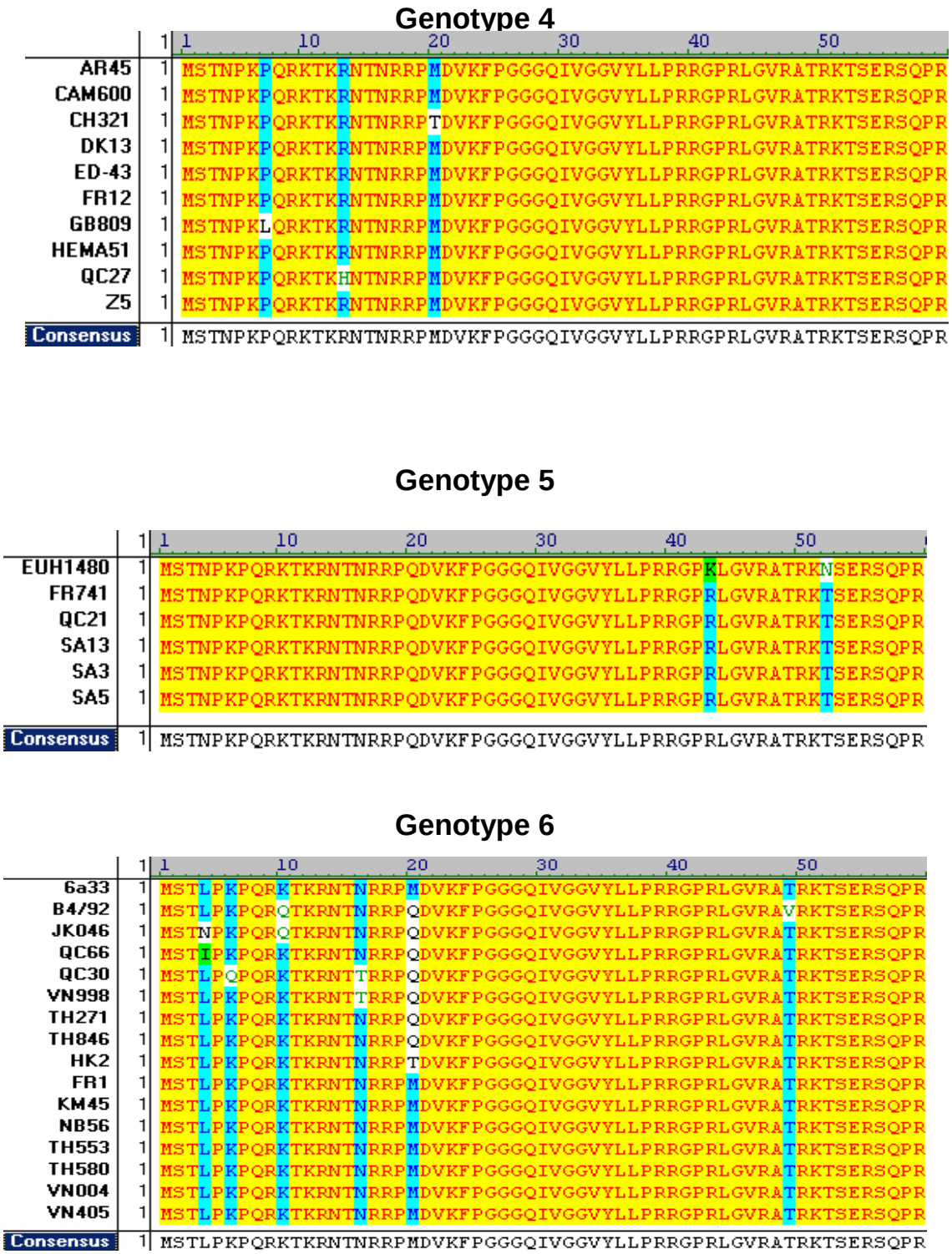


Figure 3.21: Alignment of HCV core residues 1-59. HCV core isolates were aligned by genotype using Vector NTI software (Invitrogen). Black residues on green background represent similar residues, green residues on white background represent weakly similar residues and black residues on white background represent non-similar residues.

4. Analysis of Critical HCV Core Residues in an Infectious Cell Culture System

4.1 Introduction

The lack of cell culture or small animal model for HCV infection has made studying the virus life cycle and virus-host interactions extremely difficult. Most HCV studies so far have made use of infected patients (Thimme *et al.*, 2001, Takaki *et al.*, 2000, Lechner *et al.*, 2000), chimpanzees (Logvinoff *et al.*, 2004, Bukh, 2004, Shoukry *et al.*, 2004, Thimme *et al.*, 2002) and more recently replicon systems (Ikeda *et al.*, 2002, Lohmann *et al.*, 1999). Replicon systems have allowed translation and replication studies to be performed, as well as leading to the elucidation of some of the virus-host interactions required for these processes (Gale, 2003, Katze *et al.*, 2002, Dubuisson and Rice, 1996, Kapadia and Chisari, 2005). The major drawbacks of the replicon system are that replication is very inefficient without adaptive mutations (Lohmann *et al.*, 2001, Blight *et al.*, 2000) and infectious virions are not produced. Recently however, Wakita and colleagues (Kato *et al.*, 2001, Kato *et al.*, 2003) have developed an HCV genotype 2a replicon (strain JFH-1) that can replicate efficiently in a variety of hepatic and non-hepatic cell lines without requiring adaptive mutations. Since this report, a number of publications have presented data describing robust HCV infection *in vitro*, resulting in the production of infectious virus particles (Lindenbach *et al.*, 2005, Wakita *et al.*, 2005, Zhong *et al.*, 2005). This data collectively shows that *in vitro* transcribed JFH-1 RNA can be electroporated into naïve Huh-7 cells and produce 10^4-10^5 infectious units per ml of culture supernatant. These particles can then infect naïve cells, spreading throughout the culture and can be serially passaged without loss in infectivity.

The development of this JFH-1 system has allowed most aspects of the HCV life cycle to be studied, from receptor binding, through translation and replication to particle assembly and release. Of particular interest, the consequences of interrupting virus-host interactions can now be studied in the context of the life cycle of the fully infectious virus. The previous chapter identified critical residues of core protein required for the interaction between core and DDX3. In order to study the effects this interaction has upon the virus life cycle, we aimed to introduce these critical mutations individually into JFH-1 clones. This chapter details the establishment of the JFH-1 cell culture system in our hands as well as comparing and contrasting the differences between wild type JFH-1 and JFH-1 clones containing the mutations described in the previous chapter. Site-directed mutagenesis was used to introduce specific substitutions into JFH-1 clones and viral RNA produced by *in vitro* transcription. This RNA was electroporated into naïve Huh7 cells and immunofluorescence analysis performed to study the cellular localisation of HCV core

protein and HCV mutant core proteins, as well as to look at the effect these proteins have on the cellular distribution of DDX3. Reverse-transcription PCR (RT-PCR) was used to detect negative strand HCV specific RNA in total cell extracts, indicative of viral genome replication, and Western immuno-blot analysis used to detect viral proteins in infected cell extracts. Production of infectious particles was studied by harvesting culture medium from infected cultures and using this filtered medium to infect naïve Huh-7 cells. Infection was confirmed by immunofluorescence and Western immuno-blot detection of viral proteins and detection of replicating negative strand viral RNA by RT-PCR. Finally, relative quantities of RNA were calculated using Real-Time PCR in order to detect subtle differences between the replication efficiencies of wild type and mutant viruses.

The first part of this chapter focuses on setting up the JFH-1 infectious system. HCV JFH-1 RNA was *in vitro*-transcribed by T7 polymerase from linearised plasmid DNA and electroporated into Huh7 cells. The presence of HCV proteins in Huh7 cells was confirmed by immunofluorescence and Western immuno-blot analysis while replicating HCV RNA was detected by RT-PCR. Colocalisation between HCV JFH-1 core and cellular DDX3 was also confirmed by immunofluorescence. Upon confirmation of replicating HCV RNA and HCV protein production, the secretion of infectious particles was confirmed by infection of naïve Huh7 cells with filtered medium from electroporated cells and subsequent detection of HCV specific proteins and RNA.

In the second part of this chapter, the 6 mutations found to abolish genotype 1a core-DDX3 interaction were individually transferred into JFH-1 (genotype 2a) backgrounds by site-directed mutagenesis. Mutant constructs were *in vitro* transcribed and RNA electroporated into naïve Huh7 cells. Cells carrying electroporated viral RNA were analysed for the effects on HCV core and cellular DDX3 localisation, viral RNA replication and production of infectious particles. By immunofluorescence, mutant core protein was analysed for its ability to bind DDX3 and for its ability to be targeted to the lipid droplets while replication of mutant JFH-1 RNA was studied by RT-PCR. Mutant RNA was then tested for its ability to produce infectious particles and their replication efficiency compared to that of wild type JFH-1.

This comprehensive analysis of mutant and wild-type virus will contribute to the understanding of the interaction between core and DDX3 in terms of virus replication and progeny particle production.

4.2 Results

4.2.1 Electroporation of In Vitro Transcribed JFH-1 RNA and Analysis of Viral Protein Expression and RNA Replication

Before looking at the effects the 6 HCV core mutations may have on the virus life cycle it was important to first analyse the behaviour of wild type JFH-1 in our hands. As a negative control for all experiments, JFH-1 GND was used. JFH-1 GND contains a mutation (GDD-GND) in the active site of the NS5B polymerase, blocking replication. Plasmids pJFH-1 and pJFH-1 GND (kindly donated by T. Wakita, Tokyo Metropolitan Institute for Neuroscience, Japan) were linearised (see Materials and Methods section) and 1μg of each linearised plasmid *in vitro*-transcribed using Megascript (Ambion), following the manufacturers instructions. Following *in vitro* transcription and DNA digestion, RNA was precipitated and resuspended in 20μl RNA Storage Solution (Ambion). RNA was stored at -70°C.

For each electroporation (using a BioRad GenePulser Xcell), $5x10^6$ Huh7 cells were used. Cells were trypsinised, washed twice with PBS and resuspended in 400 μl PBS before addition of 5 μg *in vitro*-transcribed RNA. Electroporation (270 V, 960 μF) was carried out using a 4 mm gap cuvette. Electroporated cells were resuspended in 10 ml DMEM (+ additives) and plated out on coverslips, 35 mm dishes and in flasks.

Seventy-two hours post-electroporation, coverslips were fixed using methanol. In order to confirm that electroporation of HCV RNA had been successful, coverslips were probed for core and E2 using appropriate antibodies. In the case of Huh7 cells electroporated with JFH-1 wild type RNA, approximately 5-10 % of cells were positive for core and E2 (Fig. 4.1). No cells electroporated with the replication deficient JFH-1 GND RNA were positive for viral proteins. To confirm that core was targeted to the lipid droplets as described previously (Moradpour *et al.*, 1996), cells were probed for core and cellular ADRP (Adipocyte differentiation-related protein) which is only found on lipid droplets (Brasaemle *et al.*, 1997) and therefore commonly used as a marker for these structures. As expected, core protein colocalised with ADRP, indicating that JFH-1 core protein is targeted to the surface of lipid droplets (Fig. 4.2). Again, no core protein was detected in cells electroporated with JFH-1 GND RNA. Finally, to study the effect JFH-1 core protein had on cellular DDX3, cells were probed for core and DDX3. Figure 4.3 confirms that JFH-1 core protein also sequesters cellular DDX3 in a similar manner while in cells

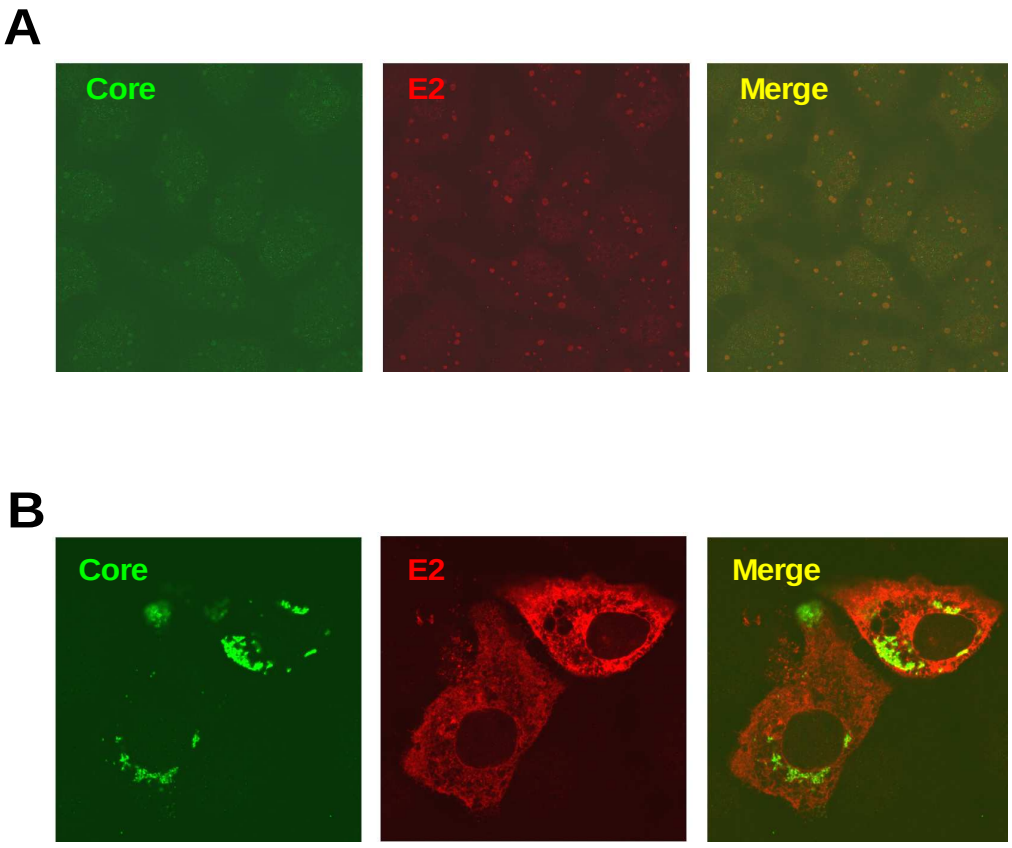


Figure 4.1: Detection of HCV structural proteins in Huh7 cells electroporated with HCV JFH-1 RNA. Naïve Huh7 cells were electroporated with 5 μg of *in vitro* transcribed JFH-1 RNA. Seventy-two hours post-electroporation, cells were fixed and probed with rabbit polyclonal anti-core antiserum (R308) and mouse monoclonal anti-E2 antibody (AP33) as in Chapter 3. A) Huh7 cells electroporated with HCV JFH-1 GND RNA. B) Huh7 cells electroporated with HCV JFH-1 RNA.

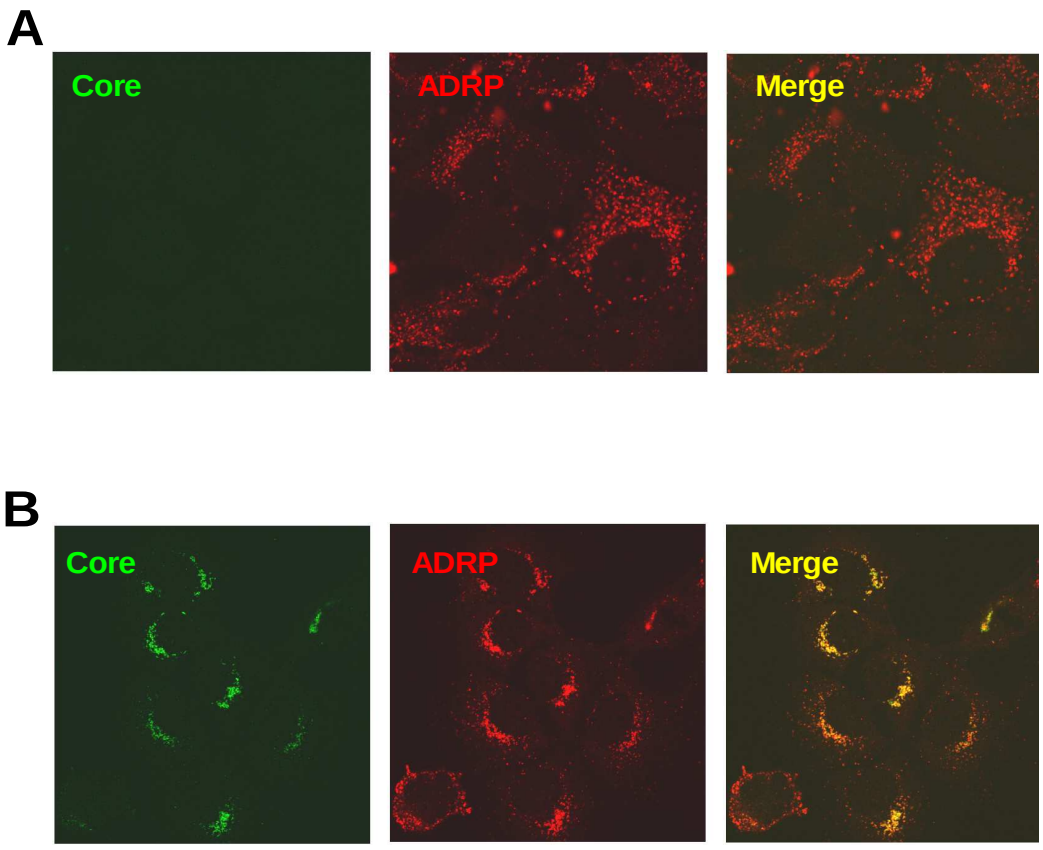


Figure 4.2: Co-localisation of core protein with ADRP in Huh7 cells electroporated with HCV JFH-1 RNA. Huh7 cells were electroporated with 5µg of *in vitro* transcribed JFH-1 RNA. Seventy-two hours post-electroporation, cells were fixed and probed with rabbit polyclonal anti-core antiserum (R308) and sheep polyclonal anti-ADRP antiserum as in Chapter 3. A) Huh7 cells electroporated with HCV JFH-1 GND RNA. B) Huh7 cells electroporated with HCV JFH-1 RNA.

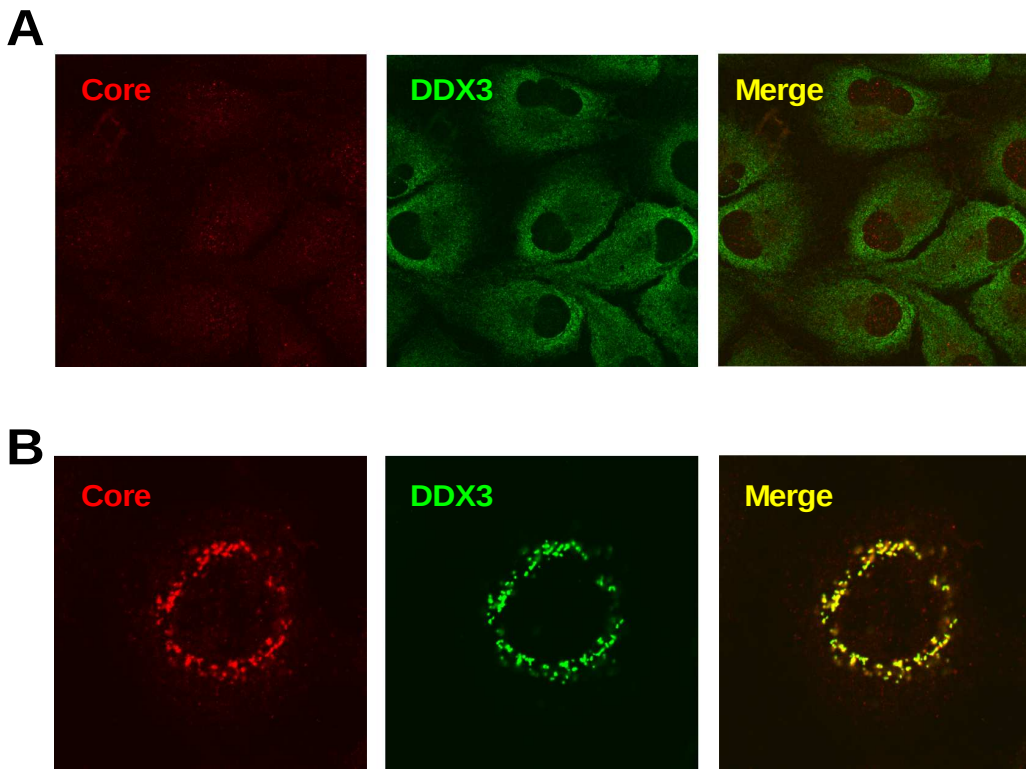


Figure 4.3: Co-localisation of core protein with DDX3 in Huh7 cells electroporated with HCV JFH-1 RNA. Huh7 cells were electroporated with 5 μg of *in vitro* transcribed JFH-1 RNA. Seventy-two hours post-electroporation, cells were fixed and probed with rabbit polyclonal anti-core antiserum (R308) and mouse monoclonal anti-DDX3 antibody (AO196) as in Chapter 3. A) Huh7 cells electroporated with HCV JFH-1 GND RNA. B) Huh7 cells electroporated with HCV JFH-1 RNA.

electroporated with JFH-1 GND RNA, DDX3 remains located throughout the cytoplasm. Due to excessive fluorescence emmited from the punctate spots of DDX3 upon colocalisation with core in wild-type samples, the gain of the FITC laser was turned down when viewing DDX3 in wild-type samples throughout the remainder of this thesis to avoid excessive fluorescence from DDX3. In doing this, diffuse cytoplasmic DDX3 staining in non-infected cells surrounding those expressing HCV proteins could not be seen. This leads to the question of whether, in the presence of core, diffuse cytoplasmic staining of DDX3 remains as well as that seen colocalising with core (suggesting an up-regulation of DDX3 expression), or if DDX3 is simply redistributed from its diffuse cytoplasmic localisation to colocalise with core. Evidence from previous studies in our laboratory indicate that DDX3 expression is not upregulated in core-expressing cells and that DDX3 is indeed sequestered from its normal cellular location. This is confirmed by the fact that DDX3 protein levels are similar in both JFH-1 wild-type and JFH-1 GND samples in this work, as shown by Western blot (Figs. 4.6, 4.9, 4.14). To confirm that JFH-1 core sequesters DDX3 to lipid droplets, cells were probed for DDX3 and ADRP. Figure 4.4 confirms that when DDX3 is found in punctate cytoplasmic spots (indicative of colocalisation with HCV core protein), DDX3 colocalises with ADRP, confirming that HCV core sequesters DDX3 to lipid droplets. In the case of the JFH-1 GND electroporated cells, there was no colocalisation between DDX3 and ADRP.

To confirm that HCV RNA was replicating in electroporated cells, confluent 35 mm dishes were harvested for total RNA 72 hrs post electroporation. Dishes were washed with PBS and cells lysed with 750 µl TRIzol. Total RNA was extracted from cells (see methods) and stored at -70°C. Reverse transcription PCR (RT-PCR) was used to detect replicating viral RNA. To confirm the RNA detected was replicating RNA as opposed to input RNA, a strand-specific primer was used for RT which binds negative strand HCV RNA. Detection of negative strand RNA provides confirmation that replication is occurring as this is only produced during replication. Using primer JFH-1 NegRT (TTGCGAGTGCCCCGGGA), identical to nucleotides 304-320 of the JFH-1 genomic sequence, cDNA was produced by reverse transcription. cDNA was then used to amplify full-length core sequence by PCR using primers JFH-1 RTPCR1 (GGTCTCGTAGACCGTGCACC) and JFH-1 RTPCR2 (GTATTCTTCACCTGGGCAGC). Figure 4.5 shows that a band of correct size (610 nt) was only detected in cells electroporated with JFH-1 RNA and not JFH-1 GND RNA, confirming that viral RNA replication occurs in JFH-1 electroporated cells.

Electroporated cells were also harvested in LB2 (see materials) 72 hrs post-electroporation for Western immuno-blot analysis. Cell lysate was separated by 10% SDS-PAGE, blotted

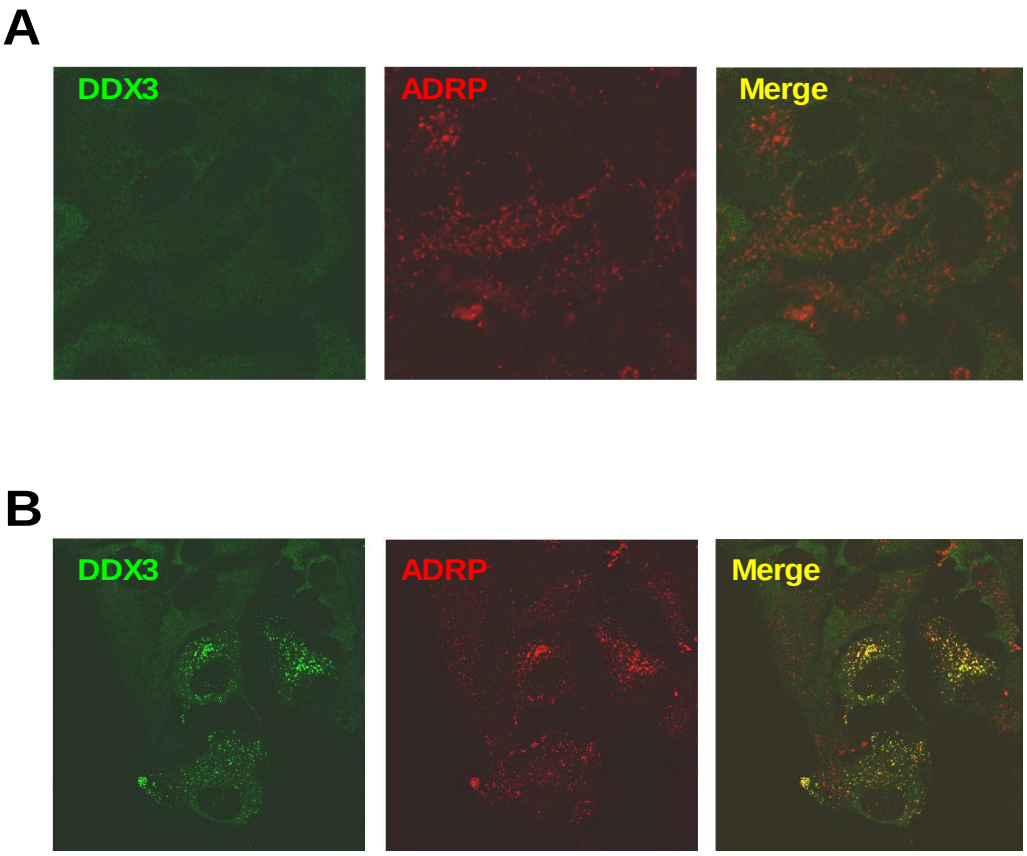


Figure 4.4: Co-localisation of DDX3 with ADRP in Huh7 cells electroporated with HCV JFH-1 RNA. Huh7 cells were electroporated with 5 µg of *in vitro* transcribed JFH-1 RNA. Seventy-two hours post-electroporation, cells were fixed and probed with mouse monoclonal anti-DDX3 antibody (AO196) and sheep polyclonal anti-ADRP antiserum as in Chapter 3. A) Huh7 cells electroporated with HCV JFH-1 GND RNA. B) Huh7 cells electroporated with HCV JFH-1 RNA.

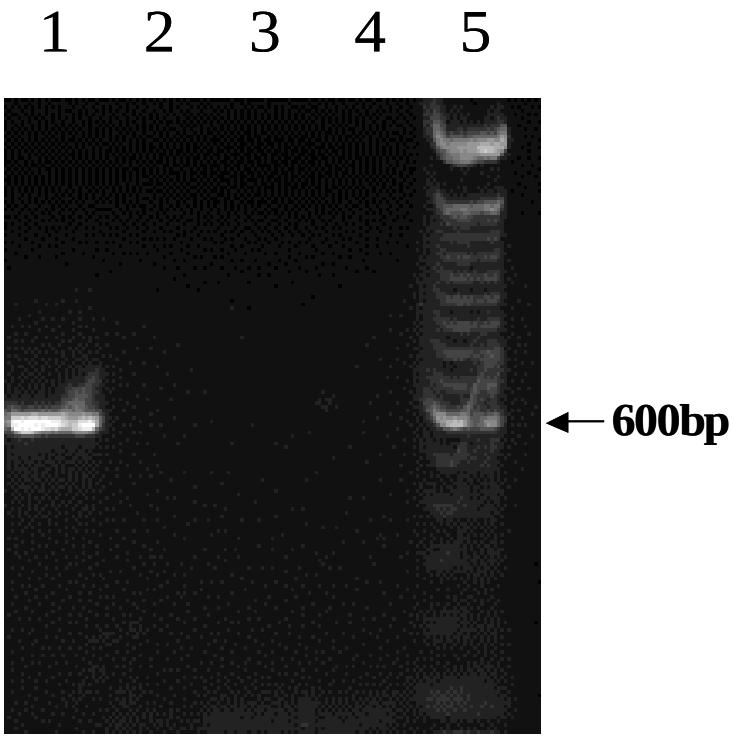


Figure 4.5: Detection of cDNA, generated from negative strand HCV RNA in Huh7 cells electroporated with HCV JFH-1 RNA. Huh7 cells were electroporated with 5 µg of *in vitro* transcribed JFH-1 RNA. Seventy-two hours post-electroporation, cells were washed with PBS, lysed using TRIzol (Invitrogen) and total RNA extracted. RNA was reverse transcribed using primer JFH-1 NegRT and resultant cDNA amplified by PCR using core primers JFH-1 RTPCR1 and JFH-1 RTPCR2. Lane 1 – cDNA generated from JFH-1 electroporated cells, lane 2 – cDNA generated from JFH-1 GND electroporated cells, lane 3 – No RT control (RNA extract from JFH-1 electroporated cells with no reverse transcription stage), lane 4 – H_2O control reaction, lane 5 – 100 bp DNA ladder (Invitrogen).

on nitrocellulose membranes and probed with various antibodies. Firstly, membranes were probed for viral proteins core, E2 and NS5A. In all 3 cases, a band of correct size was detected from the cell lysate electroporated with JFH-1 RNA but not from that electroporated with JFH-1 GND RNA (Fig. 4.6). DDX3 levels in JFH-1 and JFH-1 GND electroporated cells were also studied. DDX3 was detected in both cell lysates (Fig. 4.6).

4.2.2 Production of Infectious HCV from In Vitro Transcribed JFH-1 RNA

Wakita *et al.* (2005) showed that cells electroporated with JFH-1 RNA secrete infectious HCV particles into the culture medium and these particles are capable of infecting naïve cells. In order to confirm this in our hands, medium was collected from electroporated cells 72 hrs post-electroporation and filtered through a 0.45 μm filter to remove cells and cellular debris. Naïve Huh7 cells were plated out at $5x10^4$/ml and the following day incubated with filtered medium for 3 hrs at 37ºC before washing with PBS and overlay with appropriate culture medium. Seventy-two hrs post-infection, cells were washed in PBS and fixed with methanol. Cells were probed for core and DDX3. Cells infected with medium from JFH-1 electroporated cells were positive for core protein indicating these cells had been successfully infected with HCV particles. As expected, no core protein was present in cells infected with culture medium from JFH-1 GND electroporated cells. In cells infected with HCV particles, core protein colocalised with DDX3 in a similar way to that in electroporated cells (Fig. 4.7).

Cells infected with the same medium were also harvested 72 hrs post-infection for RT-PCR using TRIzol. Total RNA was extracted and, using primer JFH-1 NegRT to reverse transcribe negative strand, replication intermediate viral RNA, and primers JFH-1 RTPCR1 and JFH-1 RTPCR2 to amplify the resultant cDNA by PCR, replication was detected in cells infected with JFH-1 culture medium but not in cells infected with that of JFH-1 GND (Fig. 4.8).

Finally, cells infected with culture medium were harvested 72 hrs post infection using LB2 for Western immuno-blot analysis. Cell lysates were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed for viral proteins core, E2 and NS5A, followed by the appropriate secondary antibodies. As in the case of electroporated cells (Fig. 4.6), viral proteins core, E2 and NS5A could be detected in cells infected with JFH-1

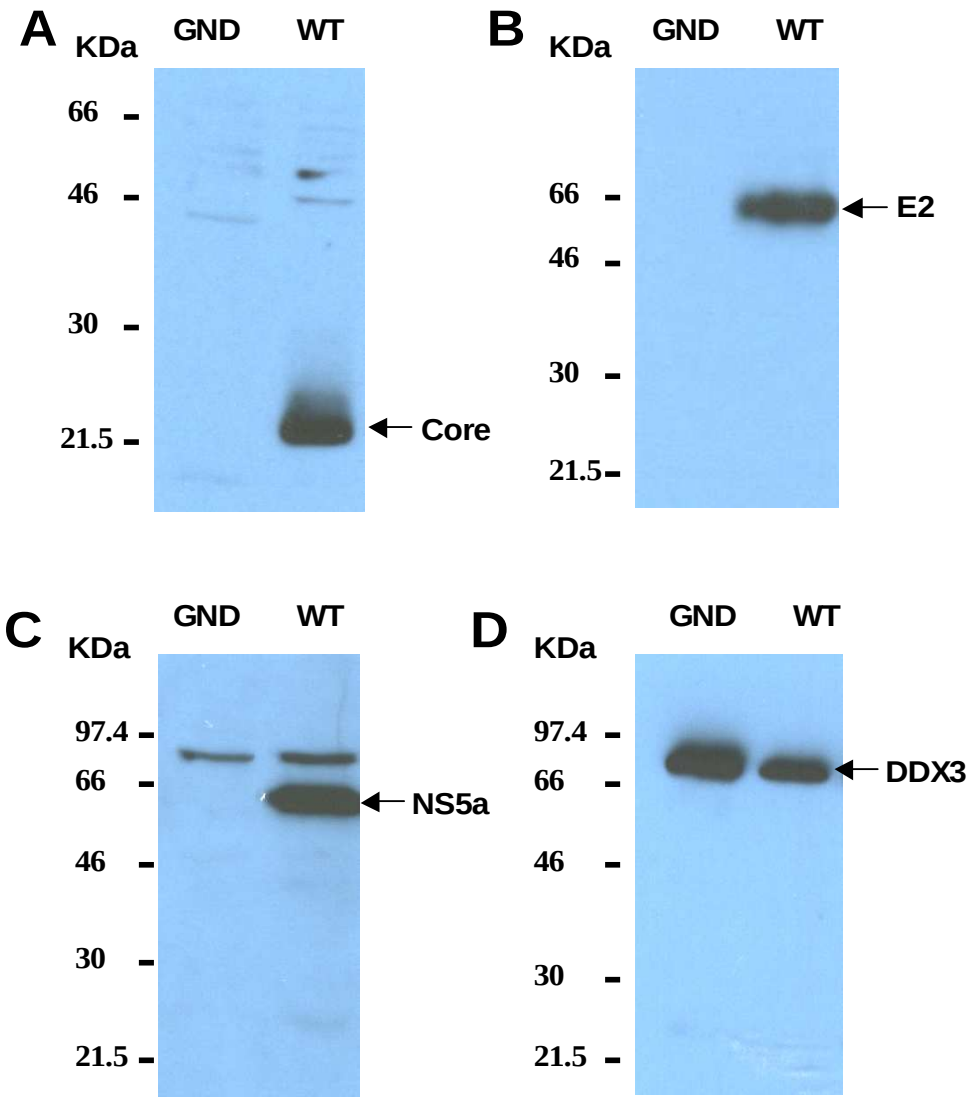


Figure 4.6: Detection of HCV viral proteins in Huh7 cells electroporated with HCV JFH-1 RNA. Huh7 cells were electroporated with 5 µg of *in vitro* transcribed JFH-1 RNA. Seventy-two hours post-electroporation, cells were washed wth PBS and harvested using cell lysis buffer LB2. Total cell extracts were separated by SDS-PAGE (10%), transferred to nitrocellulose membrane and immunoblotted with A) rabbit polyclonal anti-core antiserum (R308), B) mouse monoclonal anti-E2 antibody (AP33), C) sheep polyclonal anti-NS5A antiserum, and D) rabbit polyclonal anti-DDX3 antiserum (R648).

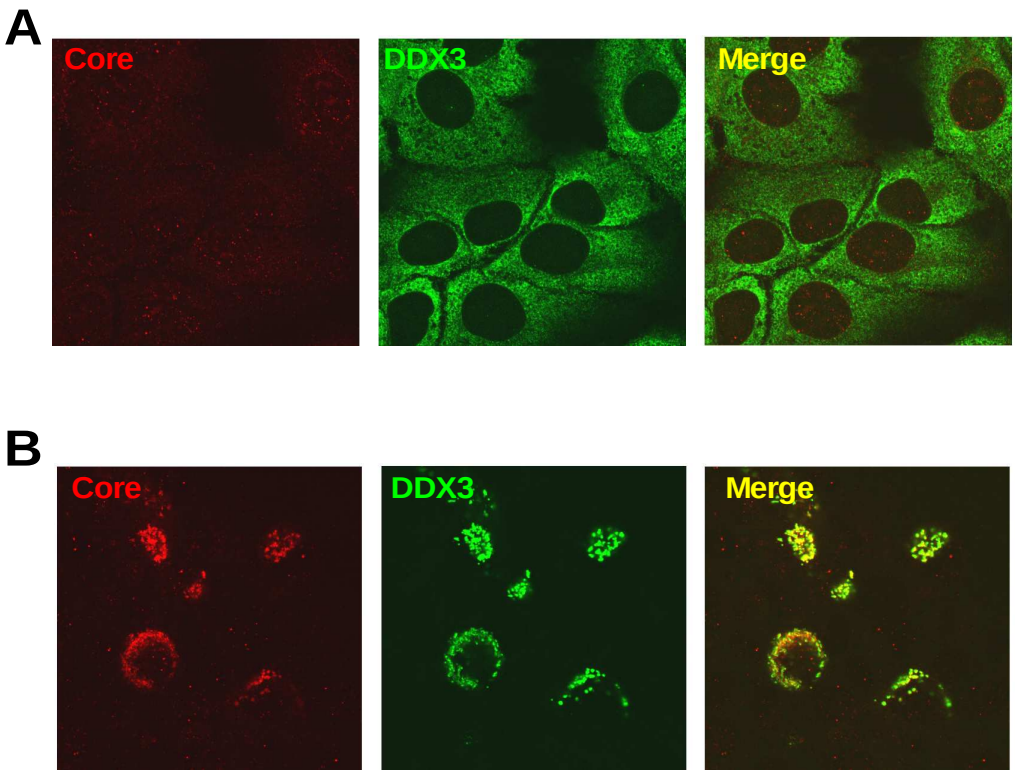


Figure 4.7: Huh7 cells electroporated with HCV JFH-1 RNA secrete infectious HCV particles into the culture supernatant. Huh7 cells were electroporated with 5 µg of *in vitro* transcribed JFH-1 RNA. Seventy-two hours post-electroporation, medium was harvested, 0.45 µm filtered and incubated with naïve Huh7 cells for 3 hrs at 37°C before washing with PBS and overlay with appropriate culture medium. Seventy-two hours post-infection, cells were fixed and probed with rabbit polyclonal anti-core antiserum (R308) and mouse monoclonal anti-DDX3 antibody (AO196) as in Chapter 3. A) Cells incubated with supernatant from HCV JFH-1 GND RNA electroporated Huh7 cells, B) cells incubated with supernatant from HCV JFH-1 RNA electroporated Huh7 cells.

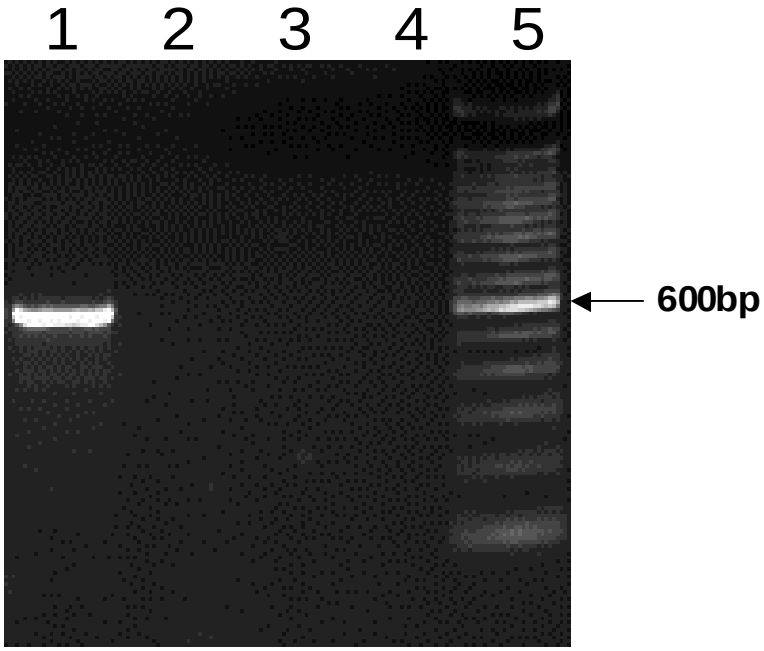


Figure 4.8: Detection of cDNA, generated from negative strand HCV RNA in Huh7 cells infected with medium from HCV JFH-1 RNA electroporated cells. Medium from Huh7 cells electroporated with 5 µg of *in vitro* transcribed JFH-1 RNA was incubated with naïve Huh7 cells for 3 hrs at 37°C followed by washing with PBS and overlay with appropriate culture medium. Seventy-two hours post-infection, total RNA was extracted, reverse transcribed and resultant cDNA amplified as in Fig. 4.5. Lane 1 – cDNA generated from JFH-1 electroporated cells, lane 2 – cDNA generated from JFH-1 GND electroporated cells, lane 3 – No RT control (RNA extract from JFH-1 electroporated cells with no reverse transcription stage), lane 4 – H_2O control reaction, lane 5 – 100 bp DNA ladder (Invitrogen).

culture medium but not in cells infected with that of JFH-1 GND (Fig. 4.9). Again, DDX3 could be detected at similar levels in both cell lysates using mouse monoclonal anti-DDX3 antibody AO196.

4.2.3 Analysis of HCV Core Mutants in HCVcc Infectious System

4.2.3.1 Cloning of Mutant Core Protein into JFH-1 Background

The 6 residues of HCV core shown to abolish core-DDX3 interaction (Chapter 3) were discovered using a genotype 1a (H77c strain) core protein. As H77c and JFH-1 have 96.6% homology within the first 59 amino acids of core, it was assumed the same residues of JFH-1 core protein would be responsible for the interaction with DDX3. To confirm this, the 6 residues of core shown to abolish core-DDX3 interaction were individually changed to alanine within the JFH-1 construct in order to study the effects of these mutations had on core localisation, core-DDX3 interaction and the life cycle of JFH-1. Site-directed mutagenesis was used to introduce alanine substitutions at the target sites in core. As site-directed mutagenesis involves amplifying the whole template by PCR, it is possible that unwanted errors occur during amplification. The construct containing the full-length JFH-1 sequence (pJFH-1) is over 12 kb in length and is therefore not suitable for nucleotide sequence analysis to check for unwanted PCR errors. To overcome this, a smaller fragment of JFH-1 core sequence was sub-cloned into pGEMT and site-directed mutagenesis carried out in this construct. Nucleotides 1-2614 were amplified from pJFH-1 cDNA by PCR using primers JFH-12 (GCCAGTGAATTCTAATACGAC, *Eco*RI restriction site underlined) and AP357 (CATATGCATGAATTCTCTAGATTATGCTTCGGCCTG GCCCAA, *Eco*RI restriction site underlined). Upon restriction digest with *Eco*RI, the PCR product was cloned into pGEMT (pGEMT JFH-1 1-2614) and sequence confirmed by nucleotide sequence analysis. The 6 alanine substitutions (F24A, G27A, I30A, G33A, V34A and Y35A) were then individually introduced into pGEMT JFH-1 1-2614 by site-directed mutagenesis and confirmed by nucleotide sequence analysis. Nucleotides 1-1369 of JFH-1 (including desired mutation) were then sub-cloned back into pJFH-1 using restriction sites *Eco*RI and *Bsi*WI. Mutant pJFH-1 was then linearised, *in vitro* transcribed and electroporated into naïve Huh7 cells (see Materials and Methods).

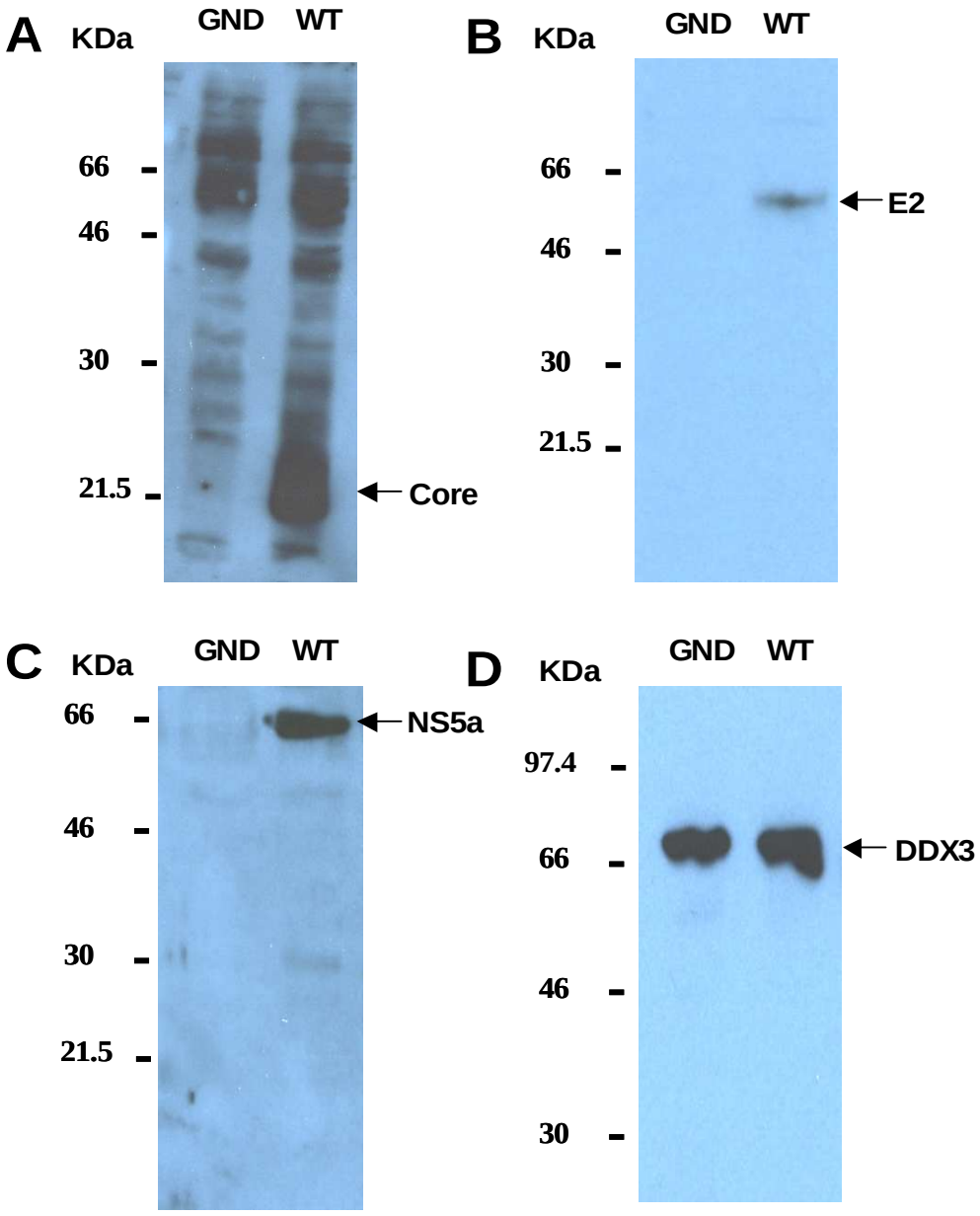


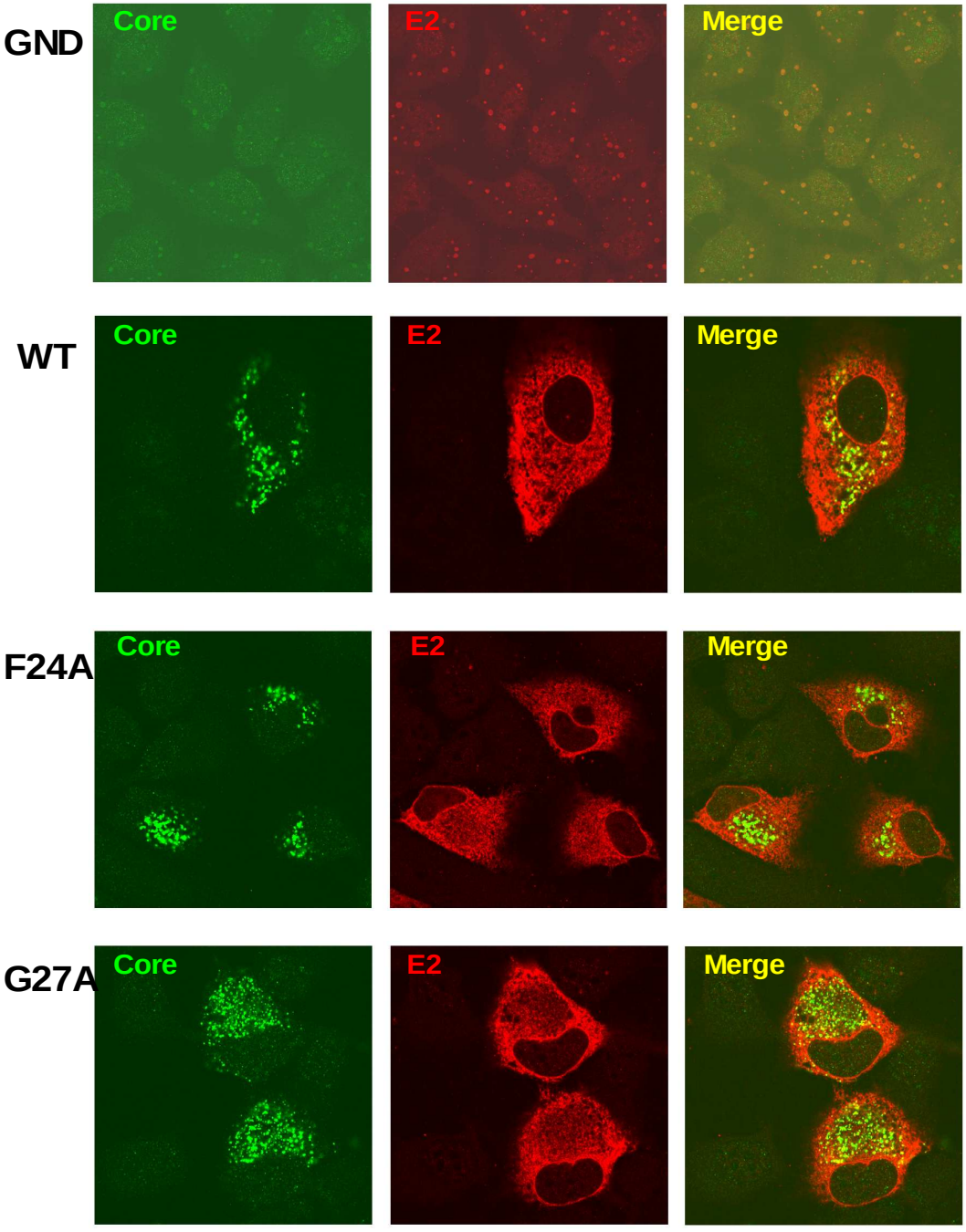
Figure 4.9: Detection of HCV proteins in Huh7 cells infected with medium from HCV JFH-1 RNA electroporated cells. Medium from Huh7 cells electroporated with 5 µg of *in vitro* transcribed JFH-1 RNA was incubated with naïve Huh7 cells for 3 hrs at 37°C followed by washing with PBS and overlay with appropriate culture medium. Seventy-two hours post-infection cell lysate was used for Western immunoblotting as in Figure 4.6. A) rabbit polyclonal anti-core antiserum (R308), B) mouse monoclonal anti-E2 antibody (AP33), C) sheep polyclonal anti-NS5A antiserum, and D) rabbit polyclonal anti-DDX3 antiserum (R648).

4.2.3.2 Cellular Localisation of Mutated JFH-1 Core Protein and Interaction with DDX3

Naïve Huh7 cells, electroporated with 5 μg of mutant HCV JFH-1 RNA and plated on coverslips, were washed with PBS and fixed in methanol 72 hours post-electroporation. To confirm that electroporation of mutant JFH-1 RNA had been successful, cells were permeablised with PBS-T and probed for core and E2. As seen before (Fig. 4.1), cells electroporated with wild type JFH-1 RNA showed typical E2 staining throughout the cytoplasm and globular core staining, also in the cytoplasm (Fig. 4.10). Analysis of cells electroporated with mutant JFH-1 RNA showed similar results. For all 6 JFH-1 core mutants, E2 was distributed throughout the cytoplasm and core had a globular cytoplasmic localisation (Fig. 4.10). One interesting observation however, was the fact that while cultures electroporated with mutants F24A, G27A, I30A, V34A and Y35A showed clusters of infected cells, mutant G33A culture presented only with isolated groups of one or two infected cells. As expected, no viral proteins were detected in cells electroporated with JFH-1 GND cDNA.

To determine if the 6 residues of core were critical for the interaction between core and DDX3 in the context of HCV genotype 2a JFH-1 strain, electroporated cells were probed for core and DDX3. While wild type JFH-1 core protein colocalised with cellular DDX3, mutant core proteins did not (Fig. 4.11), thus confirming the results shown in the transient transfection system using genotype 1a H77c strain. In each case, mutant JFH-1 core was seen to have a typical globular cytoplasmic localisation while DDX3 was diffusely located throughout the cytoplasm similar to its distribution in naïve Huh7 cells. The exception to this was JFH-1 mutant V34A. Some cells electroporated with JFH-1 V34A showed colocalisation between core and DDX3 while others did not (Fig. 4.11 V34A (i) & (ii)).

Wild type core protein is targeted to the lipid droplets by motifs located in domain 2 of core protein (Hope and McLauchlan, 2000b). As the 6 alanine substitutions introduced here are all in domain 1 of core, these mutations are not expected to interfere with the targeting of core to the lipid droplets. To confirm this, electroporated cells were probed for core and ADRP. All 6 mutant core proteins colocalised with ADRP in a similar pattern to that of wild type core (Fig. 4.12), confirming that the mutations in core had no effect on its targeting to the lipid droplets.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E2
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WT
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F24A
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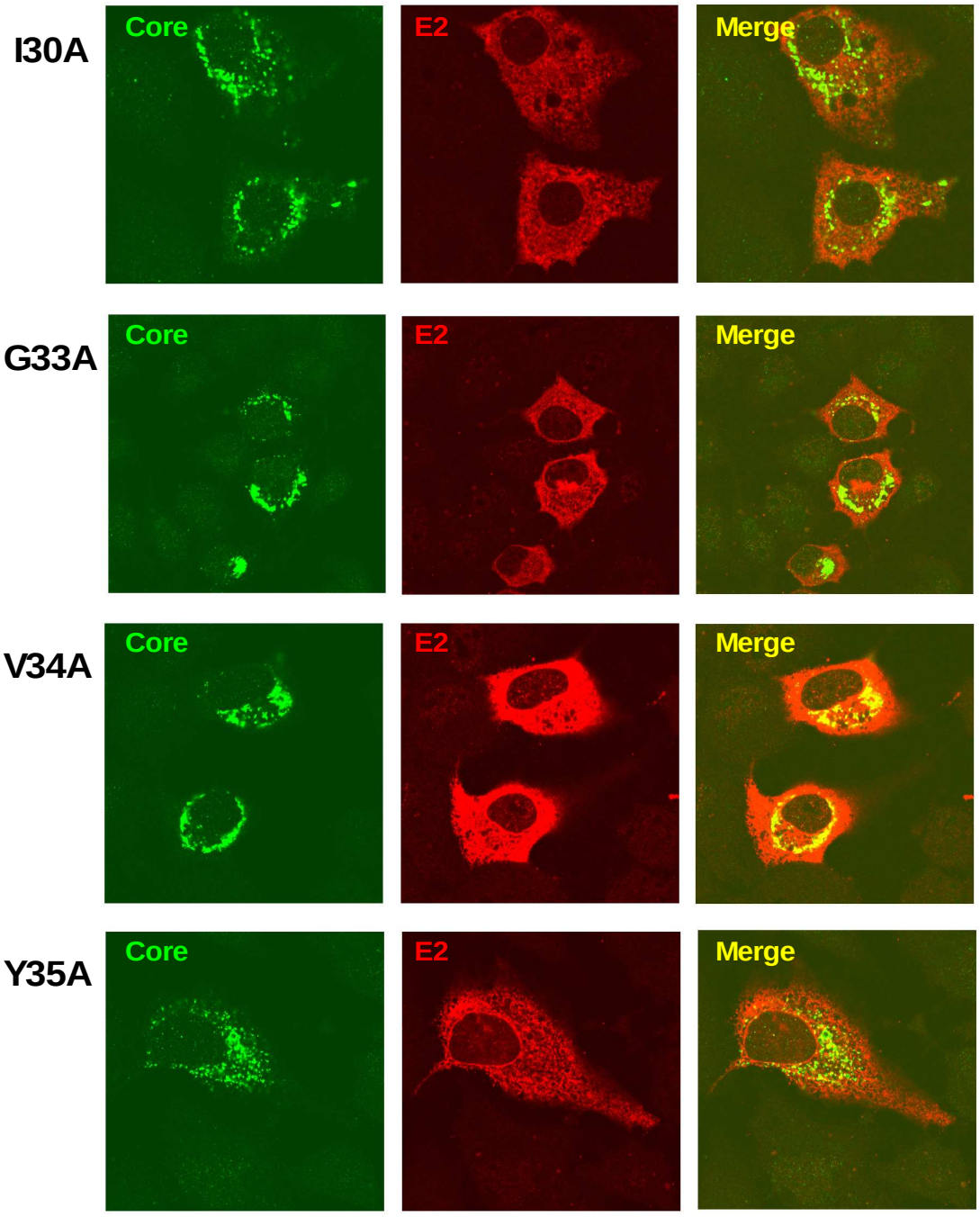
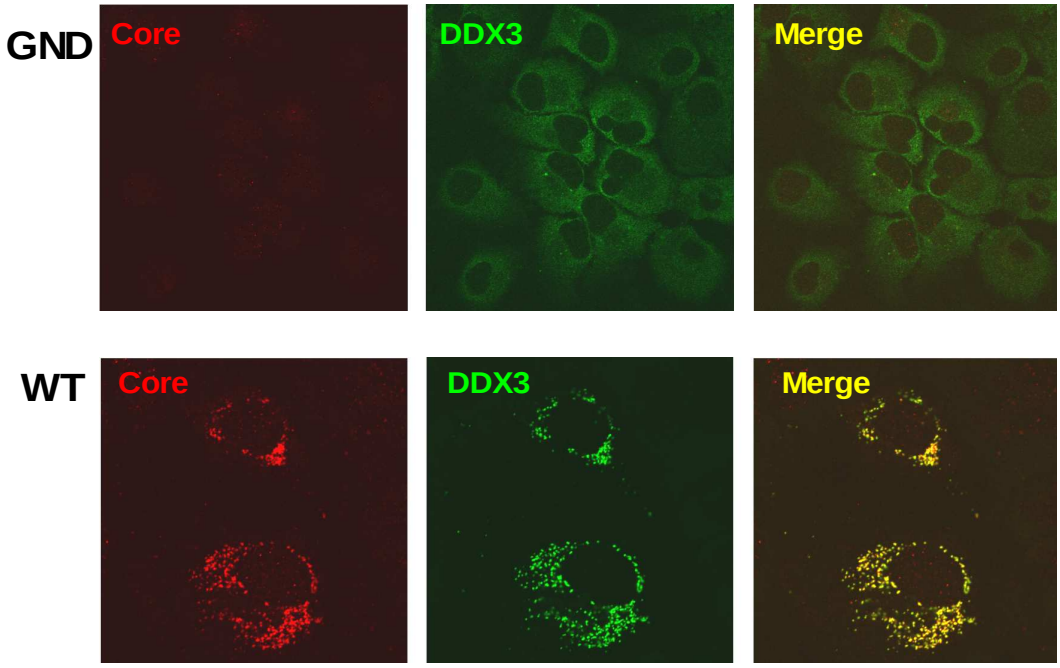


Figure 4.10: Detection of HCV structural proteins in Huh7 cells electroporated with mutant HCV JFH-1 RNA. Huh7 cells were electroporated with 5 µg of *in vitro* transcribed mutant JFH-1 RNA. Seventy-two hours post-electroporation, cells were fixed and probed with rabbit polyclonal anti-core antiserum (R308) and mouse monoclonal anti-E2 antibody (AP33) as in chapter 3.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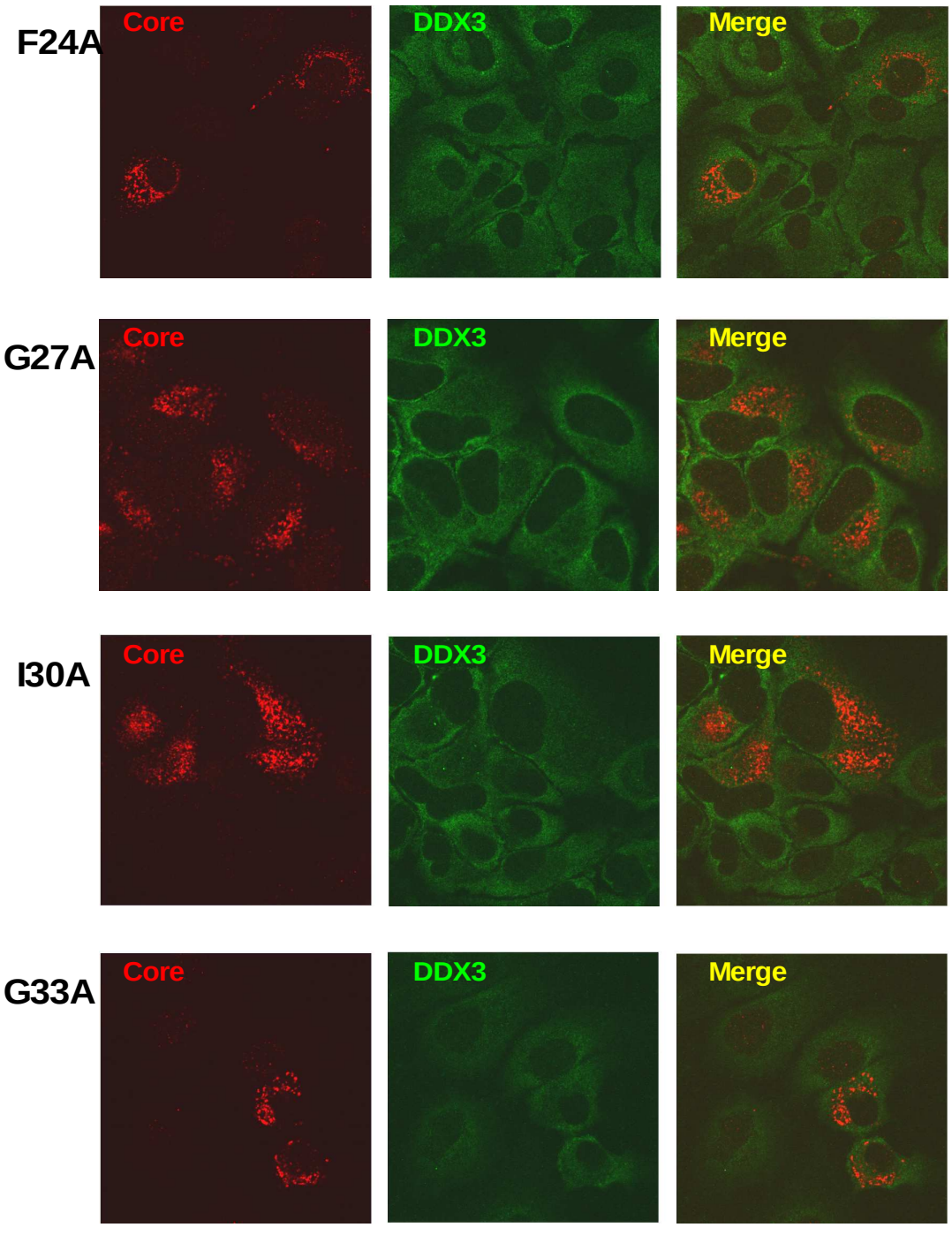
F24A
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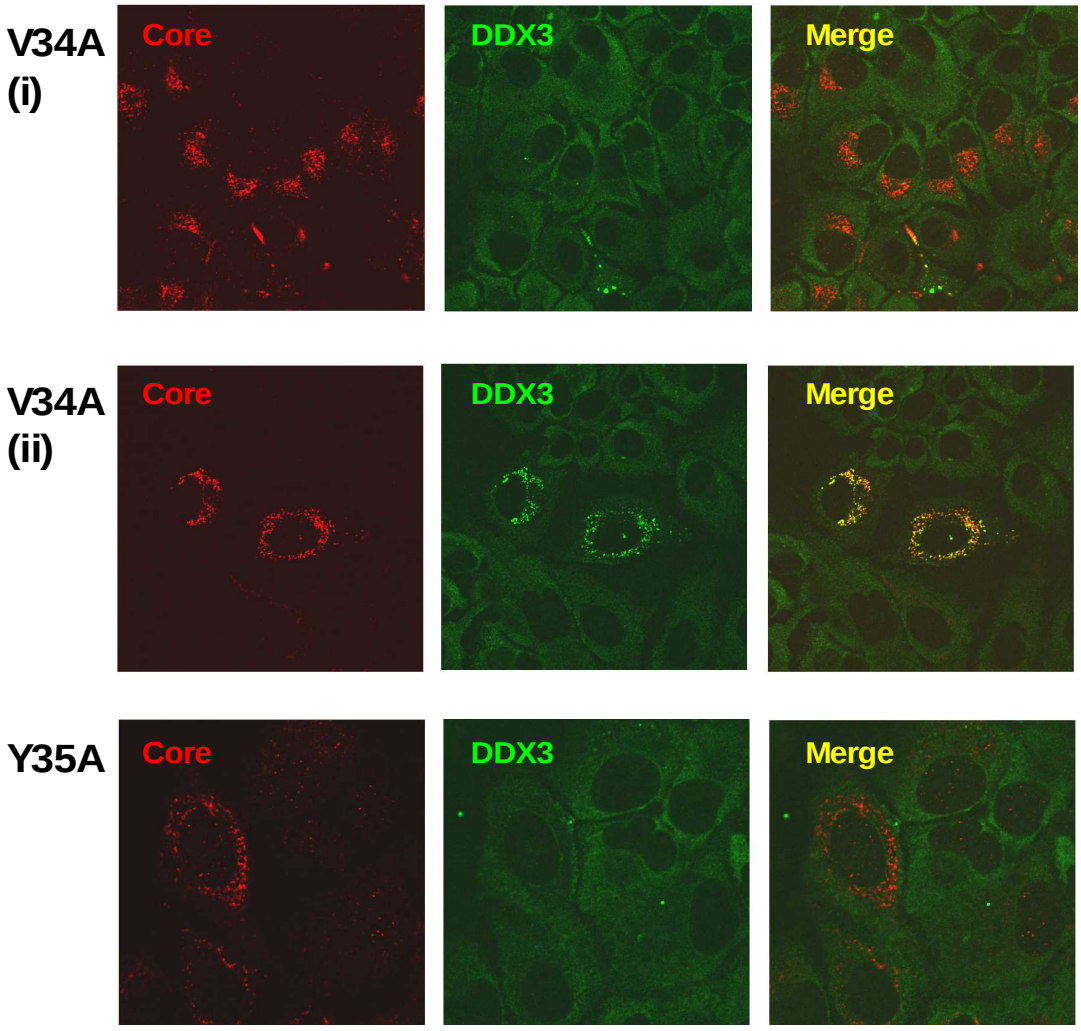
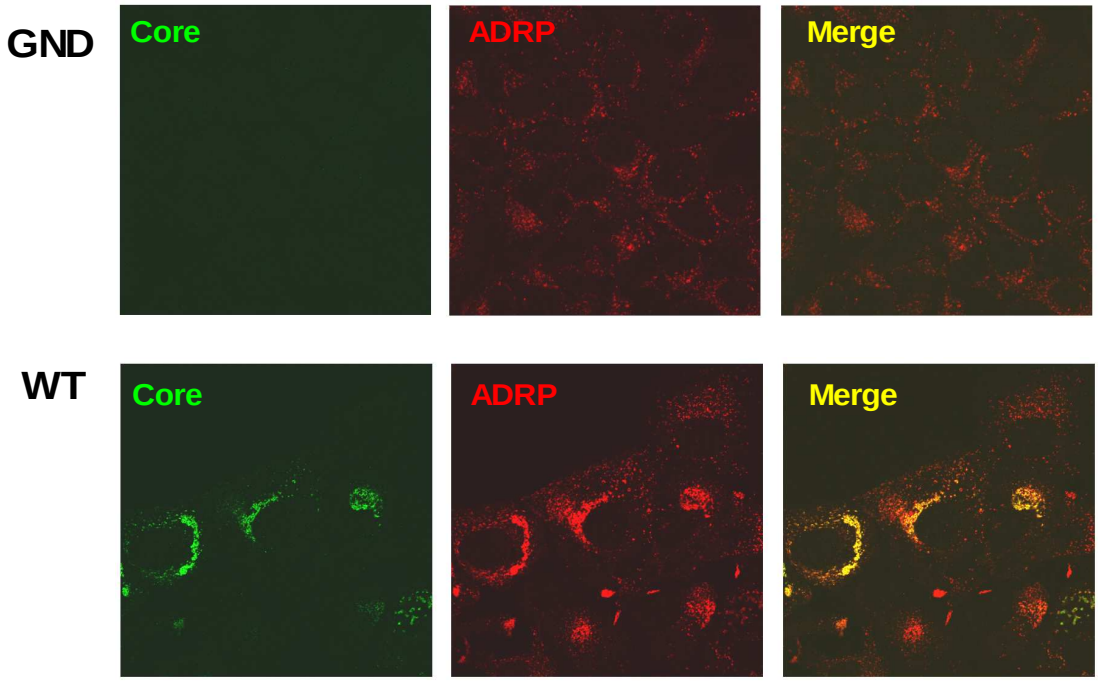


Figure 4.11: Co-localisation of core protein with DDX3 in Huh7 cells electroporated with mutant HCV JFH-1 RNA. Huh7 cells were electroporated with 5 µg of *in vitro* transcribed mutant JFH-1 RNA. Seventy-two hours post-electroporation, cells were fixed and probed with rabbit polyclonal anti-core antiserum (R308) and mouse monoclonal anti-DDX3 antibody (AO196) as in Chapter 3.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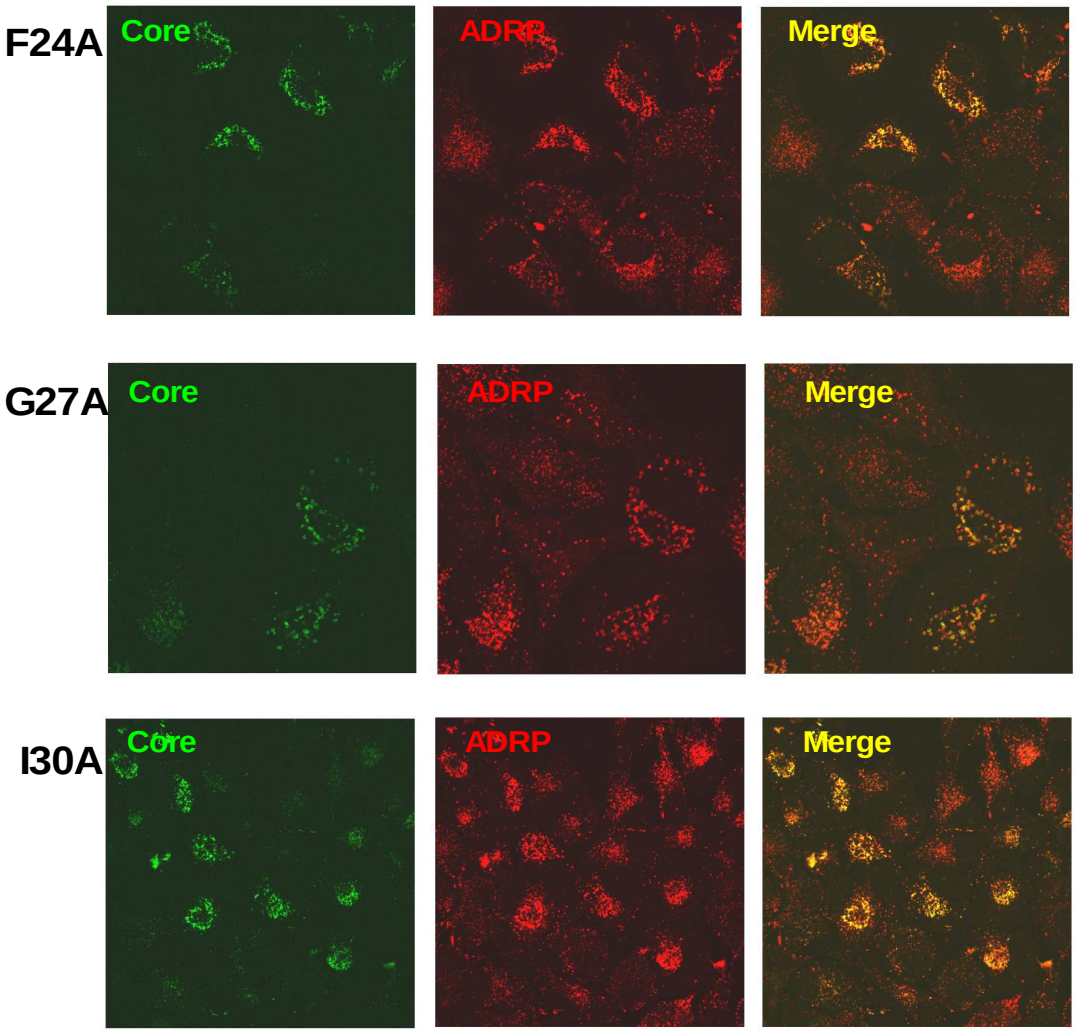
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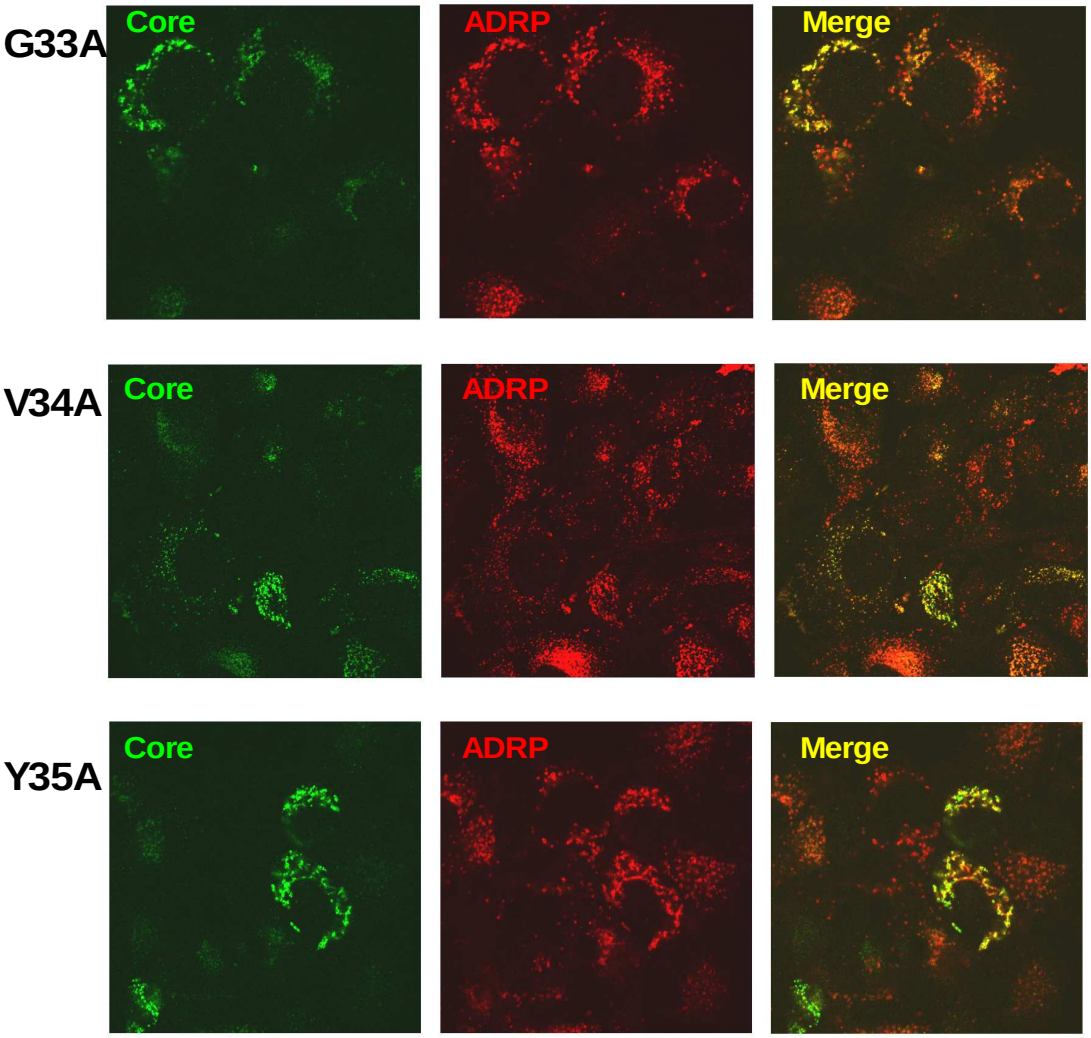


Figure 4.12: Co-localisation of core protein with ADRP in Huh7 cells electroporated with mutant HCV JFH-1 RNA. Huh7 cells were electroporated with 5 µg of *in vitro* transcribed mutant JFH-1 RNA. Seventy-two hours post-electroporation, cells were fixed and probed with rabbit polyclonal anti-core antiserum (R308) and sheep polyclonal anti-ADRP antiserum as in Chapter 3.

4.2.3.3 Replication of Mutant JFH-1 RNA

To confirm that mutant JFH-1 RNA was replicating in electroporated cells, RT-PCR was used to detect negative strand, replication intermediate viral RNA. Electroporated cells were washed with PBS, lysed in TRIzol and total RNA extracted for RT-PCR (see methods). Using primer JFH-1 NegRT to reverse transcribe negative strand RNA and primers JFH-1 RTPCR1 and JFH-1 RTPCR2 to PCR amplify the resultant cDNA, replication intermediate negative strand JFH-1 RNA was detected in the case of all mutants (Fig. 4.13), suggesting that abolishing the interaction between HCV core and DDX3 does not prevent replication of viral RNA.

4.2.3.4 Detection of Viral Proteins by Western Immuno-Blot Analysis

To confirm that viral proteins were being processed correctly from translated mutant JFH-1 RNA, Western immuno-blot analysis was performed to study the expression of viral proteins core, E2 and NS5A. As shown in Fig 4.14 (A-C), correctly sized bands representing core, E2 and NS5A were seen in all mutant JFH-1 electroporated cell lysates. Interestingly, lysate from cells electroporated with JFH-1 G33A RNA showed reduced levels of core, E2 and NS5A. The lower levels of viral proteins observed in mutant G33A lysate may be accounted for by the observation that while large clusters of infected cells were seen in other mutant transfected cell cultures (by immunofluorescence), only isolated groups of one or two cells were seen with G33A cultures (section 4.2.3.2). This would explain the differences observed by Western blot analysis, as it would seem there are less infected cells in the G33A culture resulting in less total viral protein expression. This difference was not observed when analysing viral RNA replication, possibly due to excessive PCR cycles being used, thus masking the result. Cellular levels of DDX3 were similar for all samples, ruling out any possibility of experimental error (Fig. 4.14D).

4.2.3.5 Analysis of Mutant Virus Infectivity

The above results suggest that the interaction between core and DDX3 is not essential for viral RNA replication or polyprotein processing. It is possible however that the interaction is required for either the production of progeny particles or their ability to infect naïve

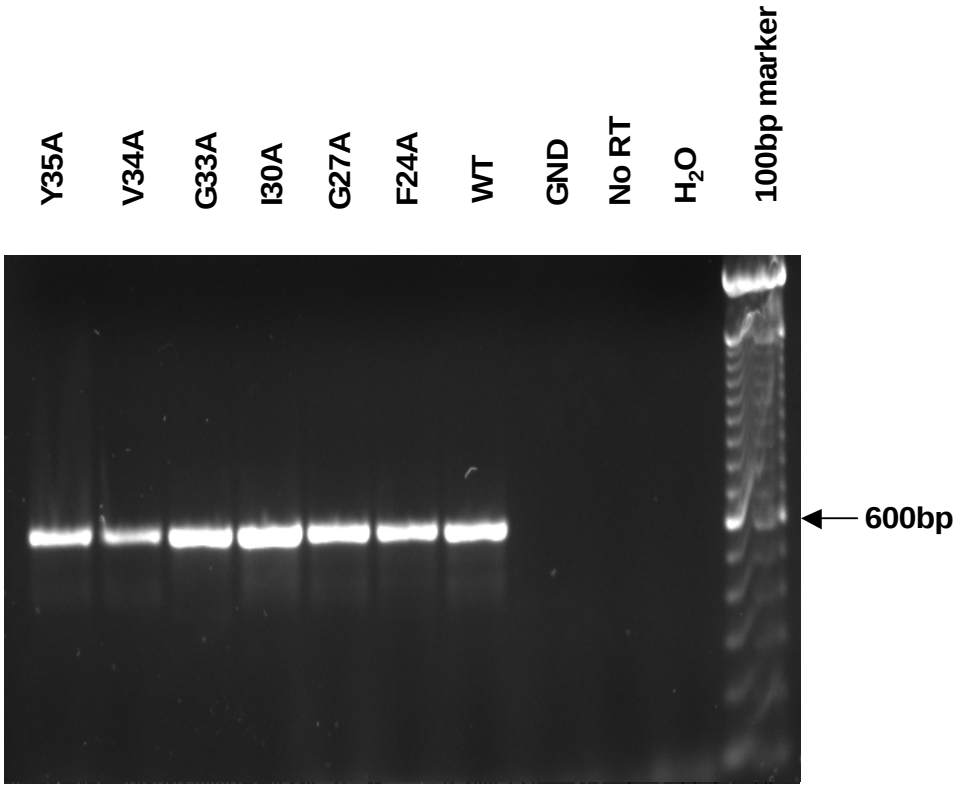
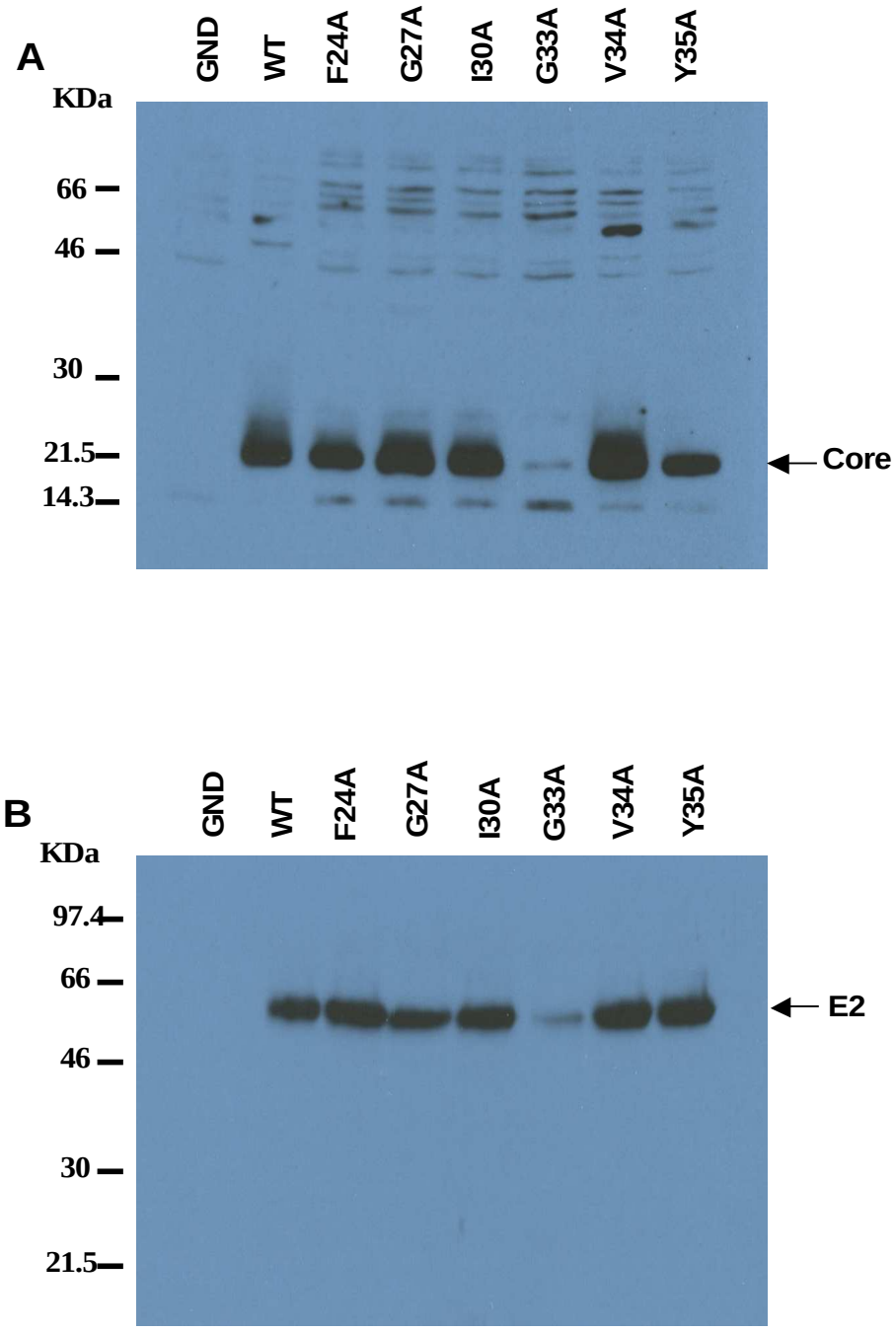


Figure 4.13: Detection of cDNA, generated from negative strand HCV RNA in Huh7 cells electroporated with mutant HCV JFH-1 RNA. Huh7 cells were electroporated with 5 µg of *in vitro* transcribed mutant JFH-1 RNA. Seventy-two hours post-electroporation, total RNA was extracted, reverse transcribed and resultant cDNA amplified as in Figure 4.5. "No RT" is a PCR control with no RT stage using RNA extracted from JFH-1 electroporated cells, H_2O is a no RNA control reaction.


A
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G27A
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G33A
V34A
Y35A
KDa
66
46
30
21.5
14.3
Core
B
GND
WT
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I30A
G33A
V34A
Y35A
KDa
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46
30
21.5
E2

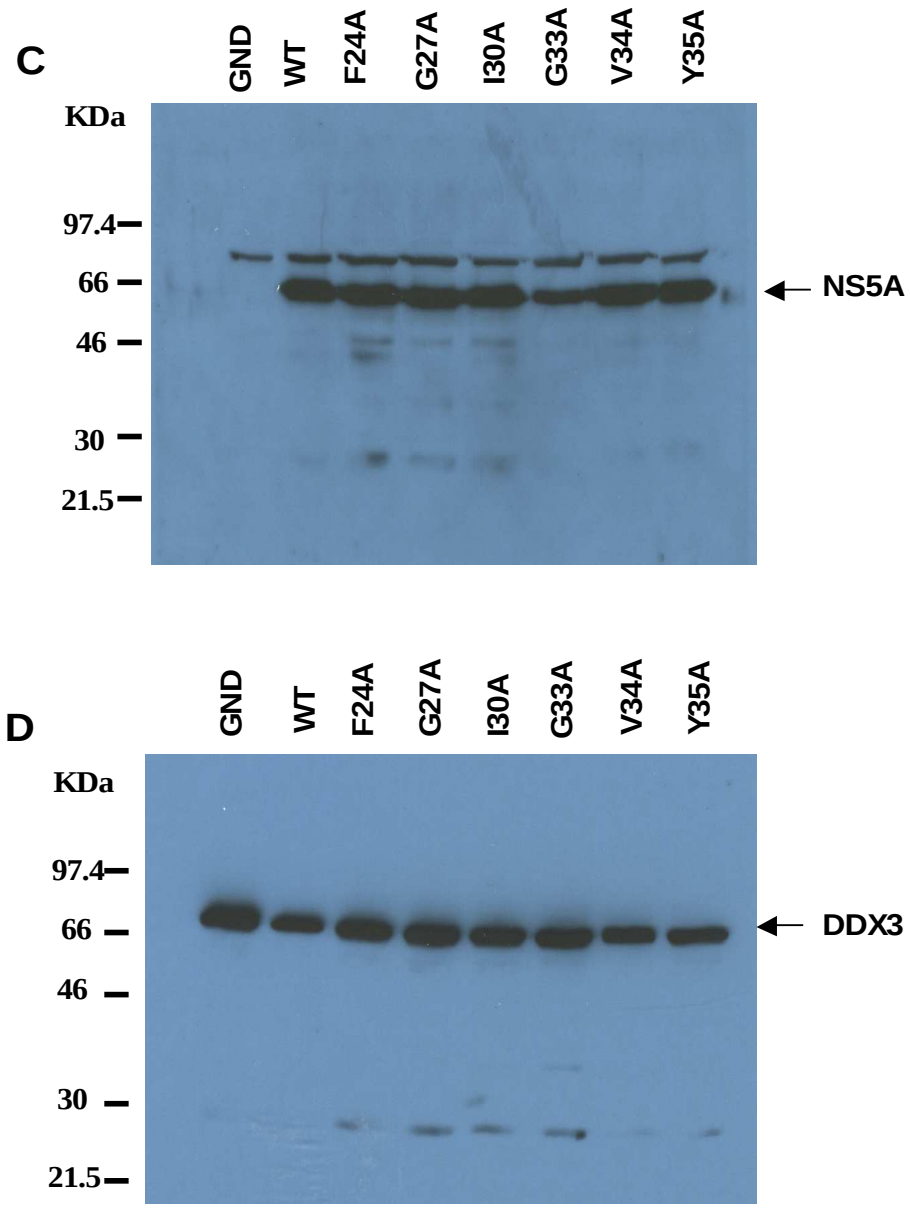


Figure 4.14: Detection of HCV viral proteins in Huh7 cells electroporated with mutant HCV JFH-1 RNA. Huh7 cells were electroporated with 5 µg of *in vitro* transcribed mutant JFH-1 RNA. Seventy-two hours post-electroporation, cell lysate was used for Western immunoblotting as in Figure 4.6. A) rabbit polyclonal anti-core antiserum (R308), B) mouse monoclonal anti-E2 antibody (AP33), C) sheep polyclonal anti-NS5A antiserum, and D) rabbit polyclonal anti-DDX3 antiserum (R648).

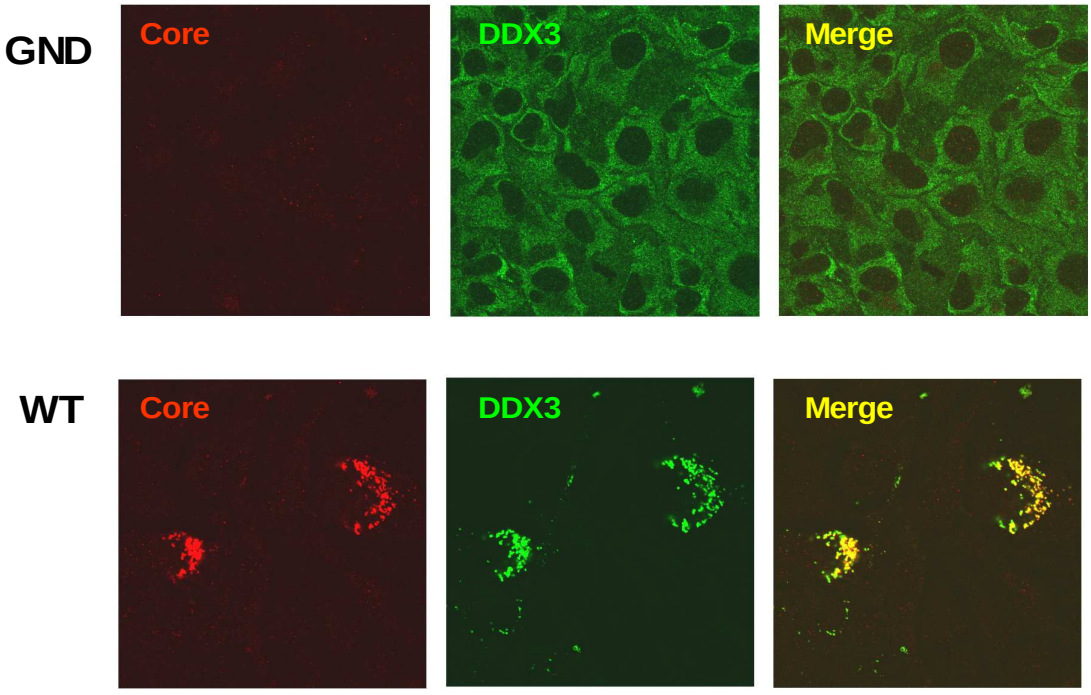
cells. To investigate this, naïve Huh7 cells were electroporated with 5 μg of *in vitro* transcribed mutant JFH-1 RNA. Seventy-two hours post-electroporation, culture medium was harvested from cells and filtered through a 0.45 μm filter. Naïve Huh7 cells were plated out at $5x10^4$ cells/ml and the following day incubated with filtered medium for 3 hrs at 37ºC. Cells were then washed with PBS and overlayed with appropriate culture medium. Seventy-two hours post infection cells were harvested for immunofluorescence and RT-PCR.

Infected cells were washed with PBS and fixed in methanol before being permeablised with PBS-T. Cells were then stained with rabbit polyclonal anti-core antiserum (R308) to confirm HCV infection and mouse monoclonal anti-DDX3 antibody (AO196) to identify any colocalisation with DDX3. By immunofluorescence, JFH-1 mutants F24A, G27A, I30A, V34A and Y35A were shown to be able to infect naïve Huh7 cells and, as seen in electroporated cells, mutant core protein had a globular cytoplasmic localisation and, as expected, did not colocalise with DDX3, which remained diffuse throughout the cytoplasm (Fig. 4.15). Also, in a similar pattern to that seen in electroporated cells, some cells infected with JFH-1 V34A virus showed colocalisation between core and DDX3 while in other cells there was no colocalisation (Fig. 4.15; V34A (i) & (ii)). Interestingly, there was no infection identified in cells incubated with culture medium from the JFH-1 G33A culture (Fig. 4.15; G33A), suggesting this mutation may have an adverse effect on the production of infectious progeny particles.

Infected cells were also analysed for replicating viral RNA. In accordance with the immunofluorescence data, mutant viruses F24A, G27A, I30A, V34A and Y35A all replicated in infected cells as determined by the presence of negative strand replication intermediate HCV RNA (Fig. 4.16). Culture medium from cells electroporated with G33A mutant RNA was unable to infect naïve Huh7 cells as shown by immunofluorescence. Similarly, no negative strand HCV RNA could be detected in infected cells, thus confirming that infectious HCV particles were not produced from the JFH-1 G33A mutant RNA (Fig. 4.16).

4.2.3.6 Mutant Viruses Continue to Produce Infectious Virus Particles

Initial experiments suggested that inhibiting the interaction between core and DDX3 does not significantly affect viral RNA replication, polyprotein processing or the production of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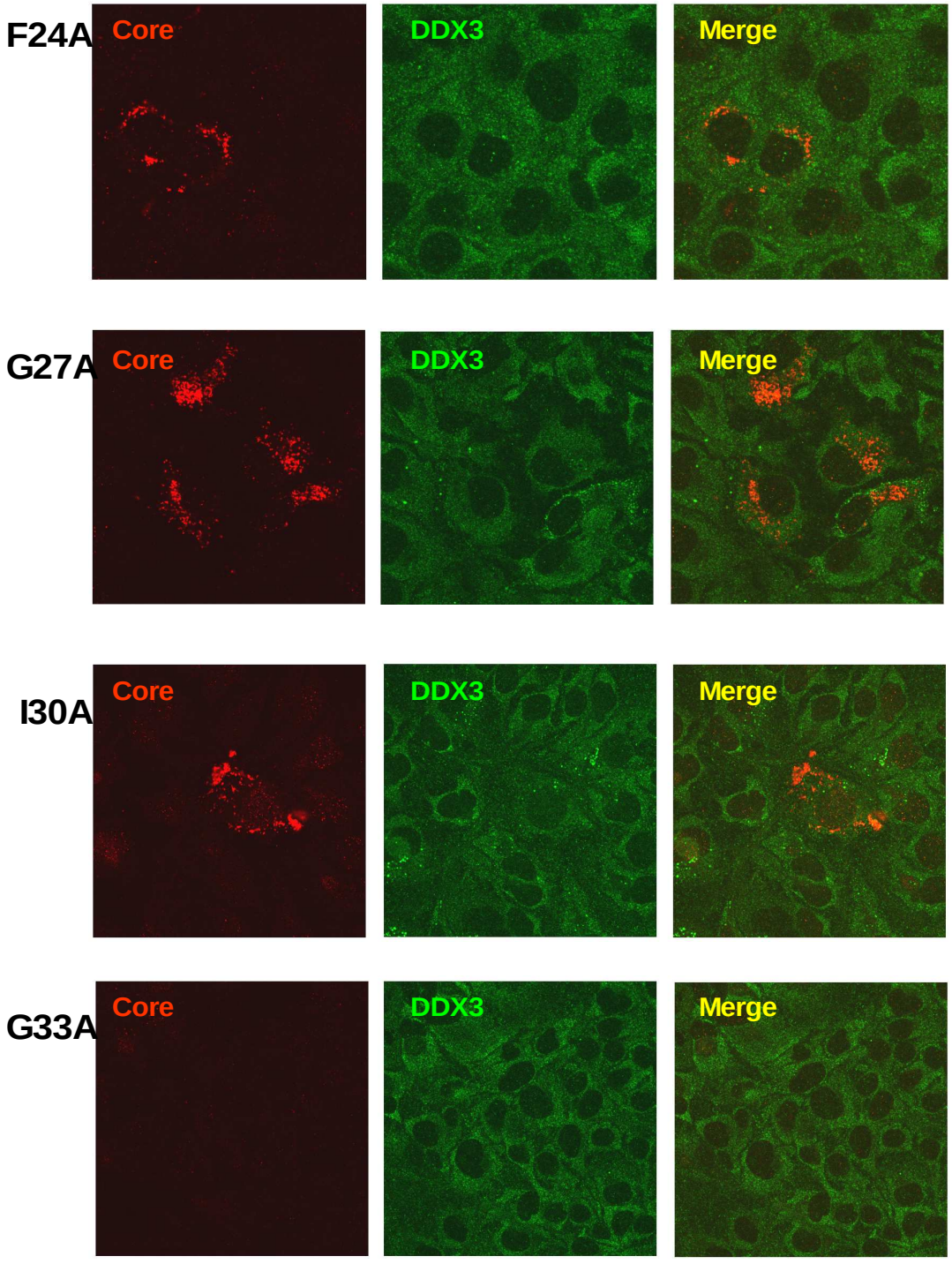
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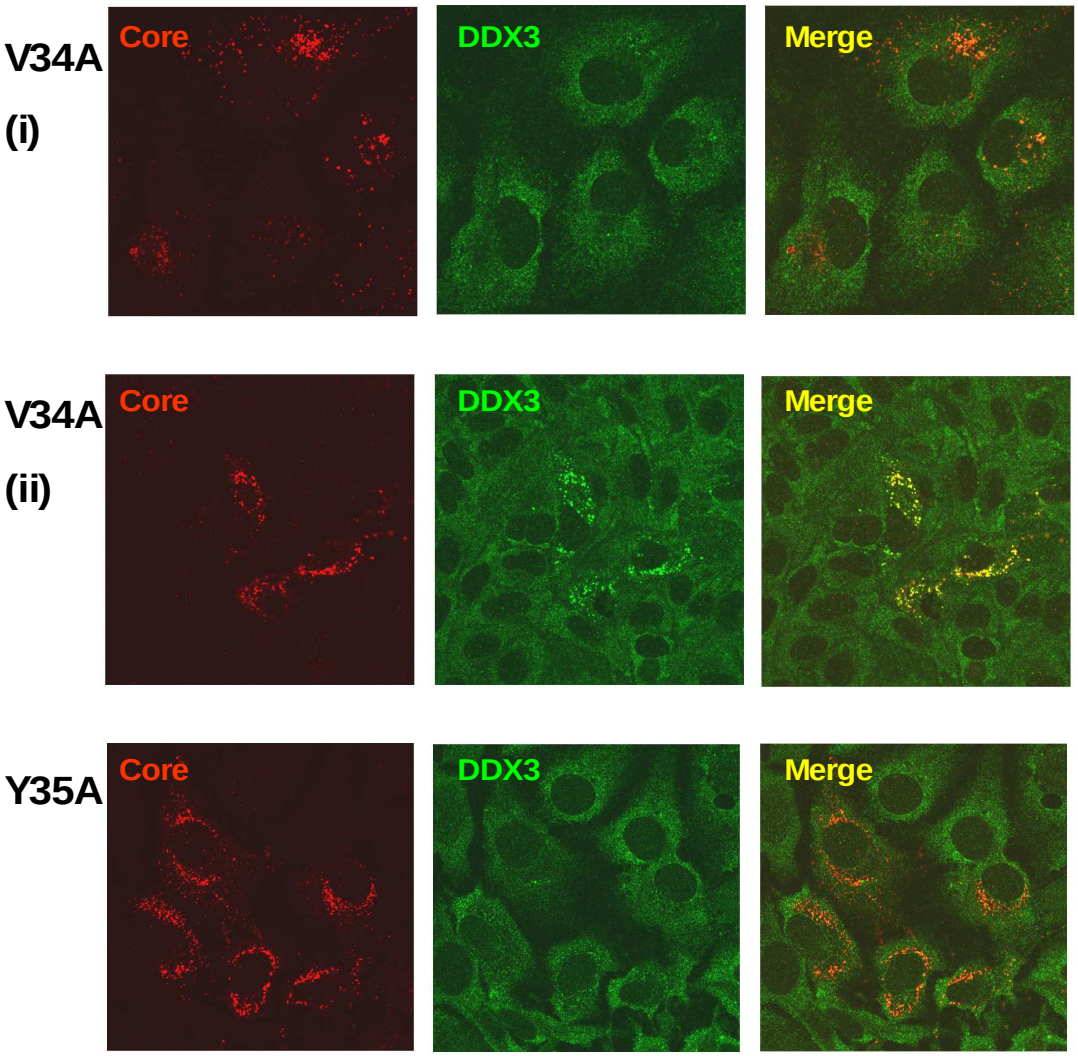


Figure 4.15: Huh7 cells electroporated with mutant HCV JFH-1 RNA secrete infectious HCV particles into the culture medium. Medium from Huh7 cells electroporated with 5 µg of *in vitro* transcribed mutant JFH-1 RNA was incubated with naïve Huh7 cells for 3 hrs at 37°C followed by washing and overlay with appropriate culture medium. Three days post-infection, cells were fixed and probed with rabbit polyclonal anti-core antiserum (R308) and mouse monoclonal anti-DDX3 antibody (AO196) as in Chapter 3.

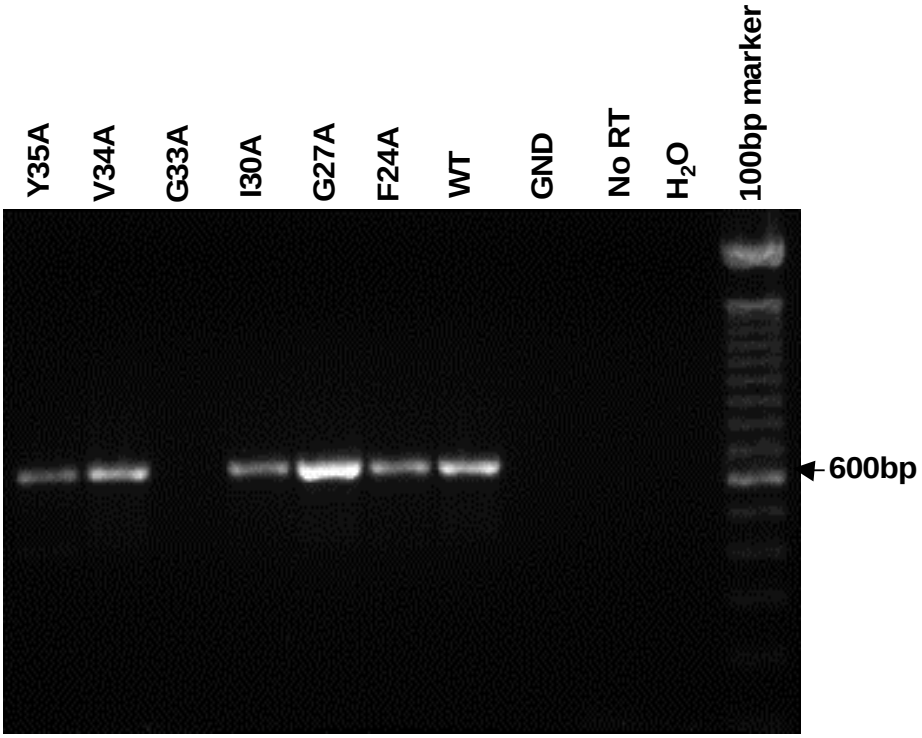
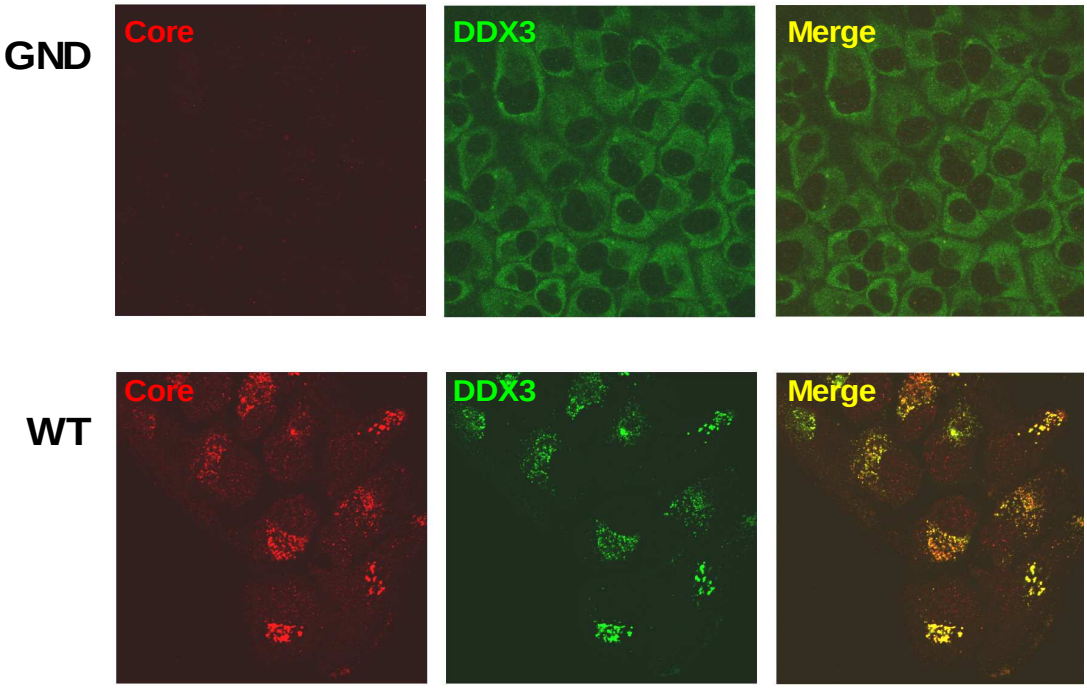


Figure 4.16: Detection of cDNA, generated from negative strand HCV RNA in Huh7 cells infected with medium from mutant HCV JFH-1 RNA electroporated Huh7 cells. Medium from Huh7 cells electroporated with 5 µg of *in vitro* transcribed JFH-1 RNA was incubated with naïve Huh7 cells for 3 hrs at 37°C followed by washing with PBS and overlay with appropriate culture medium. Seventy-two hours post-infection, total RNA was extracted, reverse transcribed and resultant cDNA amplified as in Figure 4.5. "No RT" is extract from JFH-1 electroporated cells with no reverse transcription stage, H_2O is no RNA control reaction.

infectious viral particles. It does, however, seem that the amino acid substitution G33A has an inhibitory effect on the production of infectious virus particles while not adversely affecting replication of viral RNA. To determine if mutant viruses can continue to propagate over long periods of time, 5 μg of *in vitro* transcribed mutant JFH-1 RNA was electroporated into naïve Huh7 cells. Electroporated cultures were propagated for 10 passages and re-analysed for virus infection. At each of the 10 passages, cells were plated on dishes and coverslips for analysis. To confirm the presence of virus and cellular localisation of DDX3, passaged cells were grown for 72 hrs before being washed with PBS and fixed in methanol. Cells were permeablised with PBS-T and probed for core and DDX3. HCV core protein was detected in all mutant cultures with its typical cytoplasmic globular staining, indicating that JFH-1 mutant viruses unable to bind DDX3 are still capable of propagation in cell culture (Fig. 4.17). As in electroporated cells, mutant core proteins did not colocalise with DDX3 with the exception of some cells in the V34A virus culture (Fig. 4.17; V34A). Interestingly, although cells electroporated with JFH-1 G33A RNA (which did not produce infectious particles) did not show colocalisation between core and DDX3, after 10 passages some cells contained core protein that colocalised with DDX3 while in other cells there was no colocalisation (Fig. 4.17; G33A), similar to that seen with mutant V34A. Also of note was the fact that while in G33A electroporated cells only isolated cells showed signs of HCV protein expression, at passage 10 large clusters of cells were seen which expressed mutant G33A viral proteins.

Passaged cells were also washed with PBS and harvested with TRIzol before extraction of total RNA. RT-PCR was carried out as before to detect replicating, negative strand intermediate HCV JFH-1 RNA. Using primer JFH-1 NegRT to reverse transcribe negative strand RNA and primers JFH-1 RTPCR1 and JFH-1 RTPCR2 to PCR amplify the resultant cDNA. Negative strand, replication intermediate JFH-1 RNA was detected in the case of all culture samples (including G33A), thus confirming that viral RNA replication was occurring despite the inhibition of core – DDX3 interaction (Fig. 4.18).

To confirm that the replicating mutant JFH-1 RNA detected in passage 10 cultures was producing infectious virus particles, medium from passage 10 cultures was used to infect naïve Huh7 cells. Naïve Huh7 cells were seeded at $5x10^4$ cells/ml on coverslips and 35 mm dishes. The following day, cells were incubated with filtered medium from passage 10 cultures for 3 hours before washing with PBS and overlay with appropriate culture medium. Seventy-two hours post-infection, cells were washed in PBS and fixed with methanol before being permeablised with PBS-T. To confirm infection and localisation of cellular DDX3, cells were probed for core and DDX3. As with culture medium from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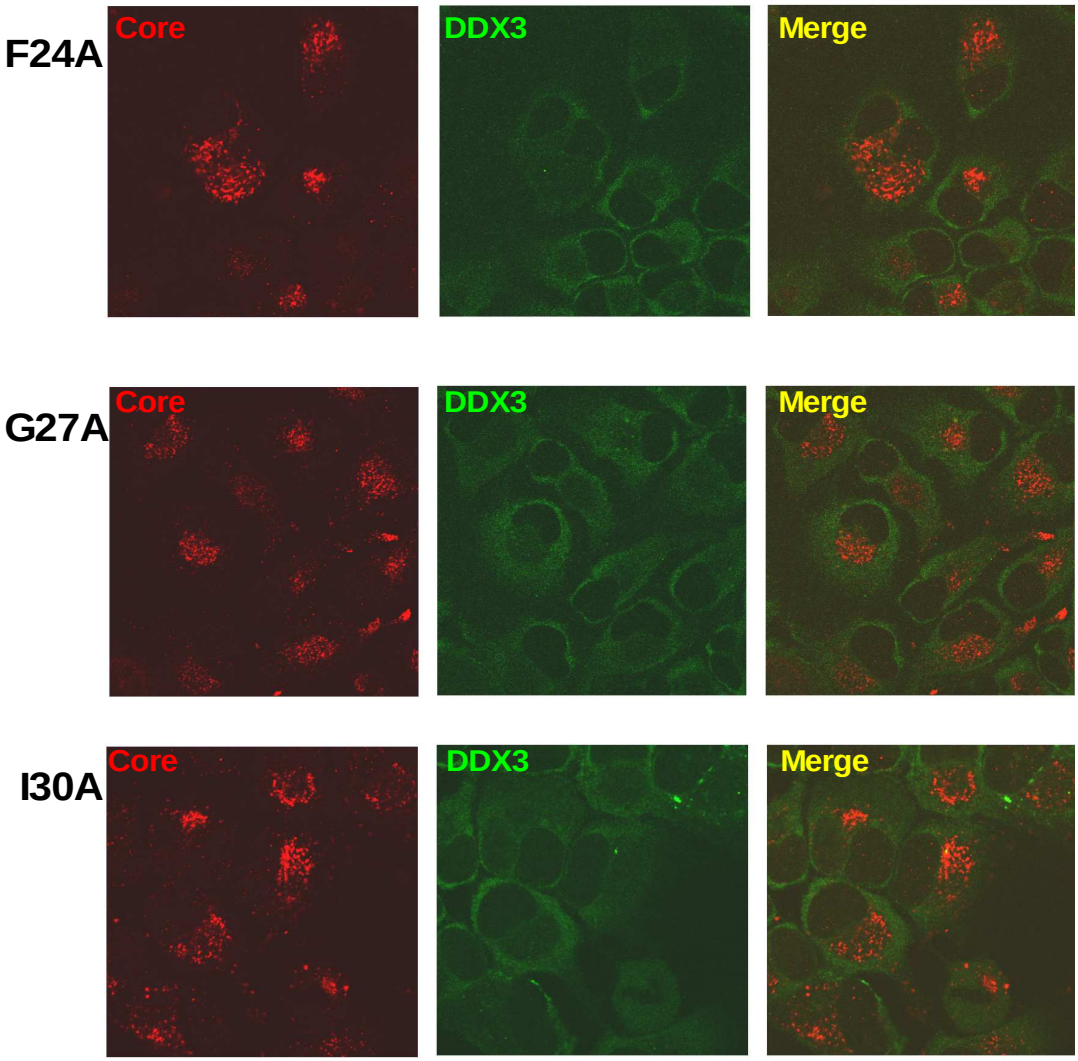
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I30A
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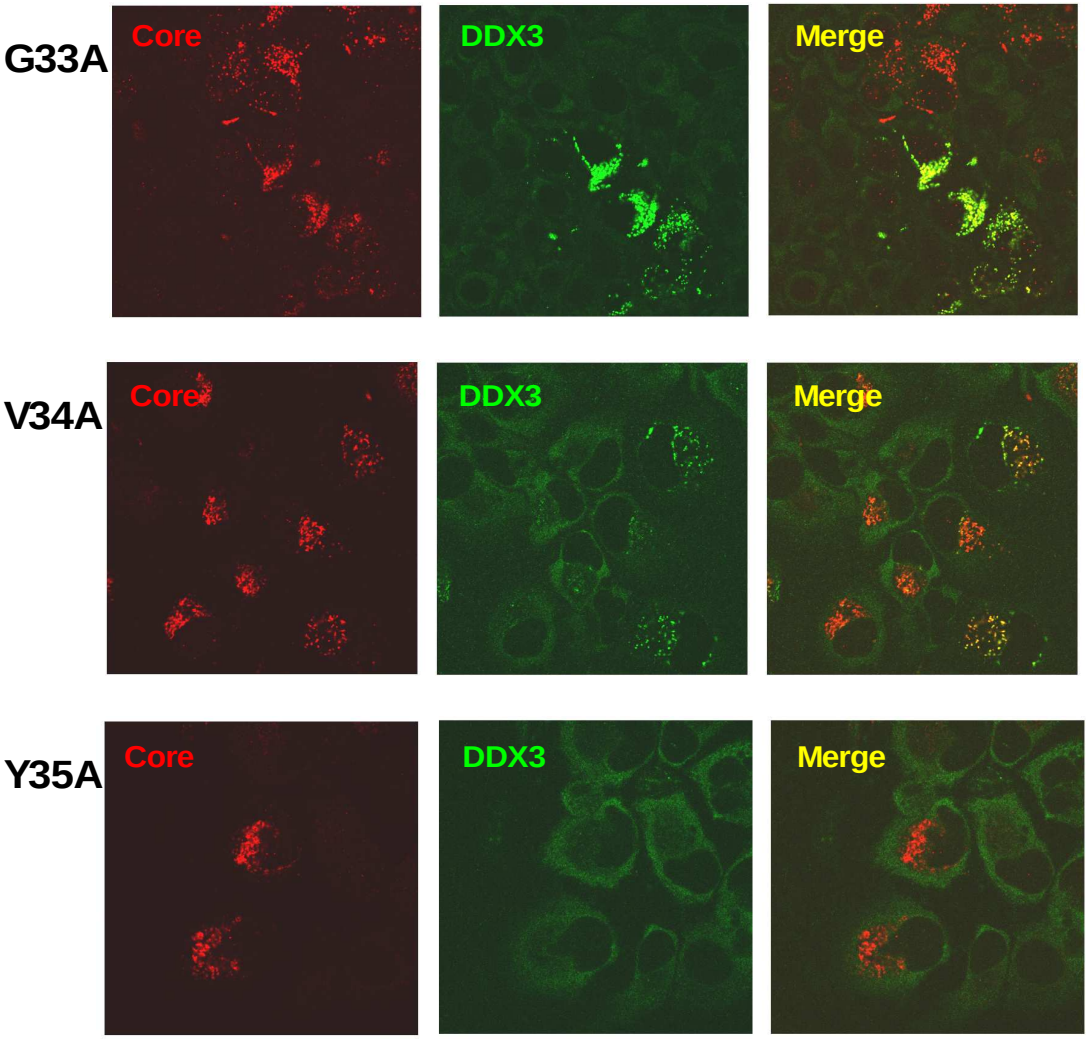


Figure 4.17: Co-localisation of core protein with DDX3 in cultured Huh7 cells. Huh7 cells were electroporated with 5 µg of *in vitro* transcribed mutant JFH-1 RNA. Cells were cultured through 10 passages then plated on coverslips. Seventy-two hours post plating, cells were fixed and probed with rabbit polyclonal anti-core antiserum (R308) and mouse monoclonal anti-DDX3 antibody (AO196) as in Chapter 3.

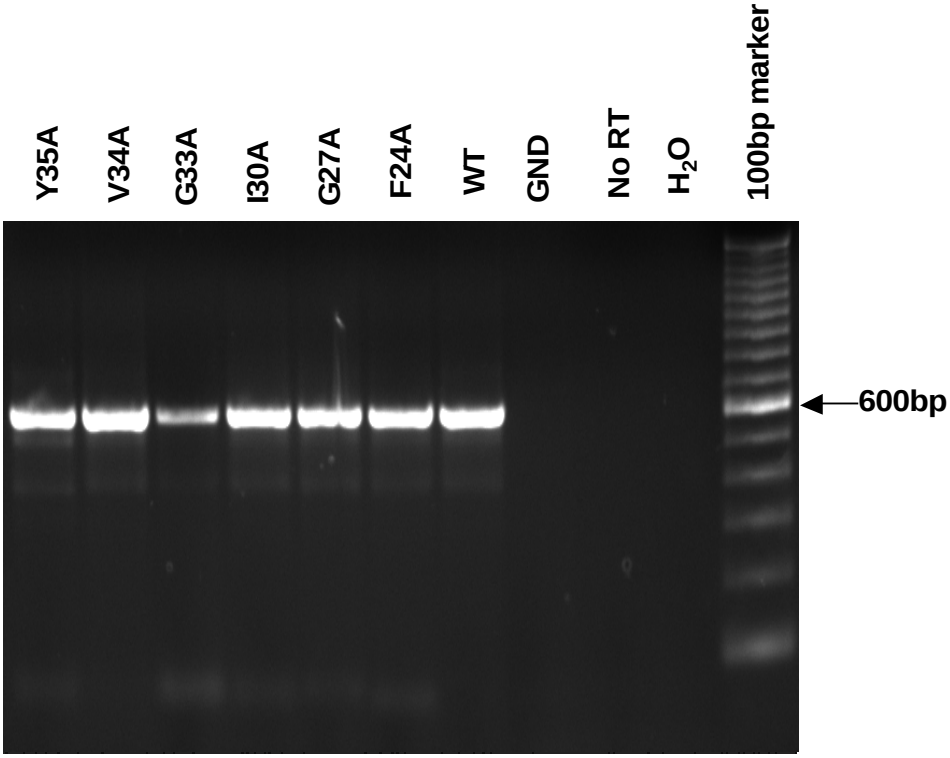


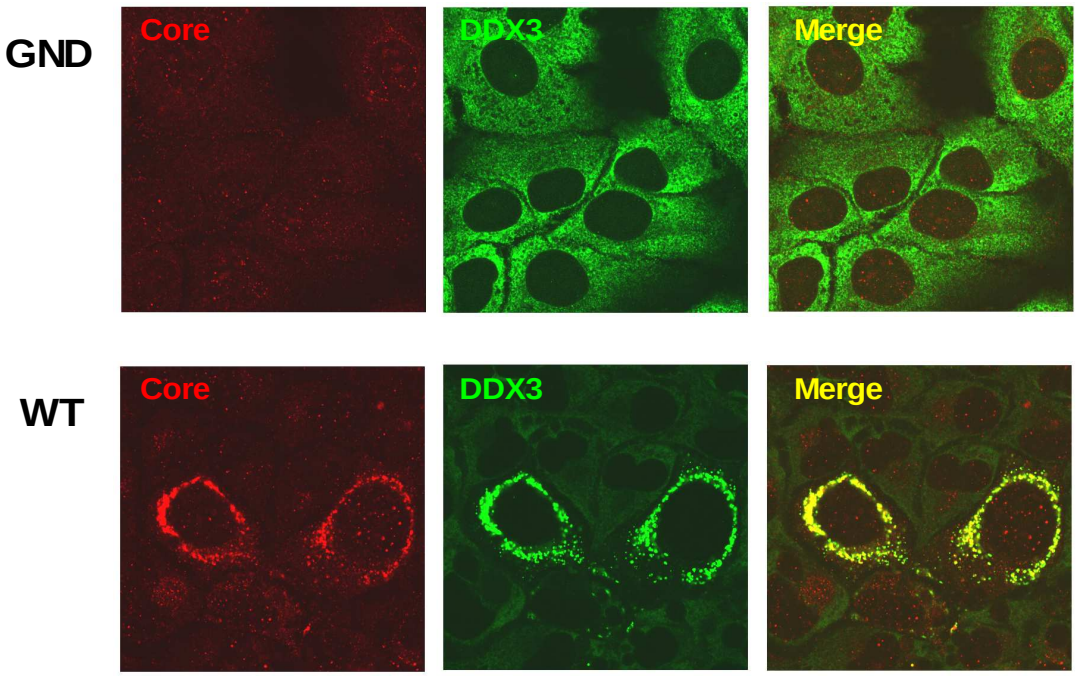
Figure 4.18: Detection of cDNA, generated from negative strand HCV RNA in cultured Huh7 cells. Huh7 cells were electroporated with 5 µg of *in vitro* transcribed mutant JFH-1 RNA. Cells were cultured through 10 passages then plated on coverslips. Seventy-two hours post-plating, total RNA was extracted, reverse transcribed and resultant cDNA amplified as in Figure 4.5. "No RT" is RNA extract from JFH-1 electroporated cells with no reverse transcription stage, H_2O is no RNA control reaction.

electroporated cells (section 4.2.3.5), medium from mutant virus cultures at passage 10 was capable of infecting naïve Huh7 cells. Again, as before, there was no colocalisation between mutant core and DDX3 except for a percentage of cells infected with medium from the V34A culture, in which some cells showed colocalisation (Fig. 4.19). Interestingly, unlike culture medium from cells electroporated with G33A RNA, supernatant from passage 10 cells carrying JFH-1 G33A RNA was capable of infecting naïve Huh7 cells. This result indicates that infectious particles were now being produced from this mutant RNA (Fig. 4.19; G33A). Also of note, the core protein produced by this infectious JFH-1 G33A mutant virus colocalised with DDX3 in a similar manner to that of wild type HCV core.

The presence of replicating JFH-1 mutant RNA in infected cells was confirmed by RT-PCR. Negative strand, replication intermediate JFH-1 RNA was detected in all culture samples, including cells incubated with JFH-1 G33A medium (Fig. 4.20). This result confirms that viral RNA replication was occurring despite the inhibition of core – DDX3 interaction.

4.2.3.7 Nucleotide Sequence Analysis of Viral cDNA

RT-PCR products from passage 10 infection experiments (Fig. 4.20) were purified using Qiagen Gel Extraction Kit and sequenced to confirm the presence of the initial alanine substitutions inserted by site-directed mutagenesis (section 4.2.3.1). Nucleotide sequence analysis confirmed that all mutants retained their initial alanine substitution after propagation for 10 passages (Fig. 4.21). Mutants F24A, G27A, I30A, V34A and Y35A had no other mutations within the JFH-1 core sequence, nor did the wild type. Interestingly however, JFH-1 mutant G33A, which did not produce infectious particles after initial electroporation, but was capable of infecting naïve Huh7 cells after propagation for 10 passages, had developed a second mutation in the amino acid sequence of core (Fig. 4.21). This mutation, at residue 32 of core (G32D), was directly upstream of the initial G33A mutation, suggesting this may be a compensatory mutation allowing production of infectious particles. This possibility is investigated further in Chapter 5.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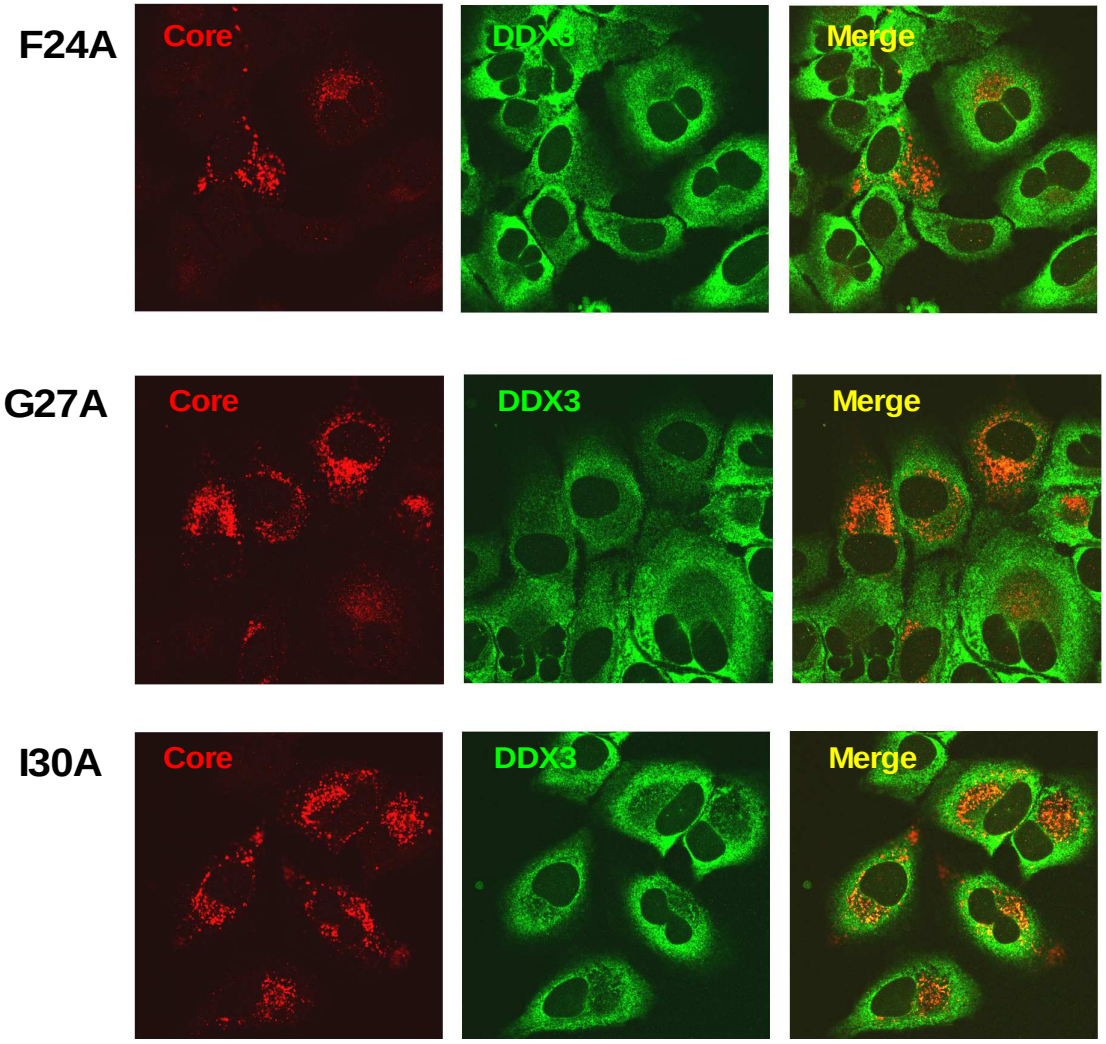
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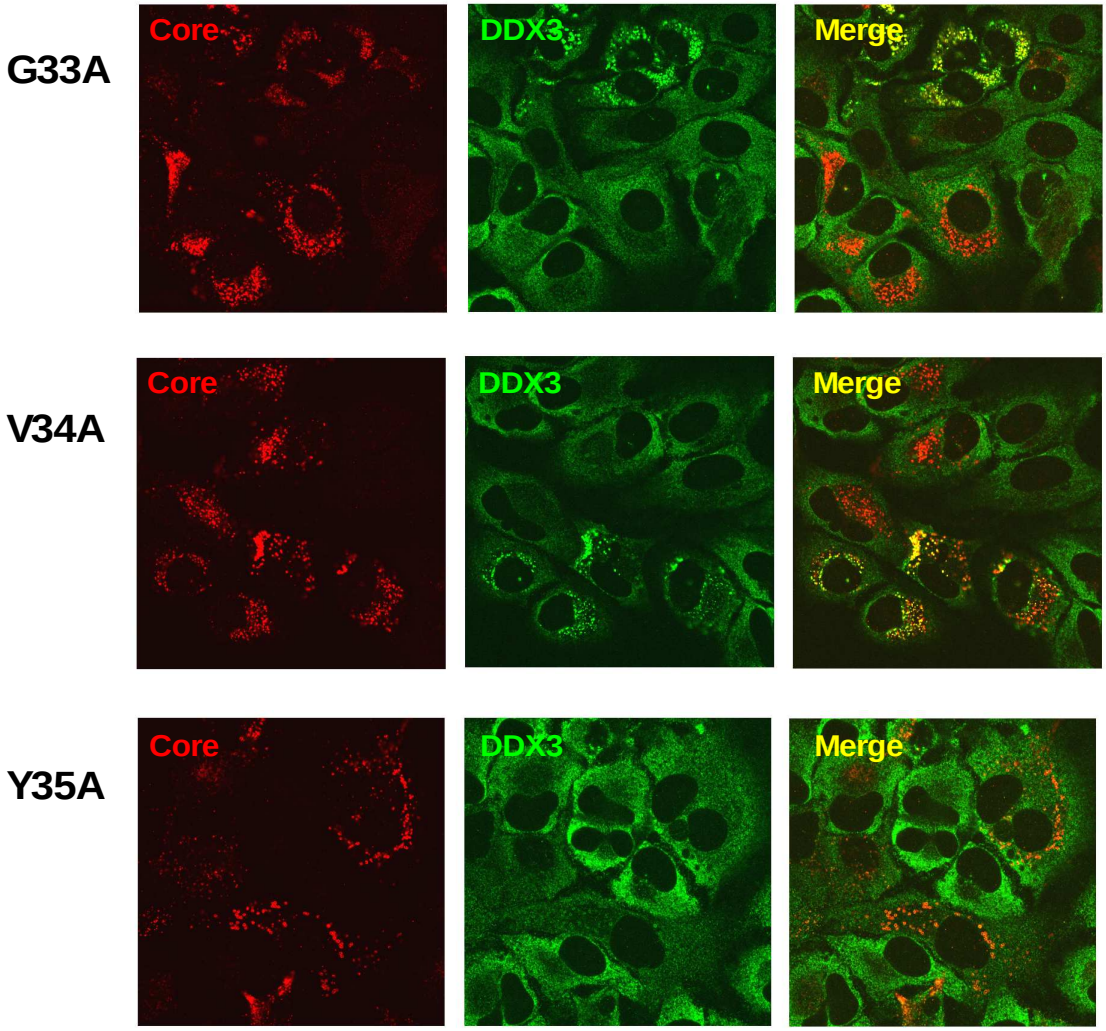


Figure 4.19: Colocalisation of core protein with DDX3 in cells infected with passage 10 supernatant. Huh7 cells electroporated with 5 µg of *in vitro* transcribed mutant JFH-1 RNA were cultured for 10 passages. Culture medium was incubated with naïve Huh7 cells for 3 hrs at 37°C before washing with PBS and overlay with appropriate culture medium. Seventy-two hours post-infection, cells were fixed and probed with rabbit polyclonal anti-core antiserum (R308) and mouse monoclonal anti-DDX3 antibody (AO196) as in Chapter 3.

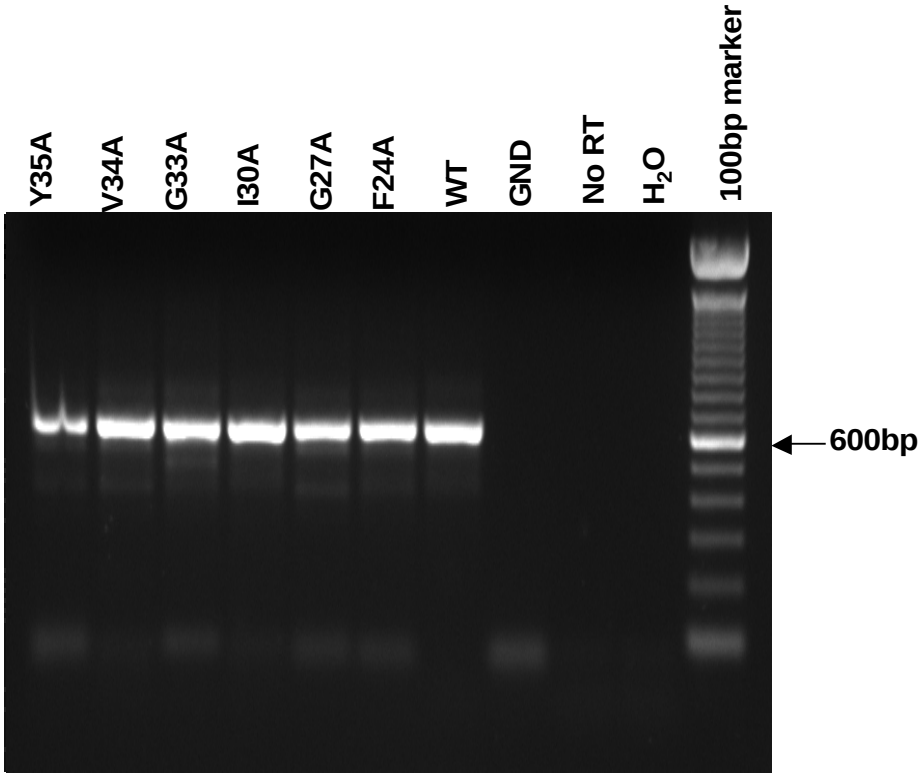


Figure 4.20: Detection of cDNA, generated from negative strand HCV RNA in cells infected with passage 10 supernatant. Huh7 cells electroporated with 5 µg of *in vitro* transcribed mutant JFH-1 RNA were cultured for 10 passages. Culture medium was incubated with naïve Huh7 cells for 3 hrs at 37°C before washing with PBS and overlay with appropriate culture medium. Seventy-two hours post infection, total RNA was extracted, reverse transcribed and resultant cDNA amplified as in Figure 4.5. "No RT" is RNA extract from JFH-1 electroporated cells with no reverse transcription stage, H_2O is no RNA control reaction.

Figure 4.21: Nucleotide sequence analysis of propagated mutant JFH-1 viruses. Huh7 cells electroporated with 5 µg of *in vitro* transcribed mutant JFH-1 RNA were cultured for 10 passages. Culture medium was incubated with naïve Huh7 cells for 3 hrs at 37°C before washing with PBS and overlay with appropriate culture medium. Seventy-two hours post-infection, cells were washed with PBS, harvested using TRIzol (Invitrogen) and total RNA extracted. RNA was reverse transcribed using JFH-1 NegRT (ttgcgagtgccccggga) and resultant cDNA amplified by PCR using core primers JFH-1 RTPCR1 (ggtctcgtagaccgtgcacc) and JFH-1 RTPCR2 (gtattcttcacctgggcagc). PCR products were gel purified using a gel extraction kit (Qiagen) and sequenced using core specific primers JFH-1 RTPCR1 and JFH-1 RTPCR2. Red residues represent original site-directed mutations, green residue represent new mutation. Only amino acids 1-59 are shown here, all mutants and wild type had identical sequence to original JFH-1 sequence from residue 60-191.

4.2.3.8 Analysis of Mutant Virus Replication and Translation Efficiencies

The previous results indicate that although the substitution mutations in core abolish the interaction with cellular DDX3, neither viral RNA replication nor production of infectious progeny particles are abolished (although efficiency may be affected). It is possible however that DDX3 is required for enhancing replication or translation efficiency and, without it, these processes continue but at a slower rate.

To determine the replication efficiency of the mutant viruses, the tissue culture infectious dose$_{50}$ ($TCID_{50}$, dilution of culture medium required to infect 50% of the wells) of each harvested virus medium was calculated and naïve Huh7 cells infected at equal $TCID_{50}$ levels. Seventy-two hours post-infection, total RNA was harvested from infected cells and Real-Time PCR conducted to determine relative viral RNA levels in each sample.

To produce sufficient virus for titration, electroporated cells were cultivated for 5 passages before culture supernatant was harvested and filtered through a 0.45 μm filter. Naïve Huh7 cells were seeded at $5x10^3$ cells/well in flat-bottomed 96-well plates. The following day, cells were overlayed with filtered virus supernatant (serially diluted 10-fold from neat to 10^{-7}, 6 wells per dilution) for 3 hrs at 37ºC before washing with PBS and addition of appropriate culture medium. Seventy-two hours post infection cells were washed with PBS and fixed in methanol. Cells were then permeablised with PBS-T and probed for NS5A.

Immunofluorescence was used to count the number of wells positive for NS5A at each dilution. From this, the $TCID_{50}$ was calculated (Fig. 4.22). As titration results for each virus were almost identical, all naïve Huh7 cells were infected with neat medium for the RNA quantitation assay. Seventy-two hours post infection cells were washed with PBS, harvested in TRIzol and total RNA extracted. Relative viral RNA quantitation was carried out using a Real-Time PCR system (Applied Biosystems). This system involves the amplification of two transcripts, one the target sequence (viral RNA), and the other an endogenous control (GAPDH) in order to normalise the samples for total RNA levels. An oligonucleotide probe is constructed with a fluorescent reporter dye bound to the 5' end and a quencher on the 3' end. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye. If the target sequence is present, the probe anneals between primer sites and is cleaved by the 5' nuclease activity of the DNA polymerase during extension. Cleavage of the probe releases the reporter dye from the quencher, thus increasing the reporter dye signal, which is measured at each cycle of the PCR reaction. With each cycle, more reporter dye molecules are cleaved from their

	WT	F24A	G27A	I30A	G33A	V34A	Y35A
Neat	**6**	**6**	**6**	**6**	**6**	**6**	**6**
10^{-1}	**6**	**6**	**6**	**6**	**6**	**6**	**6**
10^{-2}	**6**	**6**	**6**	**6**	**6**	**6**	**6**
10^{-3}	**2**	**1**	**1**	**1**	**0**	**2**	**2**
10^{-4}	**0**	**0**	**0**	**0**	**0**	**0**	**0**
10^{-5}	**0**	**0**	**0**	**0**	**0**	**0**	**0**
10^{-6}	**0**	**0**	**0**	**0**	**0**	**0**	**0**
10^{-7}	**0**	**0**	**0**	**0**	**0**	**0**	**0**
log $TCID_{50}$/ml	**-3.83**	**-3.67**	**-3.67**	**-3.67**	**-3.5**	**-3.83**	**-3.83**

Figure 4.22: Titration of mutant virus supernatant. Huh7 cells electroporated with 5μg of *in vitro* transcribed mutant JFH-1 RNA were cultured for 5 passages. Passage 5 supernatant was incubated with naïve Huh7 cells for 3 hrs before washing with PBS and overlay with appropriate culture medium. 72 hrs post infection, cells were washed with PBS, fixed in methanol and permeablised with PBS-T. Cells were then probed with sheep polyclonal anti-NS5A antiserum, followed by FITC-labelled anti-sheep secondary antibody. The number of NS5A-positive wells per dilution was counted and $TCID_{50}$ dilution calculated (see Materials and Methods).

respective quenchers resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced. In order to remove the possibility of pipetting errors when splitting samples into two reactions, both reactions (sample and endogenous control) can be carried out in the same tube using different reporter dye molecules. In the case of HCV infected cells, GAPDH has been validated as an endogenous control (Pugnale *et al.*, 2006). Reverse transcription and Real-Time PCR were carried out according to the manufacturer's guidelines (Applied Biosystems). Briefly, random hexamers were used to produce cDNA from total RNA samples. Primers RA16 (TCTGCGGAACCGGTGAGTAC) and RA17 (GCACTCGCAAGCACCCTATC) (binding the HCV 5'NCR) were used to amplify cDNA while a FAM™ labelled probe bound the 5'NCR between these primers. GAPDH was detected using Applied Biosystems Human GAPDH endogenous control primers and a VIC® labelled probe. Real-Time PCR was carried out using Applied Biosystems 7500 Fast Real-Time PCR System and data analysed using Applied Biosystems software (SDS version 1.3.1). The analysis software automatically sets the baseline and threshold and calculates the cycle threshold (CT) value of each sample relative to a positive reference and normalises samples to GAPDH. Wild type JFH-1 containing sample was used as a calibrator, against which the relative quantification of the other samples was measured. When analysing JFH-1 mutant RNA levels relative to JFH-1 wild type RNA levels, all mutants showed greatly reduced RNA levels (Fig. 4.23). Mutant viruses F24A, G27A, I30A, V34A and Y35A showed less than 3% RNA levels of that of wild type. Mutant G33A (containing the compensatory mutation G32D) showed 57% RNA levels of that of wild type, indicating that this compensatory mutation may be having an effect on viral RNA replication.

Infections at equal $TCID_{50}$ were also carried out to determine any differences in translation efficiency between wild type JFH-1 and mutant virus unable to sequester DDX3 (assuming equal uptake of virus samples). Naïve Huh7 cells were infected with passage 5 supernatant as before and 72 hrs post-infection washed with PBS and harvested in LB2. Proteins were separated by 10% SDS-PAGE and blotted on nitrocellulose membrane before being probed with various antibodies to detect viral proteins as well as cellular DDX3. Core protein was most abundant in wld-type JFH-1 infected cells while reduced in all JFH-1 mutant infected cells (Fig. 4.24A). E2 was again most abundant in wild-type JFH-1 infected cells, with mutant infected cells containing decreased levels of E2. Finally, NS5A levels were also highest in wild-type JFH-1 infected cells although mutant virus G33A had comparable levels (Fig. 4.24C). The remainder of the mutants had lower levels of NS5A in agreement with core and E2 data, and Real-Time PCR analysis. DDX3 levels were similar in all

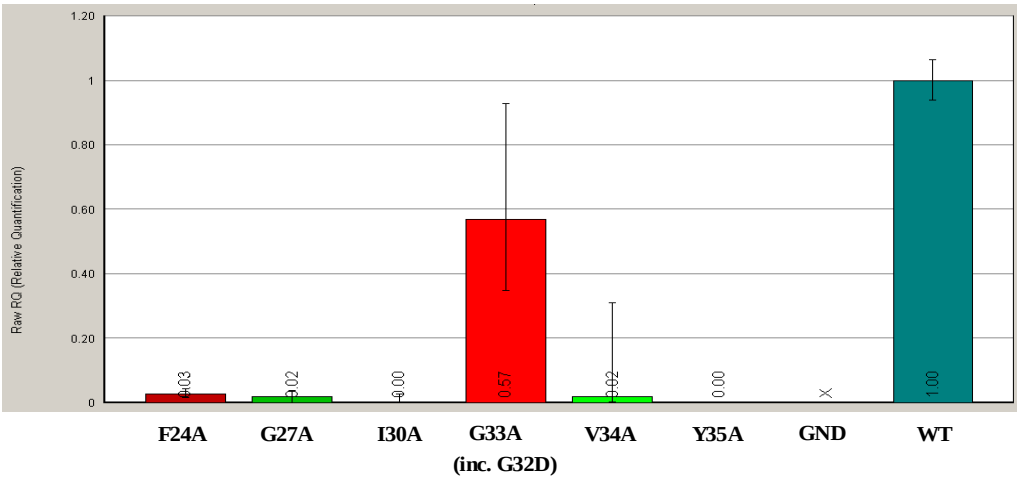
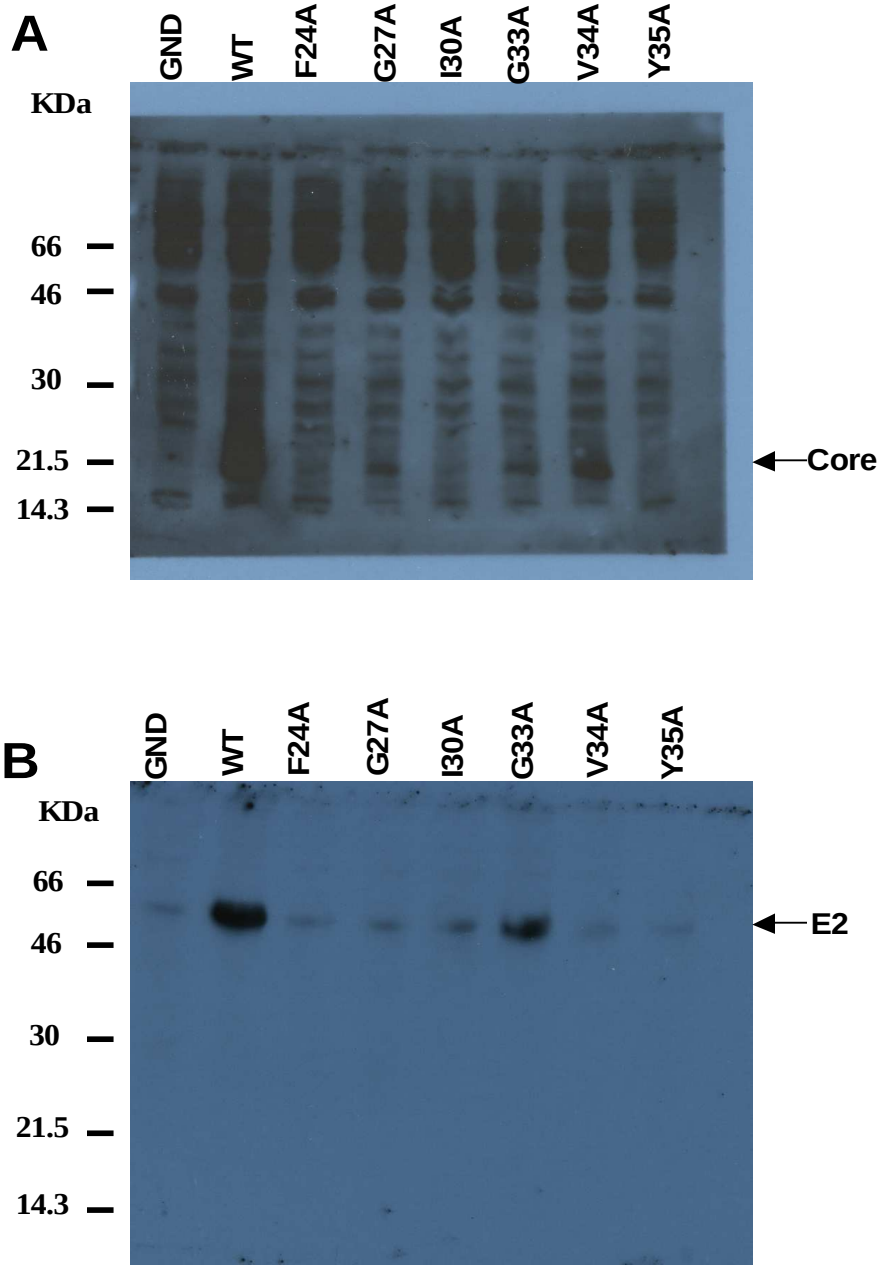


Figure 4.23: Real-Time PCR analysis. Naïve Huh7 cells were incubated with culture medium taken from mutant virus (at equal $TCID_{50}$ dilution) for 3 hrs at 37°C followed by washing with PBS and overlay with appropriate culture medium. Seventy-two hours post-infection, cells were washed with PBS, lysed using TRIzol (Invitrogen) and total RNA extracted. RNA was reverse transcribed using random hexamers (Applied Biosystems, according to manufacturers instructions) and resultant cDNAs amplified by PCR using HCV core 5'UTR specific primers and GAPDH specific primers (Applied Biosystems), in the presence of FAM and VIC probes (Applied Biosystems, according to manufacturers instructions). Relative fluorescence intensity of samples was measured using Applied Biosystems 7500 Fast Real-Time PCR System and data analysed using Applied Biosystems software. (Experiment carried out in triplicate, error bars shown).


A
GND
WT
F24A
G27A
I30A
G33A
V34A
Y35A
KDa
66
46
30
21.5
14.3
Core
B
GND
WT
F24A
G27A
I30A
G33A
V34A
Y35A
KDa
66
46
30
21.5
14.3
E2

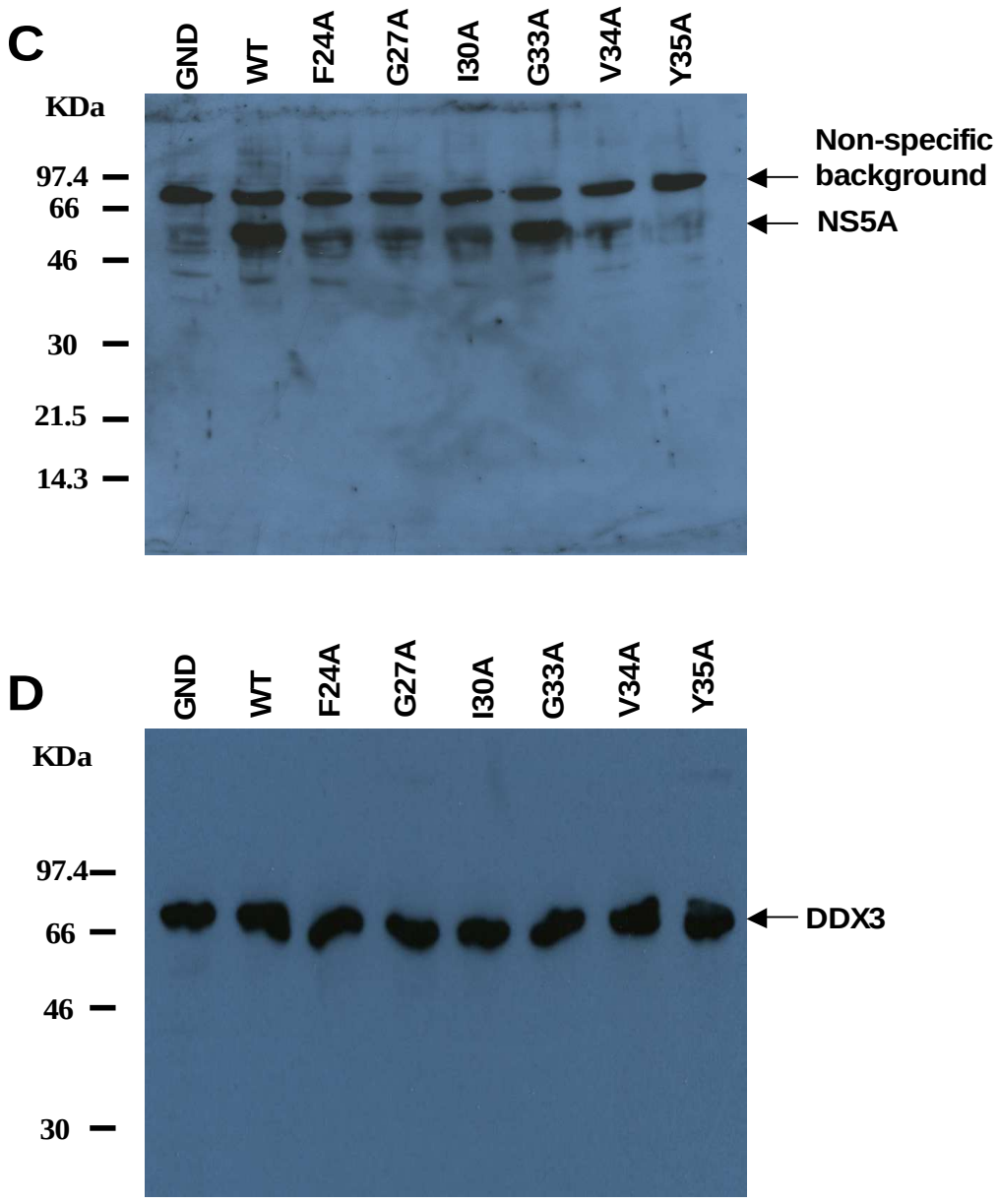


Figure 4.24: Detection of HCV viral proteins in infected Huh7 cells. Naïve Huh7 cells were incubated with culture medium from mutant virus (at equal $TCID_{50}$ dilution) for 3 hrs at 37°C followed by washing with PBS and overlay with appropriate culture medium. Seventy-two hours post-infection, lysates were used for Western immunoblotting as in Figure 4.6. A) rabbit polyclonal anti-core antiserum (R308), B) mouse monoclonal anti-E2 antibody (AP33), C) sheep polyclonal anti-NS5A antiserum, and D) rabbit polyclonal anti-DDX3 antiserum (R648).

infected cell lysates indicating that samples contained equal amount of total protein (Fig. 4.24D). Slight background bands were seen in GND samples probed for E2 and NS5A, possibly due to contamination from the wild type sample during loading of the gel since no band was seen when GND was probed for core, nor was GND positive for E2 or NS5A in previous experiments (Fig. 4.6).

4.3 Discussion

The recent discovery of a genotype 2a HCV clone (JFH-1) capable of undergoing a fully infectious life cycle in cell culture without the need for adaptive mutations (Wakita *et al.*, 2005) has allowed many previously un-investigated aspects of the HCV life cycle to be studied. Of particular interest is the interplay between viral proteins and host factors. In the previous chapter, 6 HCV core residues were identified which, when substituted for alanine, abolished the interaction between core and DDX3 in a transient transfection system. To understand more about this interaction in terms of a fully infectious replication system, these alanine substitutions were introduced individually into the JFH-1 background and their effects on the JFH-1 life cycle studied in cell culture.

Before analysing the effects of abolishing this interaction on the virus life cycle, the JFH-1 cell culture model was set up and core-DDX3 interaction confirmed in this new system. Plasmid containing full length JFH-1 sequence was linearised and RNA produced by *in vitro* transcription. This RNA was then electroporated into naïve Huh7 cells for analysis. Viral proteins were detected in electroporated cells by immunofluorescence and Western immuno-blot analysis. By immunofluorescence, core protein was detected in the cytoplasm of electroporated cells, having a globular staining pattern. Core protein was also shown to colocalise with the lipid droplet marker ADRP, indicating that it was targeted to the lipid droplets as previously described (Moradpour *et al.*, 1996). HCV glycoprotein E2 was detected throughout the cytoplasm of electroporated cells. Core protein was also shown to colocalise with DDX3. In naïve Huh7 cells, DDX3 is diffusely distributed throughout the cytoplasm and is also found in the nucleus. However, in the presence of HCV JFH-1 core protein, DDX3 is redistributed and colocalises with core. The site of colocalisation between core and DDX3 was confirmed to be lipid droplets by colocalisation between DDX3 and ADRP in core expressing cells. HCV RNA was detected in electroporated cells by RT-PCR. To confirm the RNA detected was replicating RNA and not input RNA, a primer was designed to reverse transcribe negative strand HCV RNA. Negative strand RNA is only produced during replication and can therefore be used as an indicator of viral RNA replication. Negative strand RNA was detected in cells electroporated with JFH-1 RNA, confirming that viral RNA replication was occurring. Production of infectious HCV JFH-1 particles was confirmed by infection of naïve Huh7 cells with filtered culture medium from electroporated cells. By immunofluorescence, JFH-1 core protein was detected in infected Huh7 cells and shown to colocalise with DDX3. Core, E2 and NS5A were also detected in infected Huh7 cells by Western immuno-blot analysis and the

presence of replicating RNA shown by RT-PCR, again using a reverse transcription primer against negative strand, replication intermediate HCV RNA.

To investigate the effect of abolishing the interaction between core and DDX3 on the life cycle of HCV (in a cell culture system), the 6 HCV core residues shown in the previous Chapter to abolish core-DDX3 interaction were individually substituted for alanine within the JFH-1 clone by site-directed mutagenesis. By immunofluorescence, mutant core protein was detected in the cytoplasm of electroporated cells and was shown to colocalise with ADRP on lipid droplets, similar to that seen with wild type JFH-1 core protein. In accordance with the transient transfection data in Chapter 3, mutant core protein did not colocalise with cellular DDX3. Instead, DDX3 was located diffusely throughout the cytoplasm as in naïve Huh7 cells. A percentage of core-mutant V34A did colocalise with DDX3 however. Nucleotide sequence analysis confirmed that JFH-1 V34A mutant RNA did possess the site-directed mutation introduced prior to *in vitro* transcription and RNA electroporation. It is therefore possible that in this cell culture system, HCV core mutation V34A is not as efficient at blocking interaction with DDX3 compared to the other mutations identified. Mutant G33A also showed an interesting phenotype in that while all other mutant and wild-type RNA transfected cultures showed clusters of infected cells, only isolated groups of one or two cells were seen expressing G33A mutant viral proteins. Western blot analysis also confirmed the presence of viral proteins in electroporated cells. Mutant core, E2 and NS5A were detected and all were of predicted molecular weight, although lower levels were detected in the G33A lysate, possibly due to the lack of large clusters of infected cells. One hypothesis regarding the function of DDX3 in the life cycle of HCV is that DDX3 is required for viral RNA replication. To study this, RT-PCR was used to detect negative strand, replication intermediate HCV RNA in electroporated cells. Replicating viral RNA was detected in cells electroporated with each of the 6 JFH-1 mutants indicating that core interaction with DDX3 is not essential for HCV RNA replication.

Another hypothesis to explain why core interacts with DDX3 is that the helicase activity of DDX3 is required for packaging viral RNA into progeny particles. If DDX3 is required for this purpose then blocking interaction between core and DDX3 should prevent the production of infectious progeny particles. Medium from cells electroporated with mutant JFH-1 RNA was filtered and incubated with naïve Huh7 cells. HCV infection was detected in all but one culture by immunofluorescence and RT-PCR. Mutant core protein had a typical cytoplasmic staining pattern and showed no colocalisation with DDX3 (except for a percentage of cells infected with JFH-1 V34A mutant virus). Interestingly, no core protein

was detected in the Huh7 culture incubated with medium from JFH-1 G33A RNA electroporated cells. Similarly, replicating viral RNA was detected in all infected cultures except for that incubated with medium from JFH-1 G33A RNA electroporated cells. The absence of infection in cultures incubated with G33A culture medium suggests that infectious viral particles are not being produced and / or secreted by this viral RNA. This observation ties in with the fact that no clusters of infected cells were seen upon electroporation with G33A RNA, suggesting infection was unable to spread to neighbouring cells. It is also interesting to note that structural analysis of core containing the G33A mutation showed that this mutation caused a slight "kink" in the helix between residues 30 and 39 (section 3.3, Figure 3.18). One possible hypothesis for the lack of infectious mutant G33A virus is that this mutation causes some structural change in core, which then results in production of aberrant HCV particles that are either not released from the infected cell or are unable to bind and / or enter uninfected cells.

Electroporated cultures were grown through ten cell passages and re-analysed in a similar manner to determine whether the mutant virus infection could be sustained. Cells remained positive for HCV core protein as shown by immunofluorescence with infection spreading throughout the culture. While wild type core colocalised with DDX3, the mutant core proteins were still unable to colocalise with the cellular protein. However, core mutant G33A (from which electroporated RNA did not produce infectious particles), now showed colocalisation with DDX3. As no infectious particles were produce from this mutant RNA after electroporation, it would be expected that during continuous cell passaging, numbers of HCV core positive cells would diminish. Instead however, G33A mutant virus had spread throughout the culture, similar to the other mutants and wild type virus. Infection studies on medium from these passage 10 cultures indicated (by immuno-staining for core and negative strand RT-PCR) that all mutants (including G33A) were capable of infecting naïve Huh7 cells. Sequence analysis of viral RNA in these infected cells showed that all mutants still possessed their original mutation in core. In addition, mutant G33A RNA had a second mutation, located one amino acid upstream at residue 32 of core (G32D). It is possible that this is a compensatory mutation selected in the replicating G33A RNA by its ability to produce infectious HCV particles. It is not clear whether the mutation at residue 33 of core is blocking particle production within the cell or if particles are being secreted but are unable to enter or uncoat within naïve cells. Further investigation of mutant G33A is described in Chapter 5.

As DDX3 is not essential for viral RNA replication or production of infectious particles, more detailed analysis of mutant virus replication and translation efficiencies were carried

out to detect any subtle differences compared to wild type virus. Naïve Huh7 cells were infected at equal $TCID_{50}$ levels (after titration of culture medium) and cells harvested for total RNA and protein. Using Real-Time PCR, viral RNA levels in infected cells were analysed. Compared to wild type JFH-1, total viral RNA levels in mutants were greatly reduced (approximately 50-100 fold lower). Mutant G33A however, (also containing mutation G32D), had RNA levels higher than other mutants yet still lower than wild type (approximately 2 fold lower). Similar results were obtained from Western immuno-blot analysis of viral protein levels in infected cells. Again, levels of core, E2 and NS5A were greatly reduced in mutant infected cells compared to wild type and again, mutant G33A infected cells had higher levels of viral proteins compared to other mutants. These results are in contradiction with those in Figures 4.13 and 4.14 which suggest that there is no significant difference in levels of viral RNA replication and translation between wild-type and mutant HCV RNA. One possible theory for this discrepancy is that electroporation of naïve cells with 5 μg of RNA results in cells being "overloaded" with viral RNA and as such the RT-PCR and western blot data shown are the result of saturation. Infection of naïve cells with virus-containing culture medium however, may result in less viral RNA entering cells (compared to electroporation of 5 μg RNA), therefore allowing more accurate analysis of both viral RNA replication and translation. In order to detect these differences using electroporated cells, RNA and protein extract could have been serially diluted before analysis in order to avoid saturation. Another option would have been to carry out a time-course, allowing the study of RNA replication and translation at time points earlier than 72 hours (before saturation occurred). This would have been of interest as the sensitivity of RT-PCR would allow detection of negative strand viral RNA at much earlier time points, possibly before saturation. To overcome this issue of saturation, less RNA could have been used for electroporation. However, as previous publications using the JFH-1 system have used 10 μg of RNA (Zhong *et al.*, 2005), our starting material was not thought excessive at the time. As it has become evident that this may result in "overloading" of the cell culture model, re-analysis of RNA volumes may be required. These results suggest that abolishing the interaction between core and DDX3 may have an inhibitory effect, either directly or indirectly, on viral RNA replication and translation. The higher level of replication and translation in mutant G33A/G32D compared to other mutants may be explained in that it is possible the compensatory mutation (G32D) has, as well as enabling production of infectious particles, reversed the effects on replication and translation caused by the abolishment of the core-DDX3 interaction. This explanation would seem plausible since mutant G33A can now interact with DDX3.

The results in this chapter suggest that the interaction between HCV core and cellular DDX3 is not essential for maintenance of infectious replicating virus. From this data however, it is plausible to suggest that DDX3 is required for enhancing some part of the virus life cycle involved in replication and translation. These results will serve as a basis for future detailed investigations into the replication and translation efficiency of HCV in the absence of core-DDX3 interaction.

5. Further Analysis of HCV Core Mutant G33A

5.1 Introduction

The previous chapters identified 6 conserved amino acids of HCV core protein that are required for the interaction with cellular DDX3 and analysed the effect mutating these residues had on the virus life cycle (in the context of genotype 2a JFH-1 strain). Although the 6 mutant core proteins did not colocalise with cellular DDX3, the viruses were able to replicate and produce infectious virus particles. The exception to this was the discovery that mutation of HCV core amino acid 33 from glycine to alanine inhibited the production of infectious virus particles, while the viral RNA was still capable of replication. Western blot analysis of this mutant indicated that mutant core was being processed correctly as it was of similar molecular weight to all other mutant core proteins as well as wild type (Figure 4.14A). The fact that there was significantly less core protein in this sample compared to wild type and other mutants may be explained by the fact that in the 72 hours between electroporation of RNA and harvesting of samples, wild type and mutant viruses have infected neighbouring cells in the culture while mutant G33A remained only in the initially electroporated cells. However, a slower replication rate may also be a factor. From these initial experiments it cannot be confirmed whether this mutation is preventing the assembly of virus particles or if particles are being secreted but are non-infectious. After culturing electroporated cells for 10 passages, it was noted that culture medium from mutant G33A culture could infect naïve Huh7 cells. Subsequent nucleotide sequence analysis of the core gene from this infectious G33A mutant identified a second mutation that had arisen one residue upstream from the original mutation, at amino acid 32 (glycine – aspartic acid). The appearance of a second mutation, along with infectious particle production, suggested that this might be a compensatory mutation, responsible for the production of infectious particles. In order to study this further, each mutant RNA was freshly electroporated and culture medium analysed at each cell passage for infectious particles, and nucleotide sequence analysis carried out to identify any other compensatory mutations in core. Presented here is a detailed analysis of compensatory mutations arising in HCV JFH-1 mutant G33A and their effect on the production of infectious virus particles.

5.2 Results

5.2.1 Analysis of Infectious Particle Production by Mutant G33A

As shown in Chapter 4, JFH-1 G33A mutant RNA did not produce infectious particles following electroporation, as measured by RT-PCR and immunofluorescence. However, after cultivation for several passages, infectious virus particles were eventually produced. In the first experiment, infectious JFH-1 G33A mutant RNA was found to possess a second mutation in core (G32D). To confirm these results and determine whether the secondary mutation was responsible for the production of infectious virus particles, further repetitions of these infection studies were carried out. *In vitro* transcribed RNA (JFH-1 wild type, JFH-1 F24A mutant and JFH-1 G33A mutant) was electroporated into naïve Huh7 cells as previously described (see methods). Seventy-two hours post-electroporation, culture medium was harvested and cells trypsinised and passaged. Naïve Huh7 cell were seeded at $5x10^4$/ml and the following day incubated with filtered culture medium for 3 hrs at 37°C before washing with PBS and overlay with DMEM. Seventy-two hours post-infection, cells were either fixed in methanol for immunofluorescence or harvested using TRIzol and total RNA extracted. This process was repeated at each of the first 5 passages of the original electroporated cultures.

Cells fixed in methanol were permeablised with PBS-T and probed in an indirect immunofluorescence assay for HCV core and cellular DDX3. Cells electroporated with JFH-1 G33A mutant RNA showed typical HCV core staining as seen before (Fig. 5.1A). HCV core mutant G33A could be detected in a percentage of cells. However, there was no colocalisation with cellular DDX3. Immunofluorescence experiments were repeated using cells infected with culture supernatant from passage 1-5. Cells infected with supernatant taken from JFH-1 G33A cultures up to and including passage 3 showed no signs of HCV infection (Fig. 5.1B), as described in Chapter 4. However, cells infected with passage 4 JFH-1 G33A culture medium were positive for HCV core protein (Fig. 5.1C). Interestingly, while core protein produced from non-infectious JFH-1 G33A RNA did not colocalise with DDX3, core protein produced from infectious JFH-1 G33A RNA did colocalise with DDX3. Culture medium from passage 5 JFH-1 G33A mutant RNA-electroporated cells showed more HCV infection (Fig. 5.1D) and again, mutant core protein colocalised with DDX3.

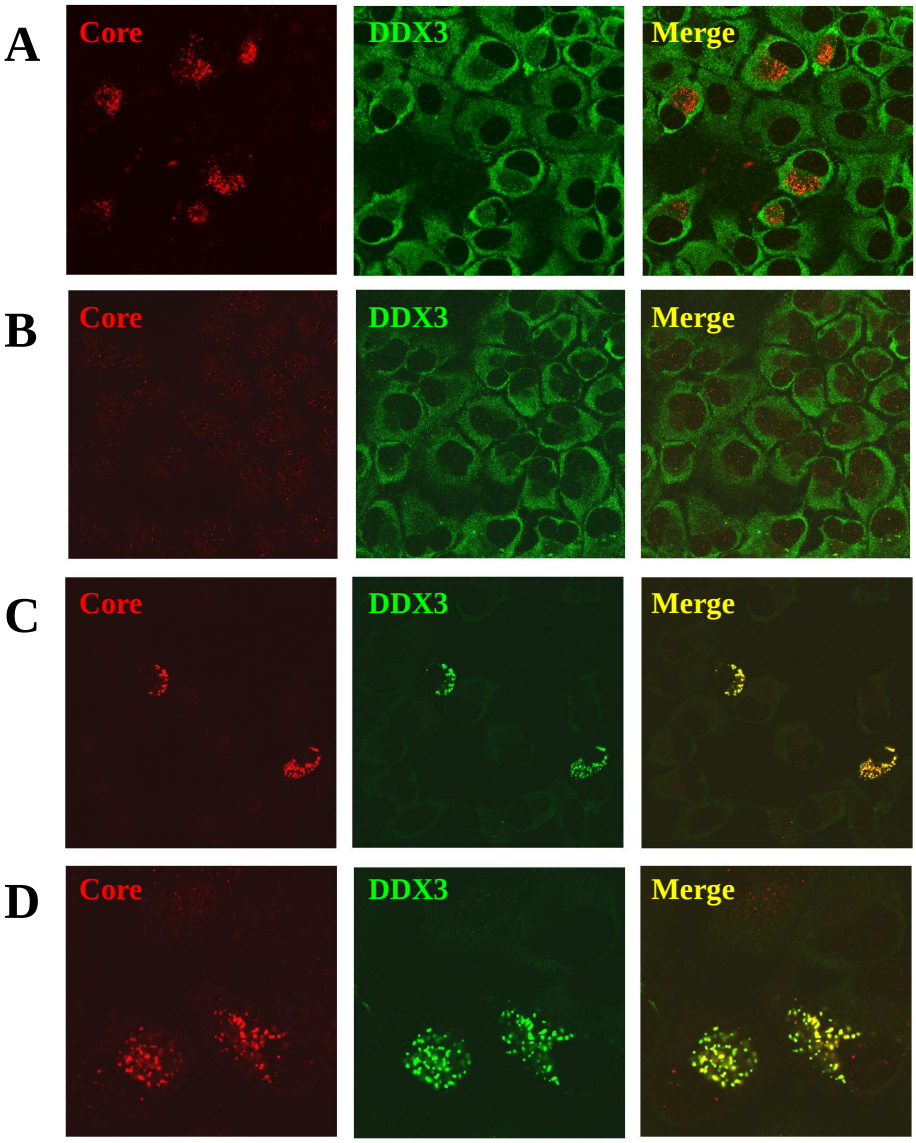


Figure 5.1: JFH-1 G33A mutant virus infections. Huh7 cells electroporated with 5 µg JFH-1 G33A mutant RNA were serially passaged. Before each passage, culture medium was harvested and incubated with naïve Huh7 cells for 3 hrs before washing and overlay with appropriate culture medium. Seventy-two hours post-infection, cells were fixed and probed with rabbit polyclonal anti-core antiserum (R308) and mouse monoclonal anti-DDX3 antibody (AO196) as in Chapter 3. A) electroporated cells, B) cells infected with passage 3 supernatant, C) cells infected with passage 4 supernatant, D) cells infected with passage 5 supernatant.

Huh7 cells infected with filtered culture medium from passages 3, 4 and 5 were also analysed for infection by the presence of negative strand replication intermediate JFH-1 RNA. Total RNA was isolated and reverse transcribed using HCV negative strand specific primer JFH-1 NegRT, and the resultant cDNA was amplified by PCR using primers JFH-1 RTPCR1 and JFH-1 RTPCR2. In accordance with the results obtained by immunofluorescence analysis (Fig. 5.1), no JFH-1 cDNA was detected in cells incubated with JFH-1 G33A mutant passage 3 culture medium, indicating there was no infection (Fig. 5.2A). Infection was clearly detected in cells incubated with passage 3 supernatant from wild type and mutant F24A cultures. Although this type of RT-PCR is not truly quantitative, cells incubated with culture medium from passage 4 G33A mutant appeared to have low levels of replicating JFH-1 RNA (Fig. 5.2B), in agreement with the immunofluorescence data. Finally, incubation of naïve Huh7 cells with passage 5 JFH-1 G33A culture medium clearly caused infection as confirmed by detection of negative strand, replicating JFH-1 RNA, with quantities appearing similar to that in wild type and mutant F24A infected cells (Fig. 5.2B).

As a final assay for detection of infectious virus particles, culture medium was titrated and $TCID_{50}$ calculated for each sample. Naïve Huh7 cells were seeded at $5x10^3$/well (96-well plate). The following day, culture medium was serially diluted 10-fold from neat – 10^{-7} and incubated with the naïve Huh7 cells. Three hours post-infection, cells were washed with PBS and overlayed with DMEM. Seventy-two hours post-infection, cells were washed with PBS and fixed in methanol. Cells were then permeablised with PBS-T and analysed by immunofluorescence for NS5A (fluorescent cells were viewed under UV light). Titration of passage 3 culture medium gave similar results to both the immunofluorescence and RT-PCR results. No infection was detected from mutant G33A culture medium while both wild type and mutant F24A supernatant was capable of infecting cells at 10^{-2} dilution (Table 5.1A). At passage 4, G33A culture medium was infectious, albeit only undiluted and at very low levels (Table 5.1B). Again, passage 4 culture medium from wild type and mutant F24A cultures was capable of infecting naïve cells at 10^{-2} dilution. Finally, by passage 5, G33A culture medium infected naïve cells at dilutions up to 10^{-2} (Table 5.1C).

During initial analysis of JFH-1 G33A mutant virus, infectious RNA was shown to possess 2 mutations in core. The first, introduced at residue 33 of core by site-directed mutagenesis and the second, inserted during replication. It is possible that this second mutation enabled the production of infectious particles from the non-infectious G33A mutant RNA. In order to identify any mutations that may have arisen during cultivation of JFH-1 G33A mutant virus, thus triggering the switch from non-infectious to infectious phenotype, nucleotide

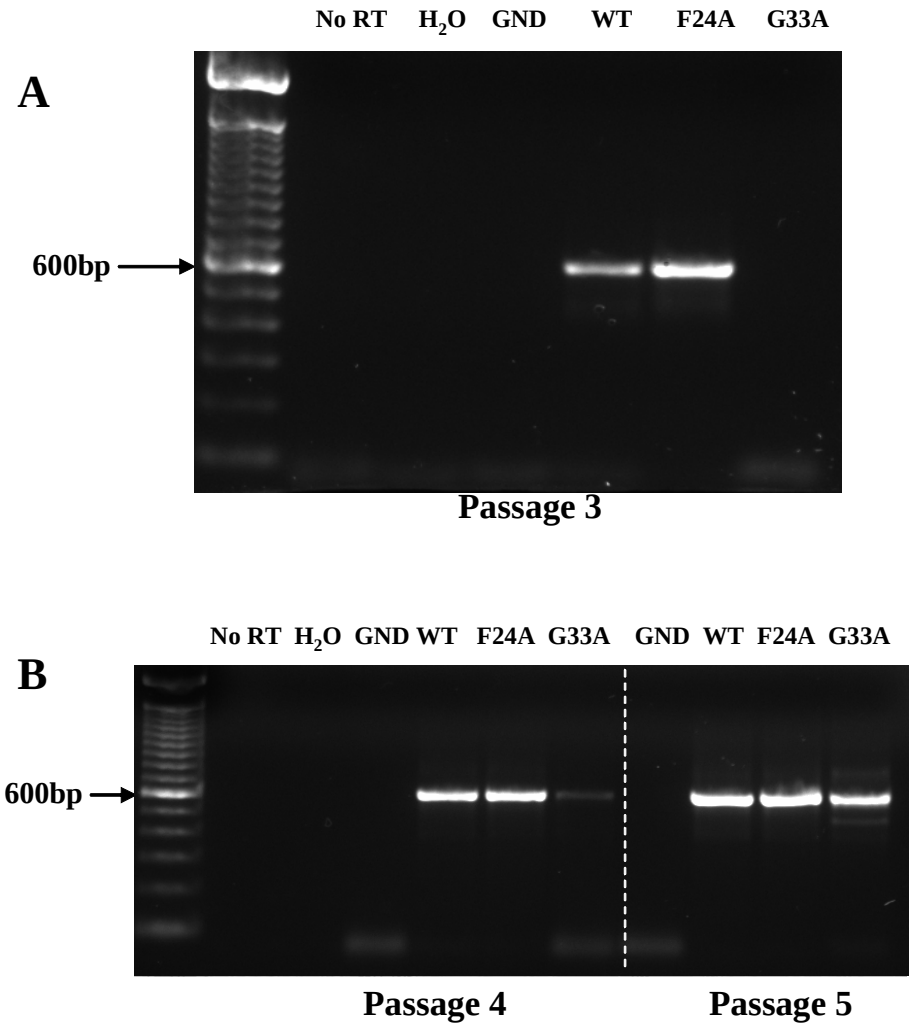


Figure 5.2: RT-PCR analysis of mutant G33A infection. Huh7 cells electroporated with G33A mutant RNA were serially passaged. Before each passage, culture medium was harvested, filtered and used to infect naïve Huh7 cells. Seventy-two hours post-infection, total RNA was extracted, reverse transcribed and resultant cDNA amplified as in Figure 4.5. A) cells infected with passage 3 medium, B) cells infected with passage 4 and passage 5 medium. No RT is control reaction using extract from JFH-1 infected cells with no reverse transcription stage, H_2O is no RNA control reaction.

A

	Wild Type	F24A	G33A
Neat	**6**	**6**	**0**
10^{-1}	**6**	**5**	**0**
10^{-2}	**4**	**1**	**0**
10^{-3}	**0**	**0**	**0**
10^{-4}	**0**	**0**	**0**
10^{-5}	**0**	**0**	**0**
10^{-6}	**0**	**0**	**0**
10^{-7}	**0**	**0**	**0**
log $TCID_{50}$/ml	**-3.12**	**-2.5**	**-**

B

	Wild Type	F24A	G33A
Neat	**6**	**6**	**2**
10^{-1}	**3**	**3**	**0**
10^{-2}	**1**	**1**	**0**
10^{-3}	**0**	**0**	**0**
10^{-4}	**0**	**0**	**0**
10^{-5}	**0**	**0**	**0**
10^{-6}	**0**	**0**	**0**
10^{-7}	**0**	**0**	**0**
log $TCID_{50}$/ml	**-2.17**	**-2.17**	**-0.83**

C

	Wild Type	F24A	G33A
Neat	**6**	**6**	**6**
10^{-1}	**6**	**6**	**6**
10^{-2}	**4**	**6**	**2**
10^{-3}	**1**	**1**	**0**
10^{-4}	**0**	**0**	**0**
10^{-5}	**0**	**0**	**0**
10^{-6}	**0**	**0**	**0**
10^{-7}	**0**	**0**	**0**
log $TCID_{50}$/ml	**-3.33**	**-3.66**	**-2.83**

Table 5.1: Mutant G33A $TCID_{50}$. Huh7 cells electroporated with G33A mutant RNA were serially passaged. Before each passage, culture medium was harvested and serially diluted 10-fold before being used to infect naïve Huh7 cells. Seventy-two hours post-infection, cells were fixed in methanol, permeablised with PBS (0.05% Tween) and probed with anti-NS5A antibody followed by anti-sheep FITC secondary antibody. Wells positive for NS5A were counted and $TCID_{50}$ calculated (see Materials and Methods). A) Cells infected with passage 3 medium, B) cells infected with passage 4 medium, C) cells infected with passage 5 medium.

sequence analysis of the full HCV core region (amplified during RT-PCR experiments) was conducted following electroporation of fresh RNA in a repeat experiment. Using core specific primers JFH-1 RTPCR1 and JFH-1 RTPCR2, viral RNA was sequenced 72 hrs post-electroporation. The only mutation present was the G33A mutation inserted by site directed mutagenesis (Fig. 5.3A). Next, the infectious JFH-1 G33A RNA (amplified from cells infected with passage 5 G33A culture medium) was sequenced. Again, the original G33A mutation was present. However, a second mutation had arisen at residue 34 with valine being substituted for alanine (Fig. 5.3E). In order to identify when this mutation arose, nucleotide sequence analysis was carried out on the JFH-1 G33A mutant RNA extracted from cultivated cells at passage 1, 3 and 5. At passage 1, the sequence of JFH-1 G33A core protein was identical to that of the electroporated G33A core sequence (Fig. 5.3B). At passage 3, mutant G33A is still unable to produce infectious virus particles as shown by immunofluorescence, RT-PCR and titration. In agreement with this, the nucleotide sequence of G33A core protein is identical to that after electroporation (Fig. 5.3C). Interestingly, at passage 5, when culture medium becomes infectious, two different HCV core nucleotide sequences can be detected in the cultured cells. As well as a thymidine at position 441 (core amino acid 34) of the JFH-1 nucleotide sequence (wild type JFH-1 sequence), a larger peak can be seen representing a cytosine at this position (Fig. 5.3D). This nucleotide mutation results in an amino acid substitution (V34A). The sequence containing the V34A mutation was the only sequence detected in cells infected with passage 5 JFH-1 G33A mutant culture medium, indicating that this mutation may be playing a role in the production of infectious virus particles. This mutation was not the same as the mutation detected in the first experiment (G32D), however, it is only situated 2 residues downstream, and only 1 residue downstream of the original G33A mutation (Fig. 5.4).

In total, this experiment was conducted 4 times, i.e. JFH-1 G33A mutant RNA was electroporated into naïve Huh7 cells on 4 separate occasions, alongside wild type, GND and F24A mutant RNA. On each occasion, infectious virus particles were not produced within the first 3 passages of the cells. Infectious particles were eventually produced from cultured cells after 4-6 cell passages. On each occasion, naïve cells successfully infected with infectious G33A virus were harvested with TRIzol and total RNA extracted. Negative strand viral RNA was detected by RT-PCR as before and resultant PCR products sequenced. The nucleotide sequence analysis results are shown in Figure 5.5. In each experiment a different mutation arose, however, each was located within the surrounding residues of the initial mutation at residue 33 (G32D, V34A, L36S and L37S). To rule out

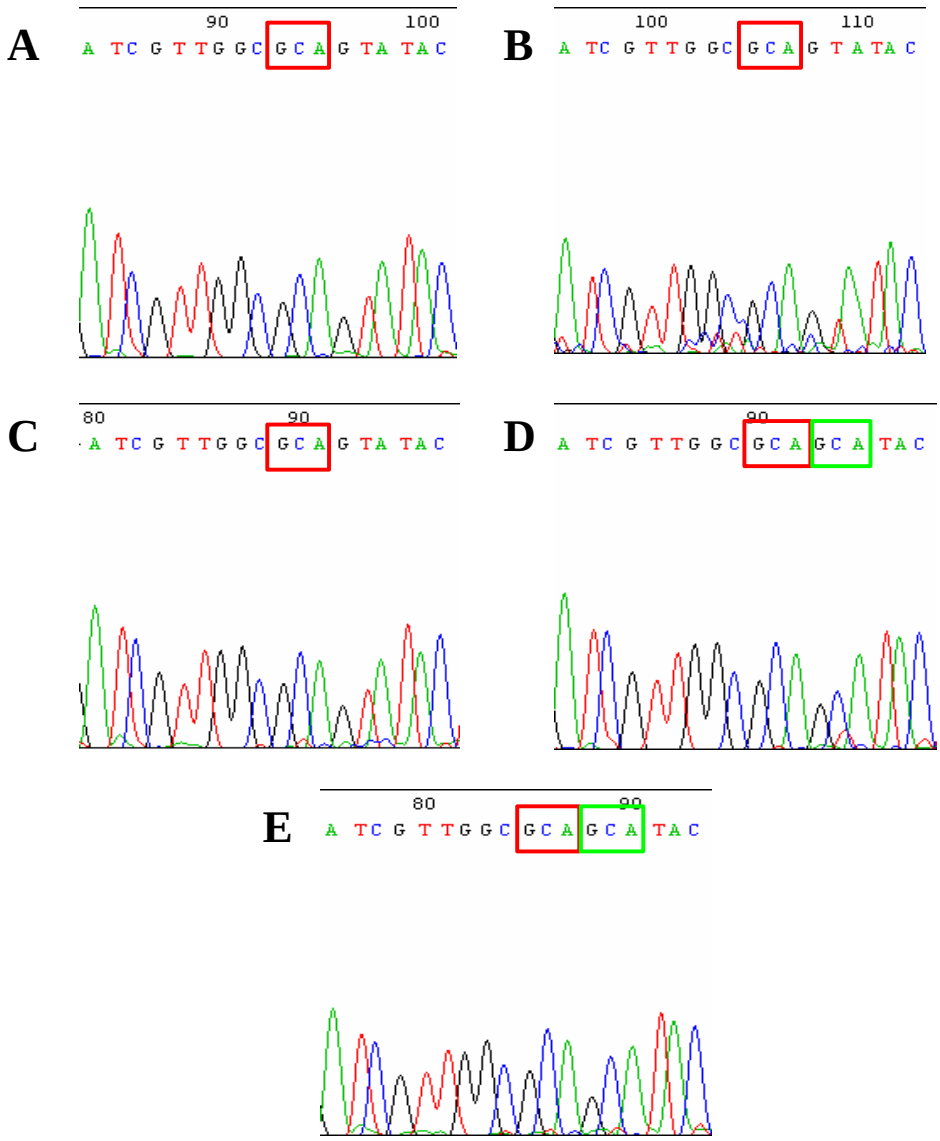


Figure 5.3: Nucleotide sequence analysis of JFH-1 G33A mutant from repeat experiment. RT-PCR products from various passages were sequenced using JFH-1 core specific primers JFH-1 RTPCR1 and RTPCR2. A) Electroporated cells, B) passage 1 cells, C) passage 3 cells, D) passage 5 cells, E) cells infected with passage 5 medium. Red box indicates original G33A mutation, green box indicates compensatory V34A mutation.

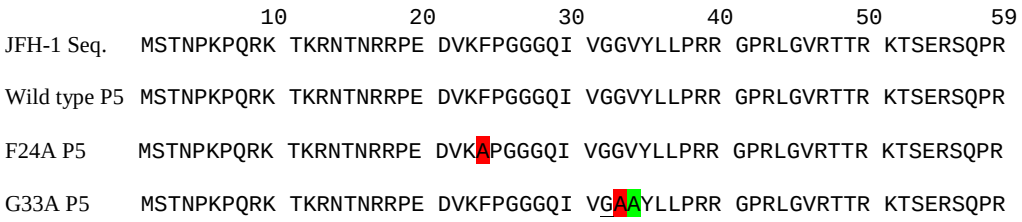


Figure 5.4: Sequence analysis of infectious G33A mutant from repeat experiment. Culture medium from passage 5 cells was used to infect naïve Huh7 cells. Cells were harvested 72 hrs post-infection and total RNA extracted. RT-PCR products were sequenced using core specific primers JFH-1 RTPCR1 and JFH-1 RTPCR2. Red residues represent original site-directed mutations, green residues represent new mutation, and underlined residue represents the amino acid changed by compensatory mutation in original experiment. Only amino acids 1-59 are shown here, all mutants and wild type had identical sequence to original JFH-1 sequence from residue 60-191.

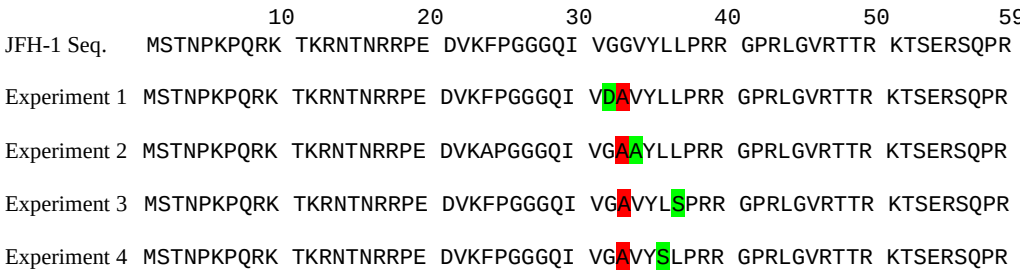


Figure 5.5: Sequence analysis of infectious G33A mutants from 4 separate experiments. G33A RNA was electroporated into 4 separate Huh7 cultures. After at least 5 passages, infectious virus was produced. Total RNA was extracted from cells infected with medium from these cultures 72 hrs post-infection. RT-PCR was carried out as before and PCR products sequenced using core specific primers JFH-1 RTPCR1 and JFH-1 RTPCR2. Red residues represent original site-directed mutations, green residues represent new mutations. Only amino acids 1-59 are shown here, all mutants and wild type had identical sequence to original JFH-1 sequence from residue 60-191.

sequencing errors, wild type RNA from each experiment was sequenced and no mutations detected. This indicates that this region of core protein may be critical for the production of infectious particles. It can therefore be hypothesised that, with the mutation of HCV core residue 33, the virus requires a compensatory mutation in the surrounding area of core in order to allow production of infectious particles. However, it cannot be ruled out that production of infectious particles is also due to other additional mutations in the virus, out-with core.

5.2.2 Identification of further Mutations arising in Mutant G33A

The above data led to the hypothesis that a compensatory mutation is required in order for JFH-1 G33A mutant RNA to produce infectious virus particles. To confirm that the compensatory mutations identified do in fact allow production of infectious JFH-1 G33A virus particles, site-directed mutagenesis was used to introduce the G33A and V34A mutations into the wild type JFH-1 sequence. The cloning strategy used was that described in section 4.2.3.1 and nucleotide substitutions were confirmed by nucleotide sequence analysis. The JFH-1 sequence (containing the double mutation) was sub-cloned back into pJFH-1 and new construct (pJFH-1 G33A-V34A) linearised, *in vitro* transcribed as before and electroporated into naïve Huh7 cells. Seventy-two hours post-electroporation, cells were washed with PBS and fixed in methanol. Cells were then permeablised with PBS-T and probed by immunofluorescence for core and DDX3. As seen before, wild type core protein colocalised with cellular DDX3 (Fig. 5.6A) while there was no colocalisation between mutant G33A core protein and DDX3 (Fig. 5.6B). In a previous experiment (section 5.2.1), a mutation at residue 34 (V34A) in JFH-1 G33A mutant RNA coincided with the production of infectious virus particles from this mutant RNA. Naïve cells infected with JFH-1 G33A mutant virus (containing V34A mutation), showed colocalisation between core and DDX3 (Fig. 5.1C/D). Similarly here, mutant core G33A-V34A colocalised with DDX3 in a manner similar to wild type core (Fig. 5.6C). To confirm that JFH-1 G33A-V34A mutant RNA produced infectious virus particles, culture medium was harvested from cells 72 hrs post-electroporation. Naïve Huh7 cells were seeded at $5x10^4$ cells/ml. The following day, culture medium was filtered and incubated with naïve cells. Three hours post-infection, cells were washed with PBS and overlayed with appropriate culture medium. Seventy-two hours post-infection cells were washed with PBS and fixed in methanol. Cells were then permeablised with PBS-T and probed by immunofluorescence for core and DDX3. As expected, medium from cells electroporated

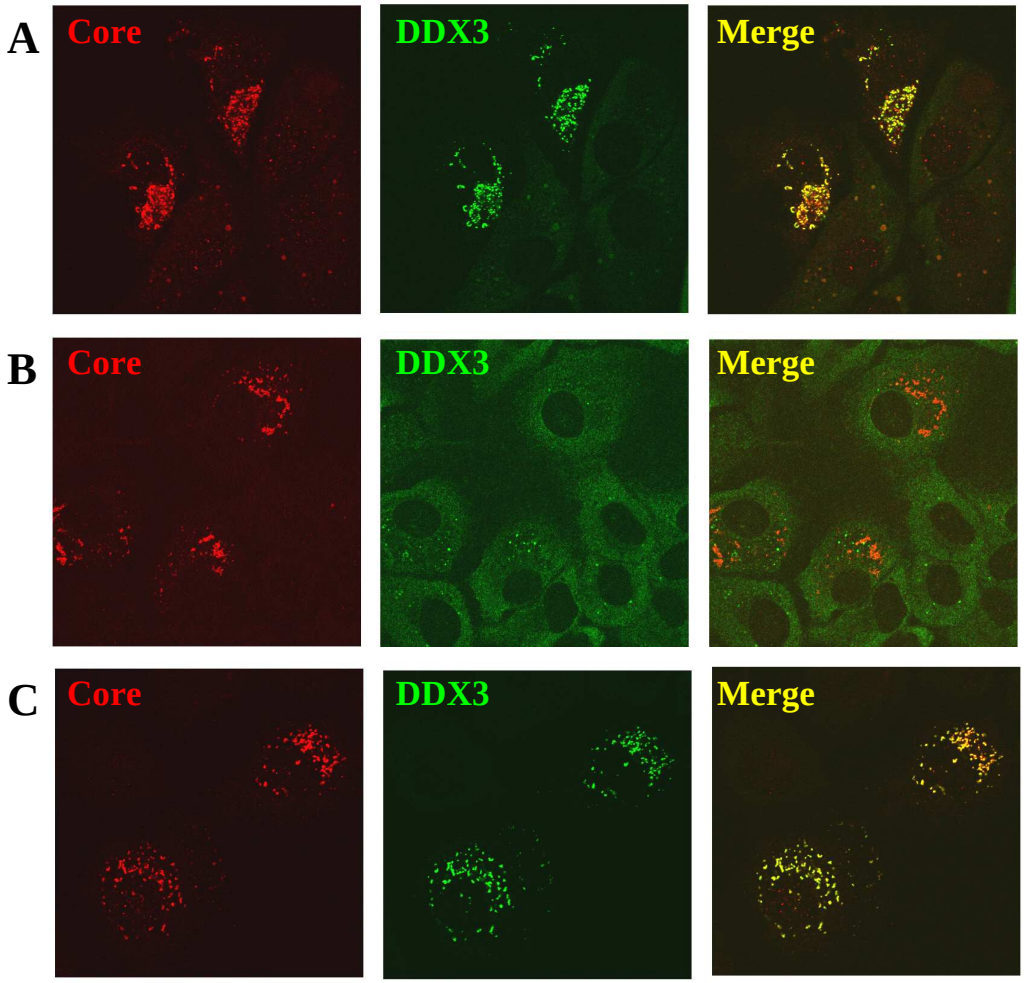


Figure 5.6: Colocalisation of G33A-V34A mutant core with DDX3. Huh7 cells were electroporated with *in vitro* transcribed JFH-1 mutant RNA G33A-V34A. Seventy-two hours post-electroporation, cells were fixed and probed for core and DDX3 as in Chapter 3. A) Cells electroporated with wild type JFH-1 RNA, B) cells electroporated with G33A mutant RNA, C) cells electroporated with G33A, V34A mutant RNA.

with wild type JFH-1 RNA was capable of infect naïve Huh7 cells, with core protein colocalising with DDX3 (Fig. 5.7A). Again, as seen previously, the absence of core staining in cells incubated with medium from the JFH-1 G33A mutant RNA electroporation confirmed infectious virus was not produced from JFH-1 G33A mutant RNA (Fig. 5.7B). Somewhat surprising however was the absence of core staining in cells incubated with supernatant from the JFH-1 G33A-V34A mutant RNA electroporation (Fig. 5.7C), thus indicating that infectious virus particles were not produced despite the compensatory mutation at residue 34. Therefore, it appears that the compensatory mutation was required to allow colocalisation with DDX3 but not production of infectious virus.

Electroporated cultures were cultivated until passage 5 when immunofluorescence analysis was again carried out. Cells cultured for 5 passages were washed in PBS and fixed in methanol. Cells were then permeablised with PBS-T and probed for core and DDX3. As before, wild type core protein colocalised with DDX3 (Fig. 5.8A). Previous analysis of mutant G33A showed that G33A mutant core protein may colocalise with DDX3 after introduction of a second mutation. On this occasion, mutant G33A core protein colocalised with DDX3 in some cells (Fig. 5.8B) while in others there was no colocalisation (Fig. 5.8C). No G33A-V34A mutant core protein was detected in cells cultured for 5 passages (Fig. 5.8D). It is possible that this is due to a lack of compensating mutation arising before cells carrying replicating G33A-V34A RNA were diluted out of the culture. Culture medium was harvested from passage 5 cultures and incubated with naïve Huh7 cells as before. Seventy-two hours post-infection, cells were washed with PBS and fixed in methanol. Cells were then permeablised with PBS-T and probed for core and DDX3. Infection was achieved using wild type culture medium (Fig. 5.9A) and also that from G33A (Fig. 5.9B and C). As with the G33A virus cultured for 5 passages (Fig. 5.8B and C), infectious JFH-1 G33A mutant virus showed a mix of core that did and did not colocalise with DDX3.

To confirm these results, RT-PCR was carried on cells at each stage to detect replicating viral RNA. Replicating HCV RNA was detected in wild type, G33A and G33A-V34A electroporated cells 72 hrs post-electroporation (Fig. 5.10A). However, after 5 passages, replicating JFH-1 G33A-V34A mutant RNA was no longer detectable (Fig. 5.10A). In accordance with the immunofluorescence data, negative strand RNA was not detected in cells incubated with G33A or G33A-V34A medium (harvested 72 hrs post-electroporation) (Fig. 5.10B). Replicating RNA was detected however, in cells infected with culture medium from JFH-1 G33A mutant virus that had been cultured for 5 passages, but not from JFH-1 G33A-V34A mutant virus cultured for the same period of time (Fig. 5.10B).

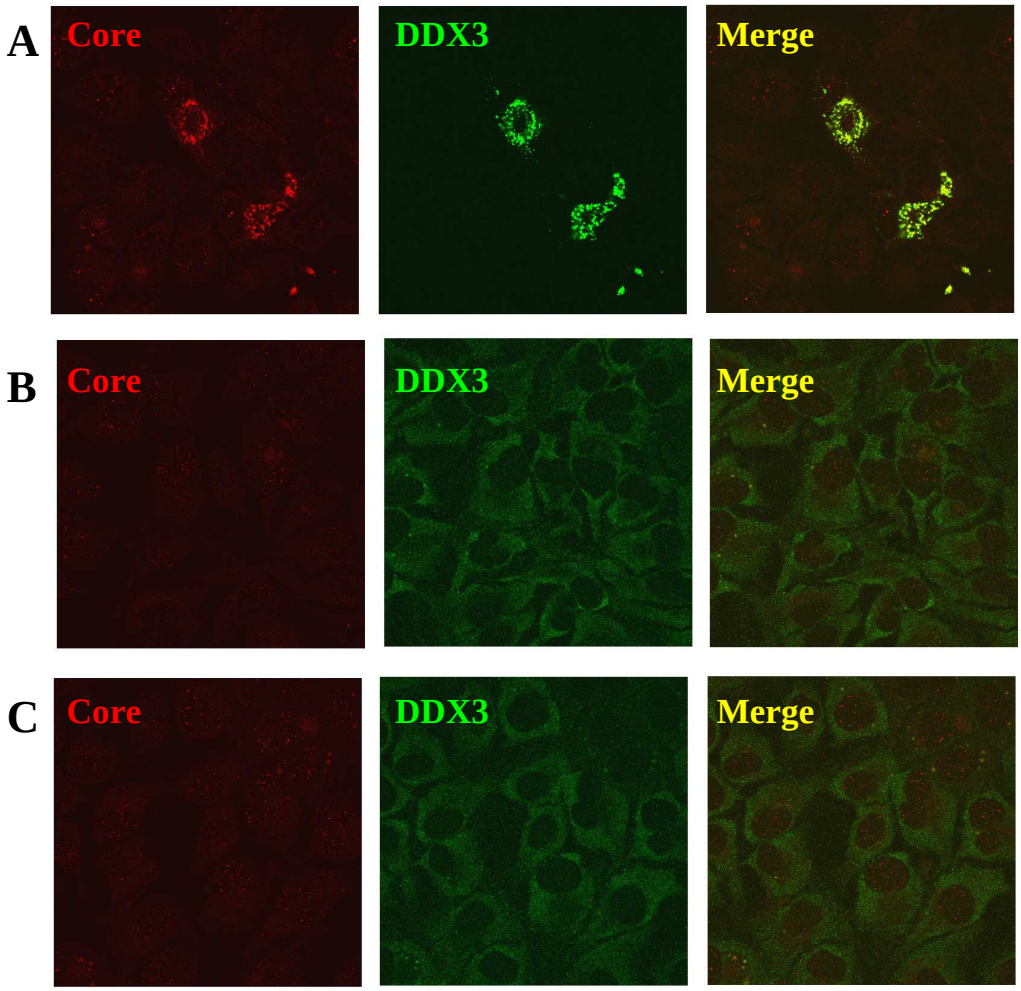


Figure 5.7: Mutant G33A-V34A infection studies. Culture medium was harvested from electroporated cells 72 hrs post-electroporation and used to infect naïve Huh7 cells. Seventy-two hrs post-infection, cells were fixed and probed for core and DDX3 as in Chapter 3. A) wild type, B) G33A mutant, C) G33A-V34A mutant.

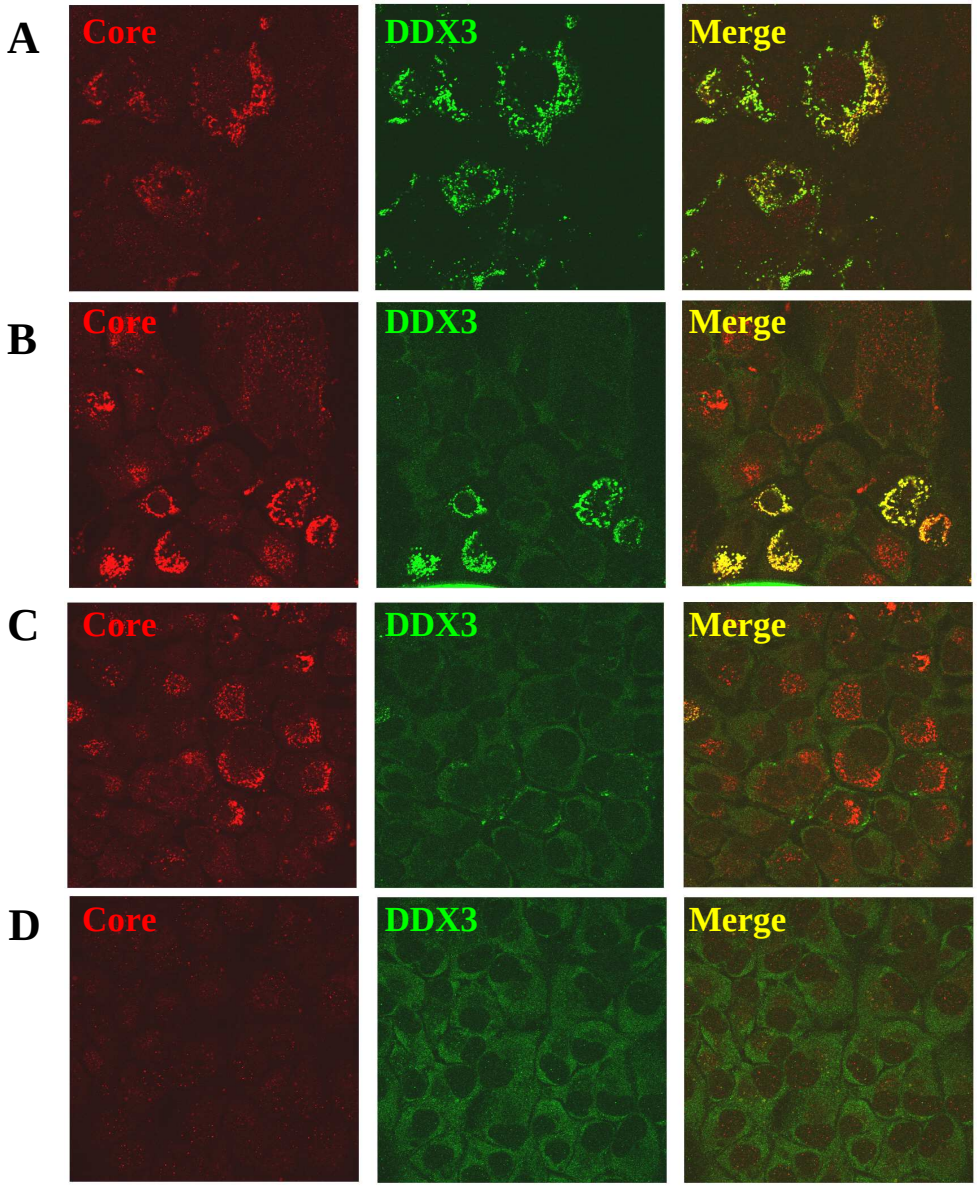


Figure 5.8: Passaging of Mutant G33A-V34A electroporated cells. Cells electroporated with *in vitro* transcribed RNA were passaged 5 times and seeded on coverslips. Seventy-two hours post-seeding, cells were fixed and probed for core and DDX3 as in chapter 3. A) wild type, B) G33A mutant, C) G33A mutant, D) G33A-V34A mutant.

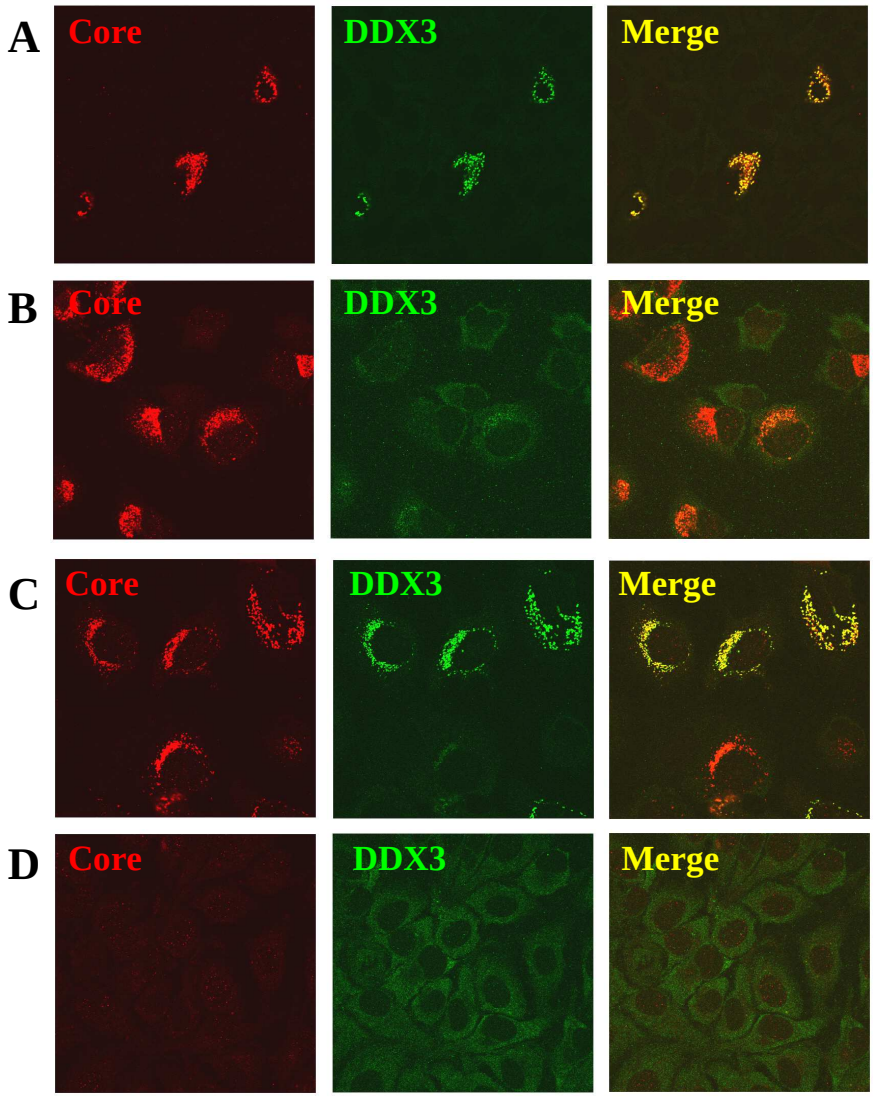


Figure 5.9: Mutant G33A-V34A infection studies. Cells electroporated with *in vitro* transcribed RNA were passaged 5 times. Culture medium was then harvested and used to infect naïve Huh7 cells. Seventy-two hours post-infection, cells were fixed and probed for core and DDX3 as in Chapter 3. A) wild type, B) G33A mutant, C) G33A mutant, D) G33A-V34A mutant.

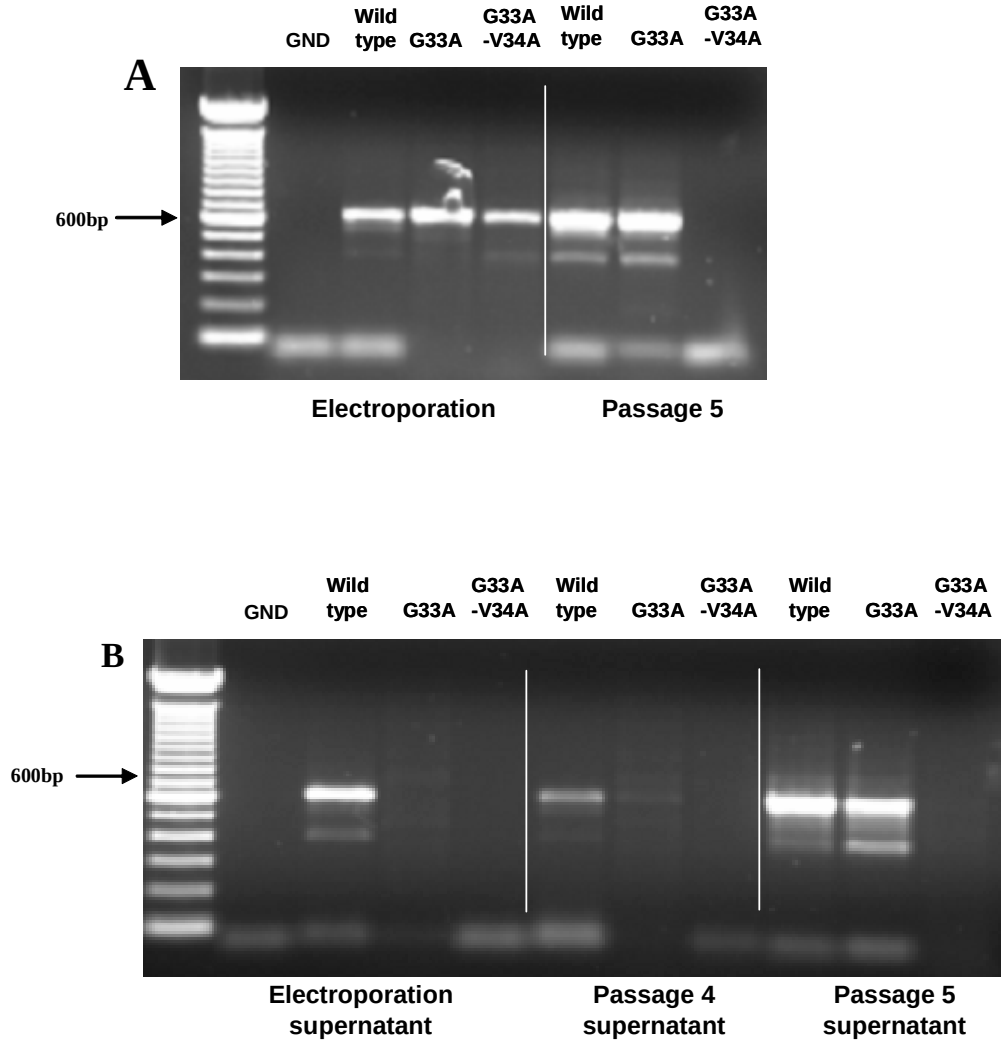


Figure 5.10: Replication of mutant G33A-V34A RNA. RT-PCR was carried out on cells harvested using TRIzol, using primer JFH-1 NegRT to detect negative strand replication intermediate viral RNA and primers JFH-1 RTPCR1 and JFH-1 RTPCR2 to amplify the subsequent cDNA. A) electroporated cells and passage 5 cells, B) cells infected with supernatant from various passages.

5.3 Discussion

The previous chapter showed that, when electroporated into naïve Huh7 cells, *in vitro* transcribed JFH-1 G33A mutant RNA replicated efficiently but was unable to produce infectious progeny virus. After cultivation of cells with replicating viral RNA, infectious particles were produced. Nucleotide sequence analysis of this infectious RNA uncovered a mutation at residue 32 of core. Further analysis in this present Chapter has confirmed that JFH-1 G33A mutant RNA alone does not produce infectious RNA. Electroporated JFH-1 G33A mutant RNA replicates within Huh7 cells and, as expected, core protein does not colocalise with cellular DDX3 as shown by RT-PCR and immunofluorescence. Medium from these cultures is not, however, capable of infecting naïve Huh7 cells. Whether or not particles are produced from this mutant RNA and are non-infectious is still unclear. Further studies are required in order to determine this, for example HCV core ELISA to detect core protein in the culture medium or perhaps RT-PCR to detect positive strand viral RNA in the medium. Care would be required in analysing supernatant by RT-PCR, however, as it is possible that *in vitro* transcribed RNA that did not enter cells during electroporation could be detected. Upon culture of electroporated cells, infectious JFH-1 G33A mutant virus particles are produced, normally after 4-6 passages. Infection was confirmed by both immunofluorescence to detect core protein and by RT-PCR detecting negative strand replication intermediate viral RNA. By immunofluorescence, core protein produced from infectious JFH-1 G33A mutant RNA was shown to colocalise with DDX3 in some cases. This is not believed to be the reason for the switch from non-infectious to infectious virus production since some infected cells did not show colocalisation between core and DDX3 and mutant F24A, which does not show colocalisation between core and DDX3, produces infectious virus following electroporation. Also, if the interaction between core and DDX3 is responsible for the production of infectious particles, then infectious particles would not have been produced from other JFH-1 mutant RNAs analysed. Nucleotide sequence analysis of infectious JFH-1 G33A mutant RNA uncovered a second mutation, the appearance of which coincided with the production of infectious particles. In total, 4 mutations were discovered in separate experiments, each of which was within 4 residues of the original G33A mutation. It is therefore possible that while the G33A mutation has no effect on HCV RNA replication, it has an adverse effect on infectious particle production, possibly by affecting the structure of core as suggested by the proposed structural difference between wild type core and mutant G33A core (Fig. 3.18). A random replication error is then required in order to compensate for the structural change caused by the G33A mutation.

To confirm that a mutation near residue 33 of core was responsible for the production of infectious particles, infection studies were carried out on JFH-1 RNA containing the original mutation G33A and a mutation at residue 34 (V34A), shown to be present in infectious JFH-1 G33A RNA. Culture medium from cells electroporated with this RNA was non-infectious for naïve Huh7 cells as shown by immunofluorescence and RT-PCR. This suggests that another compensatory mutation is required in order to allow production of infectious particles. No mutations were found in the core coding sequence other than the 4 found between residues 32 and 37, indicating that the mutation is elsewhere within the JFH-1 genome. It is possible that a combination of mutations are required, only one of which is within the core region, or perhaps the mutation in core is not required for infectious particle production. This seems unlikely however, since no other mutant RNA developed a similar mutation (Chapter 4). With the exception of 4 genotype 3b isolates, residue 33 of HCV core is completely conserved throughout all genotypes. The 4 genotype 3b isolates with a mutation at residue 33 (glycine – glutamic acid) (Fig. 3.21) also have mutations at residues 8, 16, 20, 26, 43, 48, 50-53 and 55, any one of which could be compensating for the mutation at residue 33. It is also possible however, that these strains are not infectious.

Having discovered that residue 33 of HCV core protein is essential for the production of infectious particles, future work should concentrate on identifying the mechanism behind this block, whether it is at the particle formation stage or during the infection process.

6. Nucleo-cytoplasmic Shuttling of DDX3

6.1 Introduction

Although a function has not yet been assigned to DDX3 with regards to the life cycle of HCV, it has been shown that DDX3 is involved in the Rev-RRE (rev response element)/CRM1 pathway for the export of intron-containing HIV-1 transcripts (Yedavalli *et al.*, 2004). Chromosomal region maintenance protein 1 (CRM1) was initially discovered in *S. pombe* while analysing cold sensitive mutants and their effect on chromosome structure (Adachi and Yanagida, 1989). An accumulation of evidence suggests that this evolutionarily conserved protein is an essential factor in the nuclear export of proteins containing a nuclear export signal (Kudo *et al.*, 1997, Nishi *et al.*, 1994, Adachi and Yanagida, 1989, Wolff *et al.*, 1997). CRM1 has also been shown to be the target for leptomycin B (LMB) (Nishi *et al.*, 1994), a potent inhibitor of signal-dependent nuclear export of proteins (Kudo *et al.*, 1998). Studies on HIV-1 rev protein suggest that rev transports unspliced and partially spliced HIV-1 transcripts from the nucleus to the cytoplasm by binding CRM1 (Askjaer *et al.*, 1998, Otero *et al.*, 1998, Bogerd *et al.*, 1998), using a nucleo-cytoplasmic shuttling pathway (Fukuda *et al.*, 1997). DDX3 binds CRM1 and rev, shuttles between nucleus and cytoplasm and is required to have enzymatic activity (Yedavalli *et al.*, 2004). Leptomycin B was also shown to block DDX3 export from the nucleus, presumably by acting on CRM1 (Yedavalli *et al.*, 2004). Identification of DDX3 as a nucleo-cytoplasmic shuttling protein was carried out using a transfected, HA-tagged DDX3. Here, in order to confirm if endogenous DDX3 is a nucleo-cytoplasmic shuttling protein, Huh7 cells were incubated with LMB before analysing the cellular localisation of DDX3, using a rabbit polyclonal anti-DDX3 antiserum (R648). Using Nneo/C-5B (2-3) cells, which harbour an autonomously replicating, genome length, dicistronic, selectable HCV genotype 1b RNA (Ikeda *et al.*, 2002), the effect of LMB on the colocalisation between HCV core and cellular DDX3 was also analysed.

6.2 Results

6.2.1 DDX3 is a nucleo-cytoplasmic shuttling protein

Yedavalli *et al.* (2004) showed that exogenously expressed, HA-tagged DDX3 binds CRM1 in the nucleus and shuttles between nucleus and cytoplasm and that LMB, a potent inhibitor of the CRM1 export pathway, blocked the export of HA-tagged DDX3 from the nucleus (Yedavalli *et al.*, 2004). It was also shown that LMB blocked the export of HA-tagged DDX3 from the nucleus. In order to confirm if endogenous DDX3 acted in the same way, Huh7 cells were incubated at 37ºC with 12.5 mM, 25 mM or 50 mM LMB in the appropriate culture medium. Two hrs post-incubation, cells were washed with PBS, fixed in methanol and permeablised with PBS-T before being probed for DDX3. At concentrations of as low as 12.5 mM, a build-up of endogenous DDX3 in the nucleus was seen, indicating that, as with HA-tagged exogenous DDX3, LMB is able to block the nuclear export of cellular DDX3 (Figure 6.1). At greater concentrations of LMB, no increase in nuclear build-up was seen.

6.2.2 HCV core colocalises with DDX3 in the presence of LMB

To confirm HCV core and cellular DDX3 colocalise in the presence of LMB, 2-3 cells were incubated at 37ºC with 12.5 mM LMB in the appropriate culture medium. Two hours post-incubation, cells were washed with PBS, fixed in methanol and permeablised with PBS-T before being probed with mouse monoclonal anti-core antibody and rabbit polyclonal anti-DDX3 antiserum (R648), followed by the appropriate secondary antibodies. In the presence of LMB, a build-up of DDX3 was seen in the nucleus as before, and, similar to 2-3 cells in the absence of LMB, colocalisation was seen between core and DDX3 in the cytoplasm (Figure 6.2).

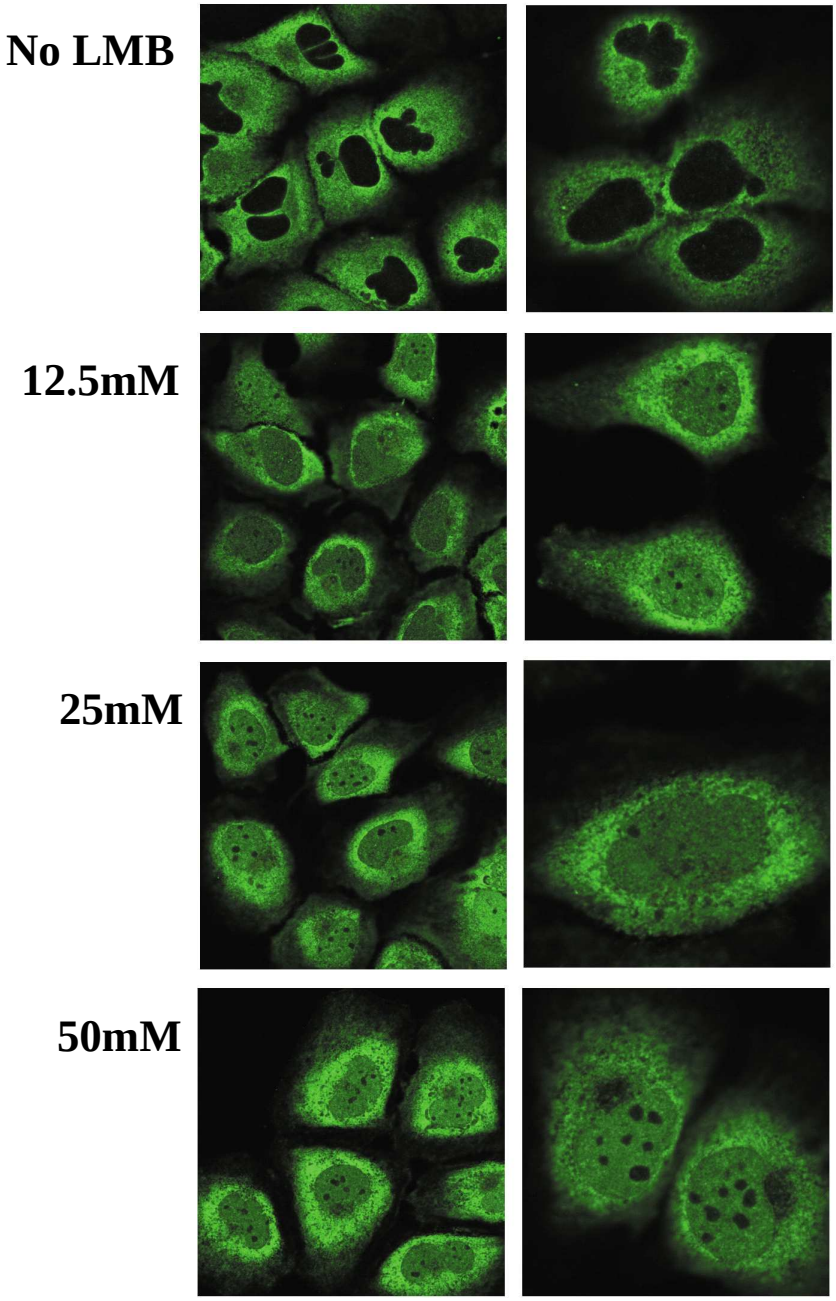


Figure 6.1: Leptomycin B inhibits DDX3 nuclear export. Huh7 cells were seeded at $5x10^4$ cells/ml. The following day, cells were treated with CRM1 inhibitor LMB at concentrations shown for 2 hrs at 37°C before being fixed in methanol, permeablised with PBS-T and probed with rabbit polyclonal anti-DDX3 antiserum (R648), followed by anti-rabbit FITC. The panels on the right show close-up images, highlighting the build-up of DDX3 in the nucleus and absence in the nucleolus.

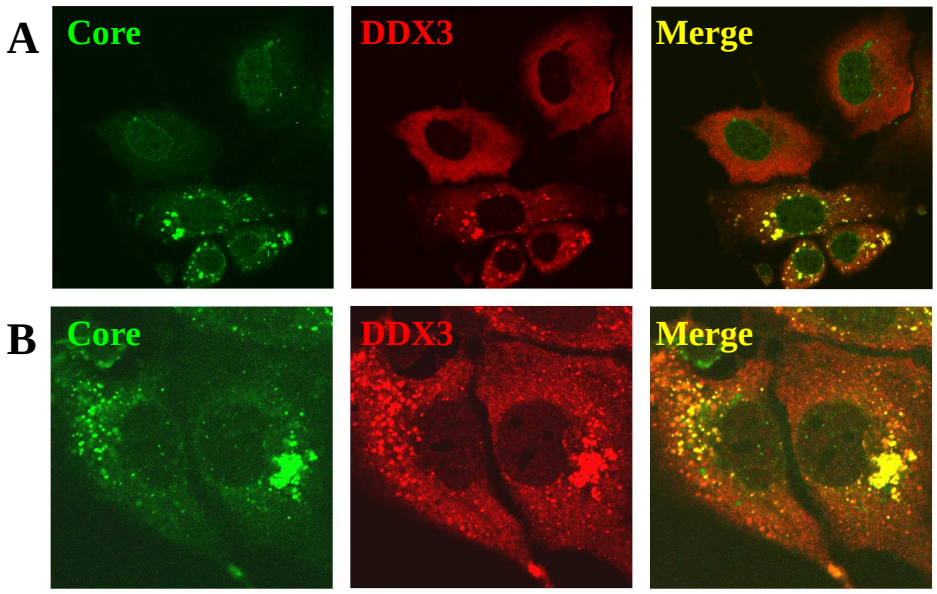


Figure 6.2: HCV core – DDX3 colocalisation in the presence of LMB. HCV replicon-harbouring Neo/C-5B (2-3) cells were seeded at $5x10^4$ cells/ml. The following day, cells were treated with LMB (12.5 mM) for 2 hrs at 37°C before being fixed in methanol, permeablised with PBS-T and probed with mouse monoclonal anti-core antibody and rabbit polyclonal anti-DDX3 antiserum (R648), followed by the appropriate secondary antibodies (anti-mouse FITC and anti-rabbit TRITC). A) No LMB, B) 12.5mM LMB.

6.3 Discussion

The results shown here using endogenous DDX3 confirm the results described by Yedavalli *et al.* (2004). Leptomycin B mediated blockage of the CRM1 export pathway causes a build-up of DDX3 in the nucleus. This confirms that DDX3 is a nucleo-cytoplasmic shuttling protein and relies on the CRM1 export pathway in order to shuttle from nucleus to cytoplasm. The hypothesis given by Yedavalli *et al.* (2004) for the involvement of DDX3 in the Rev-RRE / CRM1 pathway is that DDX3 unwinds large HIV-1 RNAs allowing them to pass through the nuclear pore and into the cytoplasm. As the HCV replication cycle takes place solely in the cytoplasm, a role for DDX3 in shuttling HCV RNA from the nucleus to the cytoplasm seems highly unlikely. However, it is possible that in the case of HCV, core sequestration of DDX3 prevents DDX3 from shuttling some cellular factor from nucleus to cytoplasm, possibly of antiviral nature.

In the presence of LMB, although a build-up of DDX3 was seen in the nucleus, colocalisation between core and DDX3 was still present in the cytoplasm of full-length replicon-harbouring cells. There are several plausible reasons for this. Firstly, colocalisation may be due to DDX3 sequestered before the addition of LMB. As LMB is toxic to cells, it was not possible to determine whether after a long-term incubation the interaction would diminish due to a limiting amount of cytoplasmic DDX3. It may also be that there are various forms of DDX3 and the form sequestered by core is not the form required for nuclear shuttling. The possibility of multiple forms of DDX3 is backed up by the discovery of an antibody that detects a nuclear form of DDX3 (A. Patel, unpublished data).

This work confirms that DDX3 is a nucleo-cytoplasmic shuttling protein, however, it is still unclear if its role in nuclear export is related to its sequestration by HCV core protein. It would be of interest to study the effects of co-infection with HIV-1 and HCV in terms of core-DDX3 colocalisation and DDX3 mediated export of Rev associated HIV-1 RNAs.

7. Conclusions

7.1 HCV core – DDX3 Interaction

Since the initial description of an interaction between HCV core protein and the cellular DEAD-box RNA helicase DDX3, little more has been reported on the significance of this interaction, while the true cellular function of DDX3 still remains undetermined. To investigate the significance of the core-DDX3 interaction in terms of the HCV life cycle, a detailed analysis of the HCV core amino acids required for interaction was carried out. What follows is a description of the major findings of this research project, culminating in a discussion on the possible functional significance of the core-DDX3 interaction.

7.1.1 Identification of Critical HCV Core Residues

The region of core involved in the interaction with DDX3 was reported to be between amino acids 1 and 59 (Owsianka and Patel, 1999) or amino acids 1-40 (You *et al.*, 1999). Analysis of a library of HCV $core_{1-59}$ mutants, each containing 0-4 amino acid substitutions, identified 9 mutants which, when fused to GFP and expressed in bacteria, showed reduced ability to bind GST-fused DDX3 in an ELISA assay (Fig 3.9). Systematic analysis of these 9 mutants expressed in Huh7 cells in the context of core, E1 and E2 resulted in the identification of a 13-residue region (amino acids 24-36), 6 residues of which were critical for interaction with cellular DDX3 (Fig 3.16). None of the 6 mutations in core affected the localisation of core to the lipid droplets (Fig. 4.12). As residues out-with positions 24-36 were not tested individually for interaction with DDX3 it is possible that other surrounding residues are also essential for the interaction, therefore it should be said that there are at least 6 residues of HCV core protein critical for interaction with cellular DDX3.

These 6 residues are highly conserved throughout all HCV genotypes, with only 4 genotype 3B isolates showing any differences, each of which has a glutamine to glutamic acid substitution at residue 33 (Fig. 3.21), however the infectiousness of these isolates is not known. Analysis of the positions of these 6 residues on a standard α-helix model unveiled their close proximity to one another (Fig. 3.17), suggesting a possible binding site for the cellular helicase DDX3.

7.1.2 Analysis of Core Mutants in HCVcc System and Effects on HCV Life Cycle

To study the role of DDX3 in the HCV life cycle, the 6 critical amino acids of core were substituted for alanine within individual JFH-1 backgrounds. As in the transient expression system, mutant core protein did not colocalise with cellular DDX3 (Fig 4.11), thus confirming the essential role these residues play in the interaction with DDX3. Replication of HCV RNA was not abolished completely in the absence of core-DDX3 interaction, suggesting DDX3 is not essential for the unwinding of highly structured HCV 5' or 3' RNA. This is of no surprise since HCV encodes its own RNA helicase which is believed to carry out this function (Banerjee and Dasgupta, 2001, Lam and Frick, 2006). It is possible however, that DDX3 plays some accessory role since RNA replication levels of mutant viruses were >50-fold lower than that of wild type virus (Fig. 4.23). A detailed analysis of wild type and mutant RNA replication levels is currently being carried out.

Ded1, the yeast homologue of DDX3, is required for the initiation step of translation (Chuang *et al.*, 1997). DDX3 rescues yeast cells with a lethal mutation in its *ded1* gene (Mamiya and Worman, 1999), and can partially relieve the block in translation from the HCV 5'NCR in Sf21 insect cells (Scott, 2002), suggesting a possible involvement of DDX3 both in cellular mRNA translation and in translation of the HCV genome. In the presence of HCV core, DDX3 was unable to rescue *ded1* deletion mutants, suggesting that core protein may sequester DDX3 in order to reduce cellular mRNA translation and/or increase translation of the viral RNA from the HCV 5'NCR. Western immuno-blot analysis did not show any differences in DDX3 levels between mutant and wild type infected cultures (Fig. 4.9), suggesting the sequestration of DDX3 does not have a major effect on cellular mRNA translation. However, differences in levels of viral proteins were seen between mutant and wild type virus infected cultures (Fig. 4.24). Upon infection with equal $TCID_{50}$ titres, all mutants showed reduced levels of core, E1 and E2 proteins as compared to wild type. This may indicate a reduction in translation due to the absence of sequestered DDX3. However, it may also be related to the reduced replication levels of the mutant viruses, which, as discussed above, may also be due to the absence of sequestered DDX3.

Similarly, interaction between core and DDX3 is not essential for production of infectious virus particles. Infectious particles were produced by all mutant JFH-1 RNAs (except for mutant G33A, discussed later), producing similar titres to that of wild type (Fig. 4.22).

Therefore, it is doubtful that DDX3 is involved in packaging viral RNA into viral particles, as such a role would have consequences on infectious titre in the absence of sequestered DDX3. It is interesting to note that, although replication levels of mutant HCV RNA were >50-fold lower than that of wild type virus, similar titres of infectious particles were produced. However, a correlation between replication level and particle production has not yet been confirmed, therefore it is possible that replication of wild type HCV RNA results in excess viral RNA molecules being produced compared to number of infectious particles produced. Mutant HCV RNA could therefore theoretically produce less HCV RNA without affecting infectious particle numbers.

From this work, it can be said that DDX3 is not essential for cell entry or virus replication, translation or release, however it is possible that DDX3 is an accessory protein which increases the efficiency of the virus life cycle, possibly by exerting some effect during replication or translation. On the other hand, the sequestration of DDX3 by core may be affecting some cellular process, thus proving advantageous to the virus, or producing some of the pathogenic effects associated with HCV infection. DDX3 has been detected in purified, functional, human spliceosome complexes (Zhou *et al.*, 2002) and also in spliceosomal B complexes which undergo catalytic activation, leading to catalysis of pre-mRNA splicing (Deckert *et al.*, 2006), suggesting an involvement in splicing. As genomic HCV RNA acts as a template for translation, splicing is not required for HCV translation. Therefore it is doubtful that DDX3 is required for some HCV-related splicing event. It is possible however, that sequestration of DDX3 by core prevents or reduces cellular pre-mRNA splicing, which may, in turn, have an effect on for example, cell cycle regulation by reduction of essential cell cycle regulators.

Yedavalli *et al.* reported the involvement of DDX3 in the nucleo-cytoplasmic shuttling of un-spliced and partially spliced HIV-1 mRNAs (Yedavalli *et al.*, 2004). Nucleo-cytoplasmic shuttling of endogenous DDX3 has been confirmed in the present study using leptomycin B to inhibit CRM1-mediated nuclear export (Chapter 6). DDX3 may therefore be involved in shuttling core protein to the nucleus. Although no nuclear staining of core protein was seen in this study, many reports have shown that core protein can have an effect on transcription from a number of cellular and viral promoters (see section 1.5.5.3), suggesting nuclear localisation. Indeed, a specific interaction between core and the proteasome activator PA28γ within the nucleus, suggested that the mature form of core localised to the nucleus where it was subsequently degraded by the nuclear proteasome (Moriishi *et al.*, 2003). The degradation of core in the nucleus would explain the lack of evidence supporting the presence of core in the nucleus. This report showed that core

localised to the nucleus both in the presence and absence of the PA28γ binding site, suggesting the possibility of alternative mechanisms for the nuclear localisation of core, other than that involving PA28γ. HCV core has several putative nuclear localisation signals (Suzuki *et al.*, 1995, Suzuki *et al.*, 2005, Chang *et al.*, 1994), suggestive of alternative mechanisms for core nuclear localisation. It is possible that DDX3 may be involved in an alternative nuclear transportation route. Shuttling of DDX3 therefore, presents a potential route for the transport of HCV core to the nucleus where it may affect cellular transcription, possibly producing a more "virus-friendly" environment within the host cell.

Finally, the sequestration of DDX3 by core may be having a direct effect on cell cycle regulation. Two independent reports have suggested that DDX3 is involved in the regulation of the cell cycle (Chang *et al.*, 2006, Chao *et al.*, 2006). Knockdown of DDX3 expression led to an accelerated proliferation rate in NIH3T3 cells (Chang *et al.*, 2006), while DDX3 expression levels were lower in HCV-infected liver tumour specimens as opposed to normal liver tumour specimens (Chao *et al.*, 2006). Core protein may therefore be sequestering DDX3 in order to control the cell cycle and in doing so, having a role in progression to tumourigenicity.

The identification of HCV core residues critical for interaction with the cellular DEAD-box RNA helicase has allowed studies on the effects DDX3 has on the life cycle of HCV. Although it has been shown here that DDX3 is not essential for propagation of the virus in cell culture, it is possible that DDX3 exerts subtle enhancing effects on either viral RNA replication or translation. However, the wealth of information regarding the effects HCV core protein has on cellular processes suggests the interaction between core and DDX3 may instead be causing some effect on the host cell. The discovery of the HCVcc system should allow future studies to analyse cellular processes in the presence of both wild type and mutant HCV, leading to a better understanding of the cellular function of DDX3 and its role in the life cycle of HCV.

7.2 Identification of HCV Core Residue Critical for Production of Infectious Particles

As described above, 6 residues of HCV core protein were identified which are critical for interaction with the cellular DEAD-box RNA helicase DDX3. Although none of the 6 residues, when mutated to alanine, prevented viral RNA replication or translation in an HCVcc system, only 5 of the 6 mutations permitted the production of infectious progeny particles. Mutation of HCV core residue 33 from glycine to alanine completely abolished the production of infectious particles without blocking replication of viral RNA or translation of viral proteins. Immunofluorescence data shows that G33A core protein localises to the lipid droplets (Fig. 4.12), indicating the correct subcellular localisation of mutant core protein. One possible explanation for the lack of infectious progeny particles is that amino acid 33 is essential for homo-oligomerisation of core protein, resulting in formation of viral capsids. Mutation of amino acid 33 would therefore result in inhibition of capsid assembly. Residues 1-75 of core have been shown to be involved in core multimerisation (Matsumoto *et al.*, 1996). However, although residues 1-68 of core were shown to be involved in assembly of capsids in a cell-free assay which were apparently indistinguishable from those isolated from HCV-infected patient serum (when analyzed by transmission electron microscopy) (Klein *et al.*, 2004), deletion of residues 27-38 did not reduce the ability of core to assemble into capsids (Klein *et al.*, 2005). The N-terminal region of core contains 2 basic regions (amino acids 6-23 and 39-67) separated by a neutral linker region (amino acids 27-38). Reducing the number of basic residues in the 2 basic regions reduces the ability of core to assemble into capsids, suggesting basic charge is important for capsid assembly (Klein *et al.*, 2005). The fact that the neutral linker region (residues 27-38) can be deleted without affecting capsid assembly, rules out an involvement of amino acid 33 in capsid assembly in this system. However, the cell-free assay is carried out in an artificial environment and therefore may not be reliable. *In vitro* capsid assembly may not mimic authentic HCV capsid assembly due to the absence of fully matured core protein and replicating viral RNA, as well as the absence of a wide array of cellular proteins that may be involved within the permissive cellular environment. Therefore, the possible involvement of amino acid 33 of core protein in the successful assembly of HCV capsids cannot currently be ruled out. Another explanation for the lack of infectious particles produced by JFH-1 mutant G33A is that residue 33 may be required for binding viral RNA. Residues 1-75 of core have been shown to bind HCV RNA (Santolini *et al.*, 1994) suggesting residue 33 may be involved in this function. However, it is likely that it is the basic residues within this region that are involved in RNA binding, as

in the case of Sindbis capsid protein (Geigenmuller-Gnirke *et al.*, 1993). It is also conceivable that amino acid 33 is required during secretion of infectious particles. Mutation of this residue would therefore block particle secretion. These possibilities are currently being investigated.

Upon passage of replicating JFH-1 G33A RNA, infectious particles are eventually produced. The viral RNA contained in these infectious particles, however, has a second mutation near the initial G33A mutation. The appearance of infectious particles coincides with the appearance of this second mutation, suggesting this is a compensatory mutation allowing production of infectious particles. However, when both the G33A mutation and the second mutation were introduced into wild type JFH-1 RNA, no infectious particles were produced following electroporation into Huh7 cells. This suggests that another mutation may have arisen elsewhere in the viral genome of the original mutant G33A mutant that is responsible for the production of infectious particles, either alone or in conjunction with the mutation identified near residue 33. Nucleotide sequence analysis of the full viral genome will be required in order to detect any further mutations responsible for the observed phenotype.

This work has provided important information regarding the interaction between HCV core protein and the cellular DEAD-box helicase DDX3. Future work can now use this information and the reagents generated to investigate such issues as the replication efficiency of HCV in the absence of core-DDX3 interaction as well as more detailed analysis on the function of HCV amino acid 33 in terms of the production of infectious progeny virus. As the results here suggest a possible role for DDX3 in HCV RNA replication, one future study should be to carry out time course analysis over the first 48 hours post RNA electroporation to detect differences in replication rates between mutant and wild type RNA before secondary infection of neighbouring cells. As the rate of secondary infection may vary between cultures, analysis at eary time points would allow direct comparison between mutant and wild type electroporated RNA. At early time points there would also be less replicating viral RNA, therefore reducing the possibility of saturation during RT-PCR analysis. Reduced replication rates may not only indicate an involvement of DDX3 in replication but also in translation of the HCV open reading frame. Time course analysis could also be used to identify differences in rates of progeny particle release. Culture medium harvested at early time points and used to infect naïve cells would allow identification of any delay in production / release of infectious particles by mutant RNA. While the work presented here has concentrated on interrupting the association between core and DDX3 by mutation of residues required for this interaction,

another method of analysis would be to knockout DDX3 protein expression using siRNA technology. Two recent publications have reported the effect knockdown of endogenous DDX3 has on HCV replication. In a study of 62 host genes shown to physically interact with HCV RNA or HCV-encoded proteins, knockdown of endogenous DDX3 by siRNA resulted in a significant reduction in both HCV RNA levels and viral titer (Randall *et al.*, 2007). Furthermore, shRNA mediated knockdown of cellular DDX3 also resulted in reduced accumulation of HCV RNA and reduced viral titers (Ariumi *et al.*, 2007). The same publication also reports a reduction in HCV RNA replication by a subgenomic replicon lacking core protein. However, no association between DDX3 and viral RNA or other HCV proteins has ever been reported. Although these reports suggest knockdown of DDX3 suppresses HCV RNA replication, no indication of the viability of the cells is given. As DDX3 is a multifunctional protein involved in splicing (Deckert *et al.*, 2006, Zhou *et al.*, 2002), cell cycle regulation (Chang *et al.*, 2006, Chao *et al.*, 2006), nucleo-cytoplasmic RNA shuttling (Yedavalli *et al.*, 2004) and RNA transport (Kanai *et al.*, 2004), knockdown of this protein may have serious consequences for essential processes within the cell which, in turn, may have a secondary effect on HCV replication. These effects may include inhibition of protein synthesis (thus limiting any cellular factors required for HCV RNA translation) or cell cycle regulation, which may result in the host entering into a stage of the cell cycle that is not optimum for HCV replication. Such an effect on cell cycle regulation was reported to occur in NIH-3T3 cells in which knockdown of mouse DDX3 led to premature entry into S-phase (Chang *et al.*, 2006). Detailed analysis of cellular functions such as cell cycle regulation, splicing and protein synthesis under DDX3 knockdown conditions will be required before direct effects of DDX3 knockdown on HCV replication can be determined. As DDX3 is constitutively expressed in all tissues and has many homologues in different species, it is also possible that it is an essential gene, without which the cell cannot survive. In this case, complete knockdown of DDX3 may not be possible without cell death. An inducible RNAi system in which the expression levels of shRNA (and thus DDX3) can be sensitively controlled may therefore be an option. Regarding non-infectious mutant G33A, further analysis is required in order to understand if infectious particles are produced that cannot exit the host cell, or, if particles are released that are non-infectious. For this, culture medium can be fractionated by sucrose density gradient centrifugation and fractions analysed by RT-PCR for the presence of G33A RNA and compared to fractions corresponding to wild type virus particles. Detection of mutant G33A RNA in the same fraction as wild type particles would indicate that non-infectious particles are being released. Mutant G33A culture medium may also be pelleted in order to detect HCV RNA and core protein, also indicative of non-infectious HCV particle

secretion. If HCV RNA and core protein cannot be detected in the culture medium, it is possible that infectious particles are being produced but cannot be secreted. To investigate this possibility, electroporated cells should be lysed and intracellular material used to infect naïve cells. Resultant infection would indicate residue 33 of core is required for secretion of infectious particles. However, if no infection occurs then it would indicate a role for residue 33 in particle assembly. If mutant G33A core protein does prevent particle assembly it would be of interest to see if this molecule could have a dominant negative effect on assembly of wild-type particles. Mutant G33A RNA should be co-transfected with wild-type core protein in order to see if the wild-type core can rescue infectious particles. More importantly however, wild-type HCV RNA should be co-transfected with mutant G33A core to see if production of infectious particles can be reduced or even completely abolished due to incorporation of G33A mutant core. If mutant G33A core did prove to have a dominant negative effect, this may have interesting applications as a therapeutic agent. However, core has been shown to have many adverse effects on the host cell therefore further modifications would be required. Ultimately, the aim of this research is to discover both the cellular role of DDX3 and its functional relevance in terms of the life cycle of HCV. The reagents and data produced during this project, in conjunction with the new HCVcc system will surely prove to be important in the progress of this project in the future.

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Appendix - Oligonucleotides

Primer	Sequence	Function
AP233	5'AAACTGCAGCACGATAATAC CATGGGC 3'	Used to amplify residues 1-59 of core (HCV strain H77c)
AP234	5'CCCAAGCTTCTATTAGGGGA TAGGCTGACGTCTACC 3'	Used to amplify residues 1-59 of core (HCV strain H77c)
Quickchange 1	5'GATGAACTATACAAACTGTA GCACGATAATACCATG 3'	Site-directed mutagenesis of pGFP-Core$_{1-59}$ to introduce stop codon at end of GFP sequence
Quickchange 2	5'CATGGTATTATCGTGCTACA GTTTGTATAGTTCATC 3'	Site-directed mutagenesis of pGFP-Core$_{1-59}$ to introduce stop codon at end of GFP sequence
Mut 99F	5'CGTCGCCCACAGGACGTCAA GTTCCCGGGT 3'	Site-directed mutagenesis of pC-E1-E2 mutant 99 to revert residue 22 back to wild type
Mut 99R	5'ACCCGGGAACTTGACGTCCT GTGGGCGACG 3'	Site-directed mutagenesis of pC-E1-E2 mutant 99 to revert residue 22 back to wild type
Mut 110F	5'CGCAGGGGCCCTAGATTGGG TGTGCGCGCG 3'	Site-directed mutagenesis of pC-E1-E2 mutant 110 to revert residue 43 back to wild type
Mut 110R	5'CGCGCGCACACCCAATCTAG GGCCCCTGCG 3'	Site-directed mutagenesis of pC-E1-E2 mutant 110 to revert residue 43 back to wild type
Mut 111F	5'ATGGGCACGAATCCTAAACC TCAAAGAAAAACCAAACGTAA C 3'	Site-directed mutagenesis of pC-E1-E2 mutant 111 to revert residue 6 back to wild type
Mut 111R	5'GTTACGTTTGGTTTTTCTTTG AGGTTTAGGATTCGTGCCCAT 3'	Site-directed mutagenesis of pC-E1-E2 mutant 111 to revert residue 6 back to wild type
Mut 115F	5'CCTAAACCTCAAAGAAAAAC CAAACGTAACACCAACCGTCG CCCACAG 3'	Site-directed mutagenesis of pC-E1-E2 mutant 115 to revert residues 10 and 12 back to wild type
Mut 115R	5'CTGTGGGCGACGGTTGGTGT TACGTTTGGTTTTTCTTTGAGG TTTAGG 3'	Site-directed mutagenesis of pC-E1-E2 mutant 115 to revert residues 10 and 12 back to wild type
Mut 125AF	5'ACCAAACGTAACACCAACCG TCGCCCACAGGAC 3'	Site-directed mutagenesis of pC-E1-E2 mutant 125 to revert residue 16 back to wild type
Mut 125AR	5'GTCCTGTTGGCGACGGTTGG TGTTACGTTTGGT 3'	Site-directed mutagenesis of pC-E1-E2 mutant 125 to revert residue 16 back to wild type
Mut 125BF	5'ACTTCCGAGCGGTCGCAACC TCGAGGTAGACGT 3'	Site-directed mutagenesis of pC-E1-E2 mutant 125 to revert residue 57 back to wild type
Mut 125BR	5'ACGTCTACCTCGAGGTTGCG ACCGCTCGGAAGT 3'	Site-directed mutagenesis of pC-E1-E2 mutant 125 to revert residue 57 back to wild type

Mut 126F	5'GGCACGAATCCTAAACCTCAAAGAAAAACCAAACGTAACACC 3'	Site-directed mutagenesis of pC-E1-E2 mutant 126 to revert residue 8 back to wild type
Mut 126R	5'GGTGTTACGTTTGGTTTTTCTTTGAGGTTTAGGATTCGTGCC3'	Site-directed mutagenesis of pC-E1-E2 mutant 126 to revert residue 8 back to wild type
Alanine 24F	5'CCACAGGACGTCAAGGCCCCGGGTGGCGGTCAG 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 24 to alanine
Alanine 24R	5'CTGACCGCCACCCGGGGCCTTGACGTCCTGTGG 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 24 to alanine
Alanine 25F	5'CAGGACGTCAAGTTCGCGGGTGGCGGTCAGATC 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 25 to alanine
Alanine 25R	5'GATCTGACCGCCACCCGCGAACTTGACGTCCTG 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 25 to alanine
Alanine 26F	5'GACGTCAAGTTCCCGGCTGGCGGTCAGATCGTT 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 26 to alanine
Alanine 26R	5'AACGATCTGACCGCCAGCCGGGAACTTGACGTC 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 26 to alanine
Alanine 27F	5'GTCAAGTTCCCGGGTGCCGGTCAGATCGTTGGT 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 27 to alanine
Alanine 27R	5'ACCAACGATCTGACCGGCACCCGGGAACTTGAC 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 27 to alanine
Alanine 28F	5'AAGTTCCCGGGTGGCGCTCAGATCGTTGGTGGA 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 28 to alanine
Alanine 28R	5'TCCACCAACGATCTGAGCGCCACCCGGGAACTT 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 28 to alanine
Alanine 29F	5'TTCCCGGGTGGCGGTGCGATCGTTGGTGGAGTT 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 29 to alanine
Alanine 29R	5'AACTCCACCAACGATCGCACCGCCACCCGGGAA 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 29 to alanine
Alanine 30F	5'CCGGGTGGCGGTCAGGCCGTTGGTGGAGTTTAC 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 30 to alanine
Alanine 30R	5'GTAAACTCCACCAACGGCCTGACCGCCACCCGG 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 30 to alanine
Alanine 31F	5'GGTGGCGGTCAGATCGCTGGTGGAGTTTACTTG 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 31 to alanine
Alanine 31R	5'CAAGTAAACTCCACCAGCGATCTGACCGCCACC 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 31 to alanine

Alanine 32F	5'GGCGGTCAGATCGTTGCTGGAGTTTACTTGTTG 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 32 to alanine
Alanine 32R	5'CAACAAGTAAACTCCAGCAACGATCTGACCGCC 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 32 to alanine
Alanine 33F	5'GGTCAGATCGTTGGTGCAGTTTACTTGTTGCCG 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 33 to alanine
Alanine 33R	5'CGGCAACAAGTAAACTGCACCAACGATCTGACC 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 33 to alanine
Alanine 34F	5'CAGATCGTTGGTGGAGCTTACTTGTTGCCGCGC 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 34 to alanine
Alanine 34R	5'GCGCGGCAACAAGTAAGCTCCACCAACGATCTG 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 34 to alanine
Alanine 35F	5'ATCGTTGGTGGAGTTGCCTTGTTGCCGCGCAGG 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 35 to alanine
Alanine 35R	5'CCTGCGCGGCAACAAGGCAACTCCACCAACGAT 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 35 to alanine
Alanine 36F	5'GTTGGTGGAGTTTACGCGTTGCCGCGCAGGGGC 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 36 to alanine
Alanine 36R	5'GCCCCTGCGCGGCAACGCGTAAACTCCACCAAC 3'	Site-directed mutagenesis of pC-E1-E2 to mutate residue 36 to alanine
Alanine J24F	5'CCAGAAGACGTTAAGGCCCCGGGCGGCGGCCAG 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue 24 to alanine
Alanine J24R	5'CTGGCCGCCGCCCGGGGCCTTAACGTCTTCTGG 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue 24 to alanine
Alanine J27F	5'GTTAAGTTCCCGGGCGCCGGCCAGATCGTTGGC 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue 27 to alanine
Alanine J27R	5'GCCAACGATCTGGCCGGCGCCCGGGAACTTAAC 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue 27 to alanine
Alanine J30F	5'CCGGGCGGCGGCCAGGCCGTTGGCGGAGTATAC 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue 30 to alanine
Alanine J30R	5'GTATACTCCGCCAACGGCCTGGCCGCCGCCCGG 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue 30 to alanine
Alanine J33F	5'GGCCAGATCGTTGGCGCAGTATACTTGTTGCCG 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue 33 to alanine
Alanine J33R	5'CGGCAACAAGTATACTGCGCCAACGATCTGGCC 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue 33 to alanine

Alanine J34F	5'CAGATCGTTGGCGGAGCATACTTGTTGCCGCGC 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue 34 to alanine
Alanine J34R	5'GCGCGGCAACAAGTATGCTCCGCCAACGATCTG 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue 34 to alanine
Alanine J35F	5'ATCGTTGGCGGAGTAGCCTTGTTGCCGCGCAGG 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue 35 to alanine
Alanine J35R	5'CCTGCGCGGCAACAAGGCTACTCCGCCAACGAT 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue 35 to alanine
Alanine J33/34F	5'CAGATCGTTGGCGCAGCATACTTGTTGCCGCGC 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue3 33 and 34 to alanine
Alanine J33/34R	5'GCGCGGCAACAAGTATGCTGCGCCAACGATCTG 3'	Site-directed mutagenesis of pGEMT JFH1 1-2614 to mutate residue3 33 and 34 to alanine
JFH 12	5'GCCAGTGAATTCTAATACGAC 3'	Used to amplify residues 1-2614 of HCV JFH1
AP 357	5'CATATGCATGAATTCTCTAGATTATGCTTCGGCCTGGCCCAA 3'	Used to amplify residues 1-2614 of HCV JFH1
JFH-1 NegRT	5'TTGCGAGTGCCCCGGGA 3'	Used to reverse transcribe negative strand HCV JFH1 core sequence
JFH-1 RTPCR1	5'GGTCTCGTAGACCGTGCACC 3'	Used to amplify HCV JFH1 core sequence
JFH-1 RTPCR2	5'GTATTCTTCACCTGGGCAGC 3'	Used to amplify HCV JFH1 core sequence
RA16	5'TCTGCGGAACCGGTGAGTAC 3'	Used to amplify HCV JFH1 5'NCR
RA17	5'GCACTCGCAAGCACCCTATC 3'	Used to amplify HCV JFH1 5'NCR