

Department of Medicine, Karolinska Institutet, Stockholm, Sweden

Molecular Studies of the Hepatitis C Virus:

**The Role of IRES Activity for Therapy
Response, and the Impact of the Non-
structural Protein NS4B on the Viral
proliferation**

Hannah Kim Lindström



**Karolinska
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To my father,
for inspiring the scientist in me

ABSTRACT

Chronic hepatitis C can lead to life threatening conditions like cirrhosis, liver failure and hepatocellular carcinoma. There are an estimated 170 million chronic carriers of the hepatitis C virus (HCV) world wide, making it a serious global health problem. 70-90 % of the newly infected individuals will fail to clear the virus and develop chronic hepatitis. There is no vaccine, and the only treatment available is a long-term combination therapy of pegylated interferon- α (peg-IFN- α) and Ribavirine (RBV). Unfortunately, this regiment cannot be given to all patients, and of the treated individuals only 50-80 % will succeed in clearing the virus. Therefore, more efficient treatment approaches are needed. To achieve them, a deeper understanding of the virus and its interaction with the host is necessary. This thesis will focus on two aspects of the HCV life cycle; the translation and replication of the virus genome.

In the first section, viral translation mediated by the internal ribosome entry site (IRES) of the HCV genotype 3 and its relation to treatment response was studied. A Pakistani cohort of approximately 144 chronic HCV patients was treated with IFN- α and RBV. Follow up one year post treatment showed 70% sustained responders (SR) and 30% non-responders (NR) (depicting if the virus was cleared or not). Substantial differences in the IRES nucleic acid sequence between the two groups were observed. This prompted analyses of translation efficiencies of IRES regions derived from 15 SR and 7 NR randomly selected patients. The SR derived IRES displayed significantly lower translation efficiencies than those of the NR group (29.7 ± 13 vs. 69.4 ± 22 ; $P < 0.01$). This difference may be a consequence of more disrupted IRES tertiary structure in the SR group compared to the NR group. The results indicate that a correlation between the IRES sequence and the therapeutic outcome may serve as a prediction model of treatment outcome for HCV genotype 3. However, further studies are needed to establish such a model, and to understand if the correlation applies to other genotypes.

In the second section, studies were undertaken to elucidate the role of the non-structural protein 4B (NS4B) in the replication of HCV. NS4B is an integral membrane protein of little known function except that it has membrane rearranging properties. Analyses of the NS4B topology when processed from the polyprotein inside cells showed that the NS4B N-terminus translocated into the ER-lumen, extending previous observations from *in vitro* experiments. The N-terminus translocation was exhibited by all the major six genotypes, manifesting a conserved function important in the viral life cycle. By immunoprecipitation and immunofluorescence experiments using a collection of NS4B mutants, we showed that NS4B homo-oligomerized and that the N-terminal translocation into the lumen was crucial for induction of the membrane associate foci (MAF) by NS4B. This provided an important insight into the events that create the very environment in which the viral RNA replication takes place. Finally, 14 point mutations located in different regions of NS4B in a HCV subgenomic replicon all affected the viral replication. Even mutations of non-conserved amino acid residues and substitutions on the

luminal side of the ER membrane displayed considerable negative effects; only one mutant exhibited increased replication. These results implied that NS4B may have a direct involvement in viral RNA replication beyond MAF induction. Together, the results emphasize a critical role for NS4B in the viral life cycle. Accordingly, elucidating interacting partners of the NS4B and intrinsic functions of the protein may offer new important insights, and possibly novel treatment strategies.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their roman numbers (I-IV).

- I. A. Yasmeeen, S. Hamid, F.N. Granath, H. Lindström, R.M. Elliott, A.A. Siddiqui, M.A.A. Persson. **Correlation between translation efficiency and outcome of combination therapy in chronic hepatitis C genotype 3.** J Viral Hepat, 2006, 13, 87–95.
- II. M. Lundin, H. Lindström, C. Grönwall, M.A.A. Persson. **Dual topology of the processed hepatitis C virus protein NS4B is influenced by the NS5A protein.** J Gen Virol, 2006, In press.
- III. H. Lindström*, M. Lundin*, M.A.A. Persson. **N-terminal translocation, oligomerization, and membrane rearrangements of the HCV protein NS4B are interlinked.** 2006, Manuscript.
- IV. H. Lindström, M. Lundin, S. Häggström, M.A.A. Persson. **Mutations of the hepatitis C virus protein NS4B on either side of the ER membrane affect the efficiency of subgenomic replicons.** Virus Res, 2006, In press.

* Both authors have contributed equally

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LIST OF ABBREVIATIONS

AH	Amphipatic helix
Alb-uPA	Urokinase-type plasminogen-activator under the albumin promoter
ALT	Alanine aminotransferase
APC	Antigen presenting cell
ATF-2	Activating transcription factor 2
BVDV	Bovine viral diarrreah virus
CD	Cluster of differentiation (e.g. CD81, CD4, CD8)
CD81	Human tetraspanin cell surface receptor
cfu	Colony forming unit
CMV	Cytomegalovirus
COPII	Coat protein II
CTL	Cytotoxic T lymphocyte
DAPI	4',6-diamidino-2-phenylindole
DC SIGN	Dendritic cell specific ICAM-3 grabbing nonintegrin (mannose binding lectin)
DMEM	Dulbecco's modified Eagle medium
DSS	Disuccinimidyl suberate
E1 and E2	Envelope protein 1 and 2
ECL	Enhanced chemiluminescence
EGFP	Enhanced green fluorescent protein
eIF	Elongation initiation factor
EIRES	Encephalomyocarditis virus IRES
ER	Endoplasmic reticulum
HCV	Hepatitis C virus
HRP	Horseradish peroxidase
hVAP	Human vesicle membrane associated protein
HVR	Hyper variable region
ICAM	Intercellular adhesion molecule
IFN	Interferon
IKK	I κ B kinase complex
IL	Interleukin
IP	Immunoprecipitation
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
IRF3	IFN regulatory factor 3
ISG	IFN-stimulated genes
ISGF3	Interferon-stimulated gene factor

ISRE	IFN-stimulated response elements
Jak1	Janus kinase 1 (Protein tyrosine kinase)
L SIGN	Liver/lymph node-specific ICAM-3-grabbing nonintegrin (mannose binding lectin)
MAF	Membrane associated foci
MHC	Major histocompatibility complex
NANB	Non-A, non-B
NE	Nuclear envelope
Neo	Neomycin phosphotransferase
NFκ B	Nuclear factor κB
NK	Natural killer cell
NR	Non-responder
NS	Non-structural
NST	Asparagine-Serine-Threonine
NTR	Non-translated region
OAS	2'-5' oligoadenylate synthetase
Oil Red O	1-([4-(Xylylazo)xylyl]azo)-2-naphthol
OST complex	Oligosaccharyl transferase complex
peg-IFN-α2a	Pegylated interferon-α2a
PKR	Double-stranded (ds) RNA-dependent protein kinase
RBV	Ribavirin
RdRp	RNA dependent RNA polymerase
RER	Rough ER
RNase L	Ribonuclease L
RT-PCR	Reverse transcription - polymerase chain reaction
SER	Smooth ER
siRNA	Small interfering RNA
SP	Signal peptidase
SR	Sustained responder
SR-BI	Scavenger receptor BI
STAT	Signal transducer and activator of transcription
T_C	Cytotoxic T lymphocytes
tER	Transitional ER
T_H	T helper cell
TNF-α	Tumor necrosis factor-α
Tyk2	(Protein tyrosine kinase)
VAMP	Vesicle-associated membrane protein

INTRODUCTION

In the seventies, an unknown, post-transfusion hepatitis causing agent, initially referred to as non-A, non-B (NANB) hepatitis, was observed in many countries (Alter *et al.*, 1978, Feinstone *et al.*, 1975). It was not until 1989 that the large majority of these cases were explained, when the infectious agent was cloned and discovered. It turned out to be a virus of the *Flaviviridae* family, and it was named hepatitis C virus (HCV) (Choo *et al.*, 1989). Soon after its discovery, it was quickly established that HCV infection was the cause of more than 90% of the previously termed NANB hepatitis cases. The HCV infection is transmitted mainly by blood, resulting in acute or chronic hepatitis. As many as 70-90 % of the newly infected individuals fail to clear the virus during the acute phase and become chronic carriers, giving an estimated 170 million chronic carriers world wide today (WHO, 1999). The chronic infection, mainly affecting the liver, may cause cirrhosis, and can ultimately lead to liver failure or hepatocellular carcinoma (Major *et al.*, 2001) (Fig 1). There is no vaccine or satisfactory cure to date; the only treatment available is the combination therapy of pegylated interferon- α 2a (peg-IFN- α 2a) and Ribavirin (RBV). Of the treated individuals, sustained responders range from 50-80 %, depending on the infecting HCV genotype (Hadziyannis *et al.*, 2004). Moreover, many patients do not qualify for or cannot tolerate this treatment (Falck-Ytter *et al.*, 2002). Accordingly more efficient and better tolerated treatment strategies based on deeper knowledge of the viral mechanisms are needed.

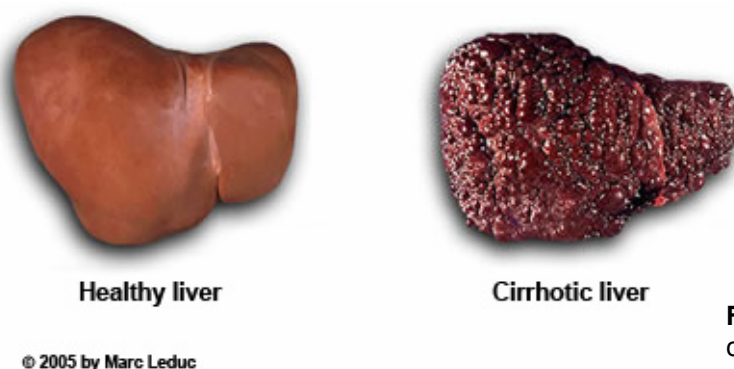


Figure 1. Healthy liver (left) versus cirrhotic liver (right)

HCV infection

The HCV is predominantly spread by blood and blood products, and very rarely by sexual transmission (Clarke & Kulasegaram, 2006). Viremia occurs around one week after the initial introduction of the virus (Shindo *et al.*, 1992). The initial infection causes an acute phase of the disease, lasting in average for 6-10 weeks. At this point most of the infected individuals (~80 %) do not show any obvious clinical symptoms (Houghton, 1996). There are cases of rapid fulminant liver failure associated with the acute phase, but these are very rare in HCV infections and seen mostly in immunosuppressed patients (Farci *et al.*, 1996, Kato *et al.*, 2001). At this initial phase, the viral infection is detectable by elevated alanine aminotransferase (ALT) levels released by the damaged hepatocytes, and by the presence of the virus in the serum detectable by RT-PCR (Marin *et al.*, 1994, Schlauder *et al.*, 1992, Shindo *et al.*, 1992).

As many as 70-90 % of the newly infected individuals will fail to clear the virus (WHO, 1999). They become chronic carriers with fluctuating or persistently elevated ALT levels, with gradual increase in necroinflammatory activity during the course of the chronic infection. About 20 % of the chronically infected individuals will develop cirrhosis that may lead to hepatocellular carcinoma and liver failure (Houghton, 1996). This makes the HCV the leading cause of liver transplantations in the developed countries today (WHO, 1999).

Genotypes and world distribution

The HCV is divided into 6 main genotypes, named genotype 1-6, each with numerous subtypes, named a, b, c, etc after their order of discovery (Salemi & Vandamme, 2002, Simmonds, 1999). The different genotypes are diversely spread throughout the world, where only the genotypes 1-3 are spread globally (WHO, 1999). The genotypes 1a and 1b is by far the most common throughout the world, accounting for about 60 % of all infections. The genotype 1a is spread predominantly in North America and Northern Europe, and the genotype 1b in Southern and Eastern Europe and Japan. Genotype 2 is also global but less frequent than the genotype 1. The genotype 3 is spread mainly in South-East Asia and quite variably in the rest of the world. The genotype 4 is found mainly in the Middle East, Egypt, and Central Africa, where the extraordinarily high infection rate may range up to 19 % of the population (Hibbs *et al.*, 1993).

The genotypes 5 and 6 are almost exclusively found in South Africa and South East Asia, respectively.

The HCV genome

The HCV is the a member of the genus *Hepacivirus* of the family *Flaviviridae*, which also includes the *Pestivirus* and the *Flavivirus* (Choo *et al.*, 1989, Choo *et al.*, 1990, Miller & Purcell, 1990, Ohba *et al.*, 1996). The HCV is a small, enveloped, positive stranded RNA virus about 50-65 nm in size (He *et al.*, 1987, Kaito *et al.*, 1994). It is similar in the genetic disposition as the other members of the *Flaviviridae*, with the closest homology to the

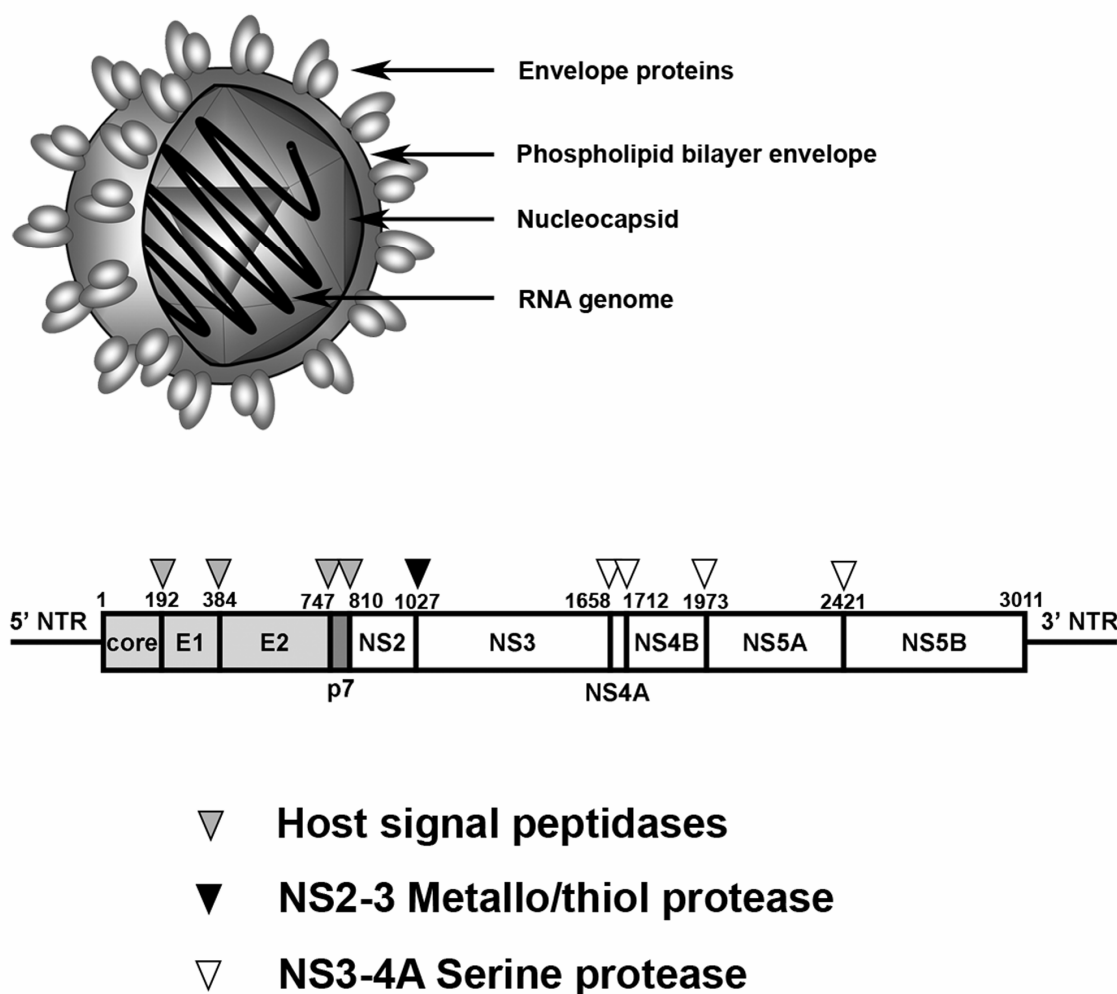


Figure 2. The HCV genome. A schematic cartoon of the virion particle and the genomic make-up is shown. The numbers above the depicted genome show the span of amino acid residues of each of the viral proteins. The triangles depict the different proteases involved in the processing of the different sites of the polyprotein.

Pestivirus (Choo *et al.*, 1991a, Choo *et al.*, 1991b, Miller & Purcell, 1990). The genome of the HCV comprises of ~9600 nucleotides, coding for a single open reading frame translated into ~3000 amino acids that are co- and post-translationally processed into at least ten proteins (Major & Feinstone, 1997, Major *et al.*, 2001) (Fig 2). The open reading frame is flanked by the 5' and 3' non-translated regions (5'NTR and 3'NTR, respectively), the structural RNA elements involved in the translation and replication of the viral genome, of which the 5'NTR and a portion of the core coding sequence form the internal ribosome entry site (IRES). The structural elements of the virus consist of the core, envelope proteins 1 and 2 (E1 and E2) (Fig 2). The core forms the nucleocapsid around the viral genome (Kunkel *et al.*, 2001). The E1 and E2 form the envelope proteins that are used for cell receptor-binding and cell fusion during the viral entry into the cell (Lagging *et al.*, 1998, Matsuura *et al.*, 2001, Meyer *et al.*, 2000, Wellnitz *et al.*, 2002). While the jury is still out on whether to group the p7 protein with the structural or the non-structural proteins, it has shown to be an integral membrane protein thought to form an ion channel (Griffin *et al.*, 2003). The non-structural (NS) proteins consist of NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Fig 2). The core, E1, E2, and p7 are processed from the polyprotein by cellular proteases. The integral protein NS2 together with the N-terminal domain of NS3 forms the zinc-stimulated metalloprotease responsible for the NS2-NS3 autocleavage (Grakoui *et al.*, 1993a, Pieroni *et al.*, 1997, Thibeault *et al.*, 2001). The NS3 consists of two domains; the N-terminus serine protease and the C-terminus NTPase/helicase (Manabe *et al.*, 1994, Suzich *et al.*, 1993, Tomei *et al.*, 1993). The NS4A, a small hydrophobic sequence acts as a membrane anchor and a co-factor to the NS3, enabling NS3-NS4A protease processing of the NS proteins 3-5B (Hahm *et al.*, 1995, Manabe *et al.*, 1994). The NS4B is an integral membrane protein involved in the induction of the membrane alteration seen during the viral infection (Egger *et al.*, 2002, Lundin *et al.*, 2003). Little else is known about this elusive protein hence much of this thesis is focused on the NS4B. The NS5A has recently been shown to be an RNA binding protein, closely associated with the NS5B protein in a regulatory fashion in the viral RNA replication (Huang *et al.*, 2005, Shimakami *et al.*, 2004). The NS5B is the key enzyme in the viral RNA replication, namely the RNA dependent RNA polymerase (Behrens *et al.*, 1996). There is also the protein F; a small 17 kDa protein encoded by ribosomal frame shift in the core coding region. Although seemingly expressed during the course of normal HCV infection, the function of this protein, if any, is unknown (Xu *et al.*, 2001). The functions of the structural and non-structural proteins will be discussed further in the context of the viral proliferation in the coming sections.

The HCV life cycle

Some light has been shed on the viral life cycle, predominantly by molecular studies of recombinantly expressed HCV proteins and by comparing with other members of *Flaviviridae* and positive stranded RNA viruses. However little is known about the exact mechanism by which the HCV proliferates in its host. Notwithstanding, a quick summary of the putative life cycle of the virus is described below (Fig 3).

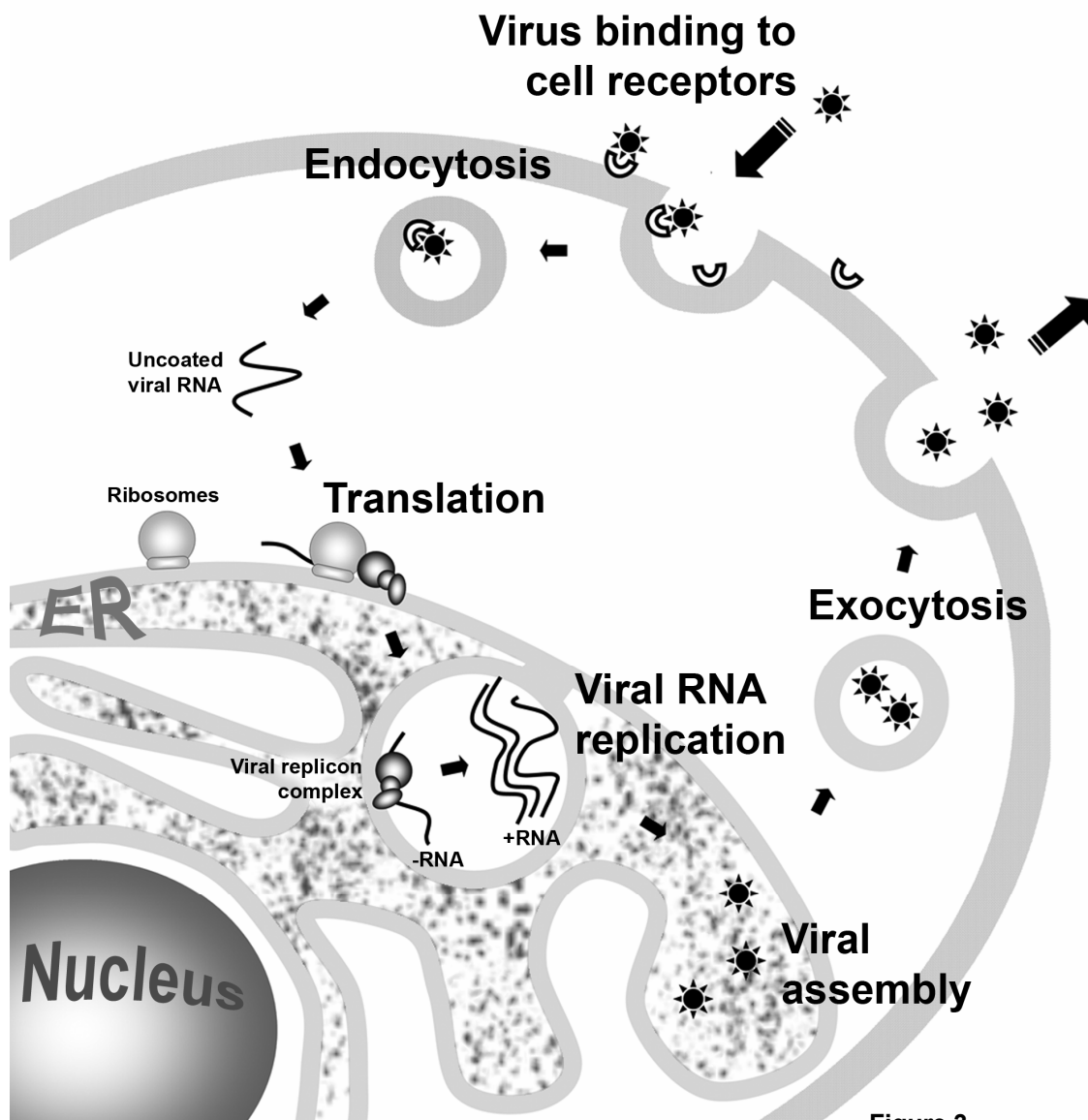


Figure 3.
The putative HCV life cycle

The viral entry presumably occurs via receptor mediated endocytosis involving the viral envelope proteins E1 and E2 and cell surface receptors. Once inside the cell, uncoating of the nucleocapsid ensues most likely by the lowering of the pH in the endosomes. This releases the viral genome into the cytoplasm to be transported to the endoplasmic reticulum (ER). Once in the ER, the positive stranded RNA genome functions as mRNA, resulting in translation of the viral proteins as a single polyprotein precursor. After translation, the polyprotein is processed by cellular and viral proteases. The resulting NS proteins then form the viral replication complex. Some of these are chambered into small ER membrane-derived spherules, referred to as the membrane associated foci (MAF), where the actual viral RNA replication takes place in an asymmetrical fashion; the positive RNA strand is used as template for the creation of its complimentary copy (the negative strand), which in turn functions as the template for the ensuing multiple copies of the positive-stranded RNA genome. The newly synthesized positive RNA is then assembled into the nucleocapsids and the progeny virus is budded off most likely into the ER and released from the cell via exocytosis.

Viral entry and exit

Entry

The viral entry is mediated by the binding of the envelope proteins E1 and E2 on the virion surface to cellular receptors (Fig 2 and 3). Many cell receptors have been implicated; the low-density lipoprotein receptor, the human tetraspanin CD81, the scavenger receptor BI (SR-BI), the mannose binding lectins DC SIGN and L SIGN, the asialoglycoprotein receptor, and glycosaminoglycans (reviewed in (Bartosch & Cosset, 2006). However no single receptor has shown to mediate viral entry by itself. Furthermore the only two cellular receptors confirmed to have functional roles in the viral entry using HCV particles and pseudoparticles are CD81 and SR-BI (Bartosch *et al.*, 2005, Bartosch *et al.*, 2003, Hsu *et al.*, 2003, Lindenbach *et al.*, 2005, Meyer *et al.*, 2000, Scarselli *et al.*, 2002, Wakita *et al.*, 2005). The complexity in finding a single cellular receptor that mediate HCV entry may be due to the heterogeneity of the viral particles found in the host serum during an infection; in “free” form, associated with high, low, or very low-density lipoproteins, complexed with immunoglobulins, etc (Bartosch & Cosset, 2006).

Once attached to the cell surface, the virion enters the cell via clathrin-mediated endocytosis (Blanchard *et al.*, 2006). As seen with the closely related Flavivirus, the HCV envelope proteins are thought to mediate the fusion between the cell and virion membranes by the lowered pH in the endosomes, releasing the capsid into the cytoplasm (Blanchard *et al.*, 2006, Bressanelli *et al.*, 2004, Hsu *et al.*, 2003, Meyer *et al.*, 2000, Modis *et al.*, 2004).

A HCV protein that may be involved in the regulation of the pH of the virion during viral entry and even exit may be the p7. This protein has been suggested to form multimeric ion channels that can be inhibited by amantadine (Griffin *et al.*, 2003, Pavlovic *et al.*, 2003). As a matter of fact, such viroporines have shown vital in the viral entry/exit of other viruses (Gonzalez & Carrasco, 2003). Case in point, the M2 viroporine of the influenza virus is essential both during the viral entry for enabling virion/endosomal membrane fusion and during the virus exit for the maturation of the virion, where it equilibrates the pH in the virion. In parallel, although the p7 has shown unnecessary for the viral RNA replication *in vitro*, it has shown vital for the HCV infectivity in chimpanzees (Lohmann *et al.*, 1999, Sakai *et al.*, 2003). Functional studies on p7 of the closely related bovine viral diarrhoea virus (BVDV) have also shown it unnecessary in replication but essential in producing progeny virus (Harada *et al.*, 2000).

Exit

The manner in which the HCV virion is assembled and released from the cell is rather enigmatic. Currently the link between the replication, virus assembly, and the viral exit from the cell is unknown. The core has shown affinity for the 5'NTR of the viral RNA (Fan *et al.*, 1999, Tanaka *et al.*, 2000) and bacterially produced core has shown to efficiently self-assemble into nucleocapsid-like particles *in vitro* (Kunkel *et al.*, 2001). However after the translation and processing of the viral proteins, the core is relocated to the membranes around the lipid droplets while the E1 and E2 are retained in the ER (Barba *et al.*, 1997, Rouille *et al.*, 2006). In cells producing infectious virions, no interaction or co-localization has been observed between the E1/E2 and the core (Rouille *et al.*, 2006). Furthermore the replicon complex and the newly synthesized viral RNA are chambered in the MAFs, where little exchange with the rest of the cytoplasm seems to occur (El-Hage & Luo, 2003, Gosert *et al.*, 2003, Gretton *et al.*, 2005, Mottola *et al.*, 2002). How the newly synthesized RNA reaches the core to be assembled into the nucleocapsid, and how the nucleocapsid then reaches the appropriate membrane where E1 and E2 reside to be enveloped remains to be seen. It was only very recently Murakami and

colleagues were able for the first time to locate assembled HCV virions to the ER membrane and the dilated cisternae of the ER using a novel 3-D radial-flow bioreactor (RFB) culture in which infectious virions could be produced (Murakami *et al.*, 2006).

Some co-localization between the core and the NS proteins 3 and 5A has been observed (Rouille *et al.*, 2006, Shi *et al.*, 2002), and interaction or co-localization between the E2 and several NS proteins such as NS2, NS3, and NS5A has also been reported (Flajolet *et al.*, 2000, Rouille *et al.*, 2006, Selby *et al.*, 1994). These observations of interactions and co-localizations consociate the core, the replicon complex hence the newly synthesized RNA, and the envelope proteins. Furthermore the existence of a small subpopulation of core that is located to the ER in cells producing infectious virions cannot be excluded. As seen in studies using recombinant polyprotein expression or genomic replicons that are unable to produce virions, some core did remain in the ER (Egger *et al.*, 2002, Matto *et al.*, 2004).

Translation initiation of the HCV RNA

Translation initiation in cells

Like the vast majority of viruses, the HCV relies on the host translational machinery at the rough ER membrane to translate its genome. In eukaryotic cells, there are two ways in which the RNA is translated; by cap-dependent or independent mechanism, where the cap-independent translation is mediated by the structural RNA elements, IRES. The vast majority of translations (95-97 %) are cap-dependent (Johannes *et al.*, 1999). In fact the cap-independent translation was first discovered by studying picornaviruses such as polio and encephalomyocarditis viruses (Jang *et al.*, 1988, Pelletier & Sonenberg, 1988). In eukaryotes, the IRES mediated translation was observed first when the cap-dependent translation was down-regulated due to cellular stress exerted by poliovirus infection (Johannes *et al.*, 1999). Since then it has shown to be involved in a variety of cellular activities including proliferation, differentiation, stress response, and the progression of the cell cycle (Stoneley & Willis, 2004, Vagner *et al.*, 2001). However translation efficiencies of the cellular IRES is very inefficient compared to that of the viral IRES, and often result in different version of the protein (Martinez-Salas *et al.*, 2001).

Cap-dependent versus cap-independent initiation

Translation in cells is accomplished by the ribosomal complex composed of two major subunits; the 40S and the 60S that are assembled by an energy demanding process (ATP) into the 80S translation initiation complex (Jang, 2006, Merrick, 2004) (Fig 4). The 40S first binds the elongation initiation factors (eIF) 1A and 3. Then the Methionine-carrying tRNA bound to the eIF2 joins the complex, forming the 43S complex. At this stage, the mRNA activated by binding to eIF4A, eIF4B, eIF4F, and eIF4H, joins the 43S, resulting in the 48S complex. This is

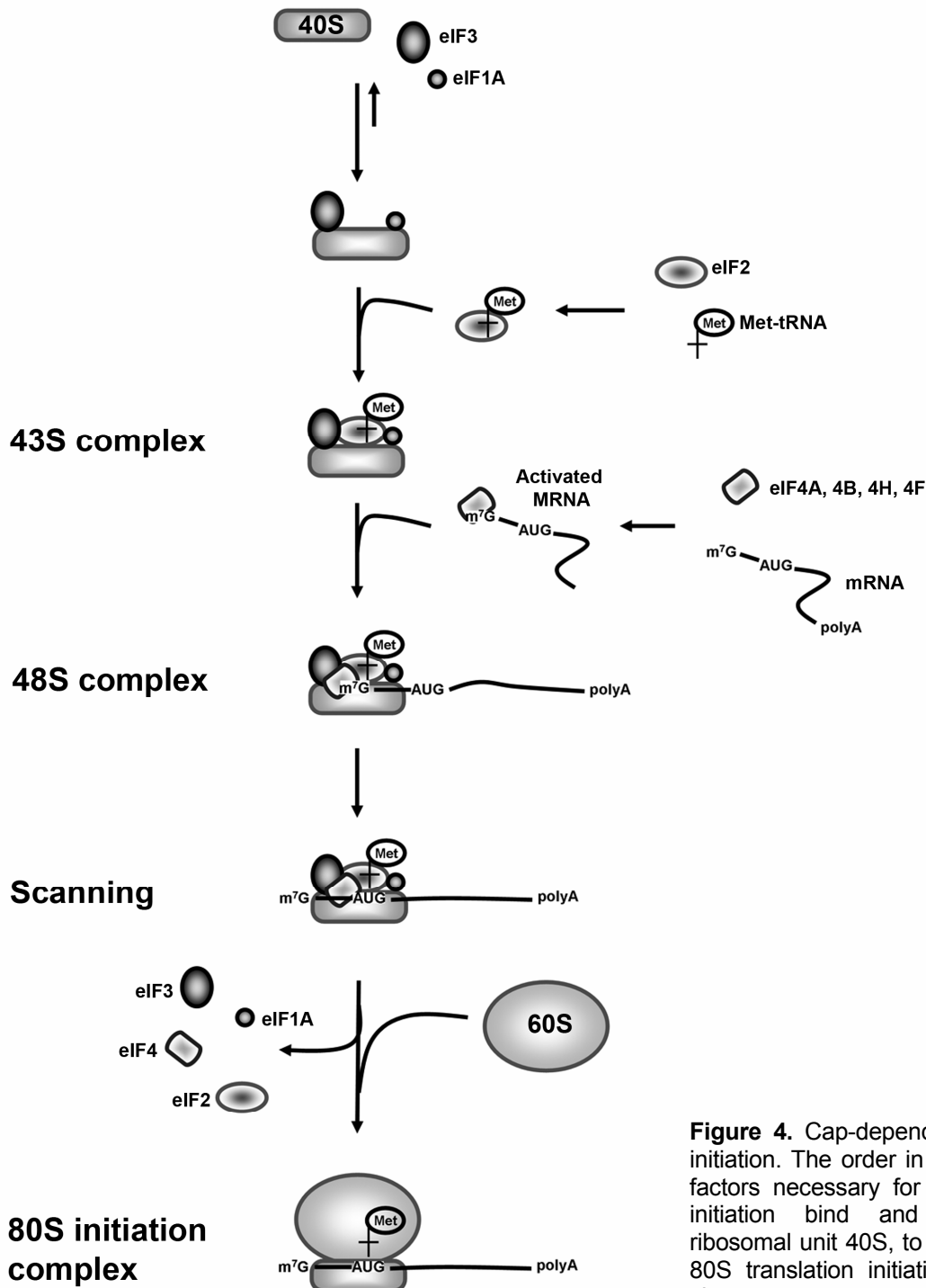


Figure 4. Cap-dependent translation initiation. The order in which different factors necessary for the translation initiation bind and release the ribosomal unit 40S, to finally form the 80S translation initiation complex is shown.

ensued by the ribosome complex “scanning” the bound mRNA necessary for the correct placement of the AUG start codon that is in a favorable Kozak context. This triggers the release of the initiation factors from the complex and the attraction of the 60S, forming the assembled 80S initiation complex. Thus begins the polypeptide synthesis.

The cap-dependent translation can be described by going through the following steps; *i)* the canonical factors eIF4A, 4B, and 4F binding to the 5' M⁷GpppG cap structure of the mRNA is necessary for the mRNA engaging the ribosome complex; *ii)* the bound mRNA has to be scanned by the 48S for placing the AUG start codon in the correct position of the ribosome.

In contrast in the cap-independent translation, the viral IRES forms complex tertiary structures that enables immediate correct positioning of the AUG start codon in the ribosome without the necessity of ribosomal scanning (Beales *et al.*, 2003). What is more, unlike capped mRNA translation initiation or IRES of picornaviruses, the HCV IRES translation initiation does not require eIF4A, 4B, and 4F (Pestova *et al.*, 1998). The HCV IRES can bind directly to the 43S ribosomal complex with the aid of eIF3 and eIF2 only, with immediate correct placement of its start codon in the ribosome (Hellen & Pestova, 1999).

Translation initiation by HCV IRES

The IRES spans the nucleotides ~1-407 of the HCV genome that includes the entire 5' NTR region and a part of the core coding sequence (Honda *et al.*, 1999, Reynolds *et al.*, 1995, Tsukiyama-Kohara *et al.*, 1992, Wang *et al.*, 2000) (Fig 5). Its structure is highly dependent on the primary sequence's ability to form stemloops resulting in complex secondary and tertiary RNA structures (Beales *et al.*, 2001). Therefore even small differences in the primary sequence of the IRES may result in differential binding of the cellular translation initiation factors required for viral translation, resulting in variations in translation efficiency. As several studies have shown, even very small nucleotide changes in the IRES stemloops could have dramatic effects on the translation efficiency (Collier *et al.*, 1998, Honda *et al.*, 1999, Kamoshita *et al.*, 1997, Lerat *et al.*, 2000, Saiz *et al.*, 1999).

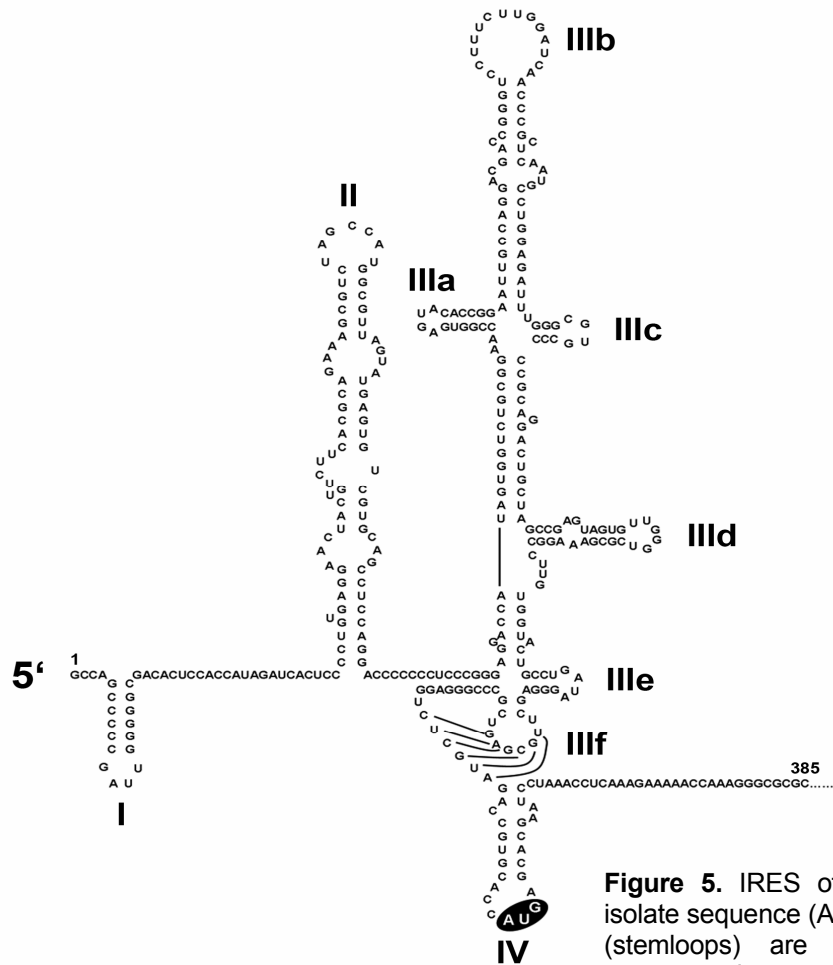


Figure 5. IRES of the HCV genotype 1b Con1 isolate sequence (AJ238799). The principal domains (stemloops) are numbered according to the consensus in current literature. The AUG start codon is marked in black.

Processing of the viral proteins

The translation of the HCV viral RNA results in one continuous polyprotein that is both co- and post-translationally processed by cellular and viral proteases into at least 10 proteins (Fig 2). Here, the ER membrane exerts an important function in the maturation of the proteins, as all of the viral proteins except the NS3 have one or more membrane-anchoring domains (Svitkin *et al.*, 2005) (Fig 6). Not all of the NS proteins' membrane anchoring domains transects the membrane; the core and NS5A utilize amphipatic helices for their anchorage (Boulant *et al.*, 2006, Elazar *et al.*, 2003).

Processing the structural proteins

Upon translation, the core is initially anchored to the ER membrane by a C-terminal transmembrane tail that also functions as a signal sequence for the ensuing E1 protein. This is cleaved off immediately after translation, resulting in the matured core that is relocated to the membranes around lipid droplets (Barba *et al.*, 1997, Hope & McLauchlan, 2000, McLauchlan *et al.*, 2002) (Fig 6B). The E1 and E2 are also anchored by a single transmembrane domain at their C-terminal ends (Cocquerel *et al.*, 2002) (Fig 6). These transmembrane domains contain ER retention signals and are also responsible for the non-covalent heterodimerization between the E1 and E2 proteins (Duvet *et al.*, 1998, Op De Beeck *et al.*, 2000, Selby *et al.*, 1994). The heterodimerization is also necessary for the correct folding and glycosylation of at least the E1 protein, (Dubuisson *et al.*, 2000, Patel *et al.*, 2001). Both proteins are heavily and heterogeneously glycosylated upon maturation (Svitkin *et al.*, 2005). The p7 has two membrane spanning domains with both N- and C-terminus in the lumen after processing (Carrere-Kremer

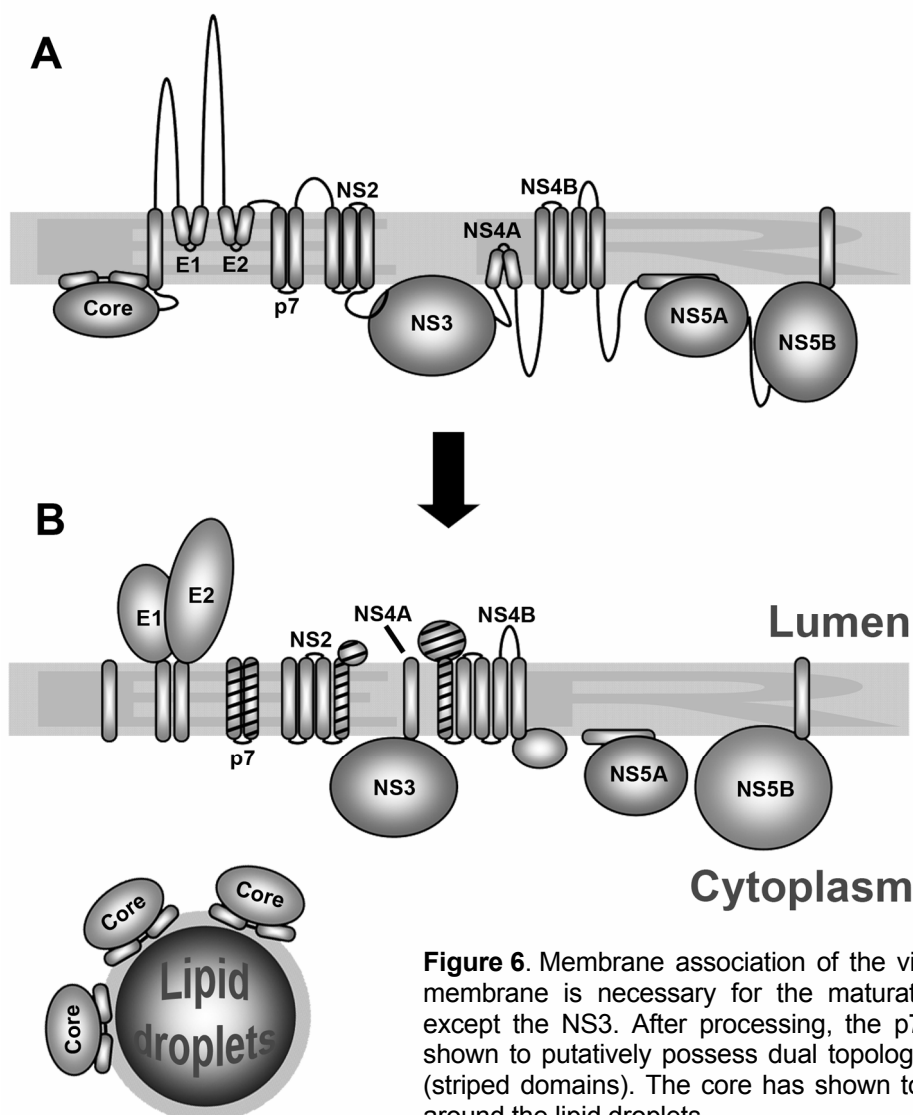


Figure 6. Membrane association of the viral polyproteins. The ER membrane is necessary for the maturation of all HCV proteins except the NS3. After processing, the p7, NS2, and NS4B have shown to putatively possess dual topologies in the ER membrane (striped domains). The core has shown to relocate to membranes around the lipid droplets.

et al., 2004) (Fig 6). However the processing between E2 and p7 has shown incomplete, resulting in E2, p7, and E2p7 variants (Lin *et al.*, 1994). What is more, E2p7 results in another topology of the p7, with both termini toward the cytoplasm (Isherwood & Patel, 2005). Both E2 and E2p7 variants seem to be included in the virion envelope, at least as observed in studies with virus-like particles, supporting the notion of viroporin action by p7 (Isherwood & Patel, 2005). However what role the dual topology of the p7 plays in the viral proliferation is unclear.

Processing the non-structural proteins

While the processing between the p7 and NS2 is mediated by cellular proteases, the processing between the NS2 and NS3 is by the autoprotease formed by these two proteins (Grakoui *et al.*, 1993a, Pieroni *et al.*, 1997, Thibeault *et al.*, 2001) (Fig 2). The NS2 is proposed to form dimers, where the C-terminal domains result in a composite catalytic site that is responsible for the autocleavage between the NS2-NS3 (Lorenz *et al.*, 2006). Strangely, this is also the only known function of the NS2 protein. The NS2 is an integral membrane protein with three or four transmembrane domain topology; with either the N-terminus in the lumen and the C-terminus in the cytoplasm (3 transmembrane domains) or both N- and C-termini in the lumen (4 transmembrane domains) (Yamaga & Ou, 2002) (Fig 6). This protein has also shown to co-localize with the replication complex to the membrane associated foci (Dimitrova *et al.*, 2003). Furthermore yeast-two-hybrid and co-precipitation studies have shown that the NS2 may interact directly with several, if not all of the NS proteins involved in the replication complex, and the E2 protein (Dimitrova *et al.*, 2003, Flajolet *et al.*, 2000, Grakoui *et al.*, 1993b, Hijikata *et al.*, 1993, Kiiver *et al.*, 2006, Lohmann *et al.*, 1999, Selby *et al.*, 1994). It is remarkable that the NS2 is not necessary in the viral RNA replication, yet interacts with both the replication complex and the E2 (Lohmann *et al.*, 1999). Perhaps this suggests a function in a latter part of the viral life cycle, such as the transition between replication and virus assembly.

The NS3 consists of two domains, the serine protease domain and the NTPase/helicase domain (Gwack *et al.*, 1996, Kim *et al.*, 1995, Kim *et al.*, 1998, Love *et al.*, 1996, Manabe *et al.*, 1994, Suzich *et al.*, 1993, Tomei *et al.*, 1993). The processing of all of the downstream NS proteins is dependent on this NS3 serine protease in cohort with the NS4A as co-factor (Hahm *et al.*, 1995, Lin, 1995 #131, Kim *et al.*, 1996, Manabe *et al.*, 1994, Tomei *et al.*, 1993). The NS4A is not only essential for the NS3 protease activity; it is the association between the NS3 and NS4A that keeps the NS3 anchored to the ER membrane (Wölk *et al.*, 2000) (Fig 6B). If expressed

alone, the NS3 is spread diffusely between the ER membrane, cytoplasm, and the nucleus. Although the NS3 is the only HCV protein that does not require membranes for maturation, it does need the membrane anchorage via the NS4A for protein stability and function (Lin & Rice, 1995, Svitkin *et al.*, 2005, Wölk *et al.*, 2000).

Due to the NS3 residing on the cytoplasmic side of the ER membrane, the processing of the ensuing NS proteins are assumed to occur on the cytoplasmic side as well. Hence the processing between NS4A-4B results in the post-translational anchorage of the NS4A, with its C-terminus toward the lumen and leaves the NS4B N- and C-termini on the cytoplasmic side of the ER membrane. Based on the above processing data and computer predictions, our group initially proposed a four transmembrane model of the NS4B. However *in vitro* studies showed that soon after translation the NS4B N-terminus was translocated into the lumen. This gave rise to a five transmembrane domain model of the NS4B, with the fifth transmembrane domain created when the N-terminal relocates into the ER lumen (Lundin *et al.*, 2003) (Fig 6B). What role this dual topology plays in the viral life cycle is currently unknown.

The NS5A is located to the cytosolic side, where it is associated to the ER membrane by an N-terminal amphipathic helix (AH) (Elazar *et al.*, 2003) (Fig 6B). The NS5B is also located on the cytosolic side of the ER membrane, anchored by a single C-terminal transmembrane domain (Ivashkina *et al.*, 2002, Schmidt-Mende *et al.*, 2001) (Fig 6B).

The NS3-5B proteins constitute the replication complex therefore they will be discussed further in the section below.

Replication of the viral genome

The ER membrane

The ER membrane is the scene of both viral translation and replication, and thus plays a central role in the HCV life cycle. It is a complex organelle, organized into numerous subdomains that perform different tasks (Baumann & Walz, 2001). It should be kept in mind that the translation and the replication of the virus seem to occur in different regions of the ER membrane. For instance the translation of the viral proteins occurs on the rough ER (see below), while on-going viral RNA replication seems to be associated to the functions of the smooth ER (Aizaki *et al.*,

2004, Bishop & Bell, 1988, Eisenhaber *et al.*, 2003, Helms & Zurzolo, 2004, Kapadia & Chisari, 2005, Rajendran & Simons, 2005, Sagan *et al.*, 2006, Shi *et al.*, 2003, Simons & Toomre, 2000, Soccio & Breslow, 2004, Su *et al.*, 2002).

Rough versus smooth ER

The ER network is dynamic, constantly in contact with other cellular organelles such as peroxisomes, mitochondrias, Golgi, etc. Although it is actually one continuous sheet of membrane enclosing a single lumen, the ER membrane is classified into three main regions; the rough ER (RER), the smooth ER (SER), and the nuclear envelope (NE) (Baumann & Walz, 2001). The NE wraps around the nucleus, where the inner nuclear membrane is undercoated by the nuclear lamina. The RER is where the majority of the cell's protein synthesis occurs. It is named by its granular appearance due to the abundance of ribosomes attached to its surface visible in electron micrographs. The RER is also enriched in other protein complexes involved in the translocation and processing of proteins. For instance the signal peptidase (SP) complex recognizes and recruits the polypeptide sequence to be translocated through the ER membrane by the translocon complex. The translocon forms the protein conducting channel for the translocation of the nascent polypeptide through the membrane. These are also in association with the oligosaccharyl transferase (OST) complex that glycosylates the nascent polypeptide chain (Johnson & van Waes, 1999). It is at the RER that the synthesis of the viral proteins also occurs. The SER lacking ribosomes also gets its name from its appearance. It is the major store and regulator of intracellular Ca^{2+} . Accordingly it exerts an extremely important function of a multitude of physiological activities within a cell, as many of them are sensitive to changes in the cytosolic Ca^{2+} levels (Pozzan *et al.*, 1994). The SER also is responsible for most of the biosynthesis the cell's membrane lipids and lipophilic compounds; e.g. lipid, cholesterol, and glycosylphosphatidylinositol syntheses (Bishop & Bell, 1988, Eisenhaber *et al.*, 2003, Soccio & Breslow, 2004). Thirdly the SER is also the site where much of the detoxification in cells takes place. The reducing environment and the presence of enzymes such as P450, flavincontaining mono-oxygenases, esterases, and epoxide hydrolases, and UDP-glucuronosyltransferases and glutathione S-transferases, help eradicate xenobiotic agents from the cell (Eisenhaber *et al.*, 2003).

The ER is a patch-work of highly specialized subdomains with different protein compositions that perform different functions. One such subdomain is the transitional ER (tER), a part of the SER; these are involved in the packaging of proteins into the ER derived coat protein II (COPII)

for the ensuing vesicular transport from the ER (de Martin *et al.*, 2005). Such microdomains consists of lipid rafts, small functional microdomains in cellular membranes (Helms & Zurzolo, 2004, Rajendran & Simons, 2005, Simons & Toomre, 2000). These microdomains have lipid and cholesterol compositions that differ from the rest of the membrane, making them insoluble to certain detergent conditions that would dissolve the other cellular membranes. These microdomains are concentrated in small invaginations of membranes that make up the sorting centers for protein transport in endocytosis and transcytosis, including the tER.

The SER and its lipid rafts have shown to play an important role in the viral replication. As seen with HCV subgenomic replicons, the HCV replication complex together with newly synthesized viral RNA is also found on lipid rafts (Aizaki *et al.*, 2004, Shi *et al.*, 2003). Furthermore subgenomic replicons also show that HCV replication may be heavily dependent on the host lipid metabolism (Kapadia & Chisari, 2005, Sagan *et al.*, 2006). This notion is also supported by microarray data of chimpanzee liver biopsies, where up-regulation of genes involved in the lipid metabolism has been reported (Su *et al.*, 2002).

Viral replication and cellular membrane rearrangement

The association of RNA virus replication to cellular membranes were first observed by studying alphaviruses (Semliki forest viruses) in the late sixties, where membrane fractionation studies showed that viral replicase activity were associated with the mitochondrial membranes (Kaariainen & Soderlund, 1978). Although the origins of the membranes may vary, rearrangement of cellular membranes seems to be a general feature in all positive stranded RNA viruses (Ahlquist *et al.*, 2003, Mackenzie, 2005, Salonen *et al.*, 2005). Furthermore, these altered membranes have also shown to be the sites for the viral RNA replication. For many RNA viruses such as the picorna-, flavi-, arteri-, and bromoviruses, the membrane is ER-derived. However other cellular membranes such as endosomes and lysosomes (togaviruses), peroxisomes and chloroplasts (tombusviruses), mitochondria (nodaviruses) and membranes of the secretory pathway are also used (Mackenzie, 2005, Salonen *et al.*, 2005). While not all RNA virus membrane rearrangements seem to result in MAF formation, this seems to be a common trait at least within the flaviviruses and the hepacivirus (Egger *et al.*, 2002, Mackenzie *et al.*, 1996, Westaway *et al.*, 1999, Westaway *et al.*, 1997b). The rearranging of cellular membranes associated with the replication of these viral genomes could have several functions, among which to; *i*) chamber off the replication complex into protective environments where the double stranded RNA replication intermediate does not trigger host defenses; *ii*) enclose thus increase

the concentration of the components necessary for the viral replication; *iii*) increase the membrane surface on which the replication complex resides.

HCV replication and ER membrane rearrangements

The proteins that form the replication complex of the HCV are located to ER membrane-derived spherules referred to as the membrane associated foci (MAF) (Egger *et al.*, 2002, El-Hage & Luo, 2003, Gosert *et al.*, 2003, Mottola *et al.*, 2002). The MAF induction is exerted by the NS4B (Egger *et al.*, 2002, El-Hage & Luo, 2003, Gosert *et al.*, 2003, Gretton *et al.*, 2005, Lundin *et al.*, 2003, Shi *et al.*, 2003). It is the most well documented molecular function of the NS4B, exerted even in the absence of the other HCV proteins (Fig 7). These foci have shown to be the site of the newly synthesized viral RNA (El-Hage & Luo, 2003, Gosert *et al.*, 2003, Shi *et al.*, 2003). Though not all MAFs may be the sites for viral replication, the lack of MAF induction capacity may disable viral replication (Elazar *et al.*, 2004, Mackenzie & Westaway, 2001, Westaway *et al.*, 1999). As observed, mutations introduced to the NS4B that abolished membrane rearranging capacity also caused the abolishment of replication when introduced to the subgenomic replicon (Elazar *et al.*, 2004).

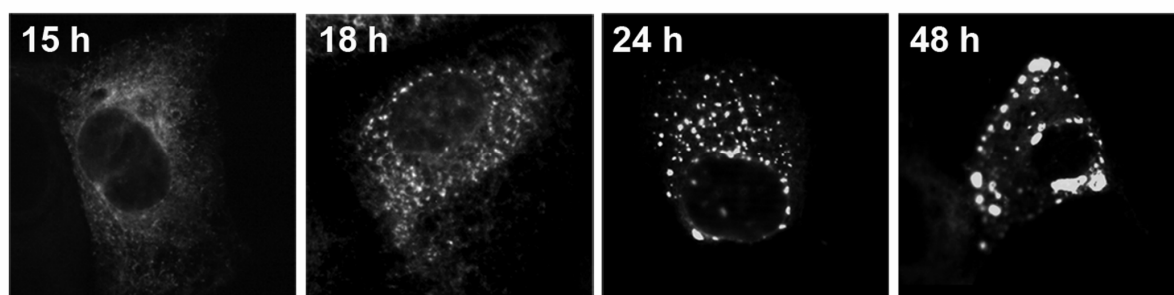


Figure 7. Induction of MAFs by recombinantly expressed NS4B-EGFP. Huh7 cells were allowed to express the recombinant NS4B protein fused to the EGFP at the C-terminus for 15, 18, 24, and 48 hours. These pictures show the step-wise increase in the MAF formation. The longer the expression, the higher the cell death could be observed. As can be seen, cells still surviving at 48 hours had very large and distinct MAFs. It should be kept in mind that despite that these images show a time-dependent gradual increase of MAF formation, all time-points studied displayed cells with varying degrees of MAF formation, though each time-point had a dominating degree of MAF induction.

The replication complex

Studies with the subgenomic replicons have shown that only NS3-5B are required to maintain the replication of the viral RNA in cells (Lohmann *et al.*, 1999). Furthermore, replicons derived from HCV as well as other *Flaviviridae* have shown that the proteins NS3-5B except the NS5A are necessary in *cis* configuration in the context of the viral polyprotein for maintaining viral replication (Appel *et al.*, 2005, Grassmann *et al.*, 2001, Khromykh *et al.*, 2000, Li & McNally,

2001, Tong & Malcolm, 2006). The NS3 helicase/NTPase and the NS5B RNA dependent RNA polymerase (RdRp) make up the central functional domain of the replication complex (Ishido *et al.*, 1998). Multimeric forms of NS5B have shown to synthesize RNA in a cooperative way by interacting with the structural element of the 3'NTR of the viral negative strand RNA (Friebe & Bartenschlager, 2002, Friebe *et al.*, 2005, Piccininni *et al.*, 2002, Tanaka *et al.*, 1996, Wang *et al.*, 2002, Yi & Lemon, 2003).

The NS3 and the NS4B in replication

The polymerase activity of the NS5B is regulated by the interaction with the other components of the replicon complex, for instance by interacting with the NS3 helicase. The NS3 helicase has a 3' to 5' directional duplex RNA unwinding action with affinity for poly U regions within the viral RNA (Gwack *et al.*, 1996, Hong *et al.*, 1996, Kanai *et al.*, 1995, Kim *et al.*, 1998, Lesburg *et al.*, 1999). The interaction between the NS3 and NS5B seems to promote the copying of the negative strand -3'NTR of the viral RNA to produce the positive stranded RNA (Piccininni *et al.*, 2002). The NS4B seems to suppress this process by partial inhibition of the NTPase activity of the NS3. This is very interesting, since besides MAF induction, the NS4B being an integral membrane protein was thought to merely provide a scaffold for the rest of the replication complex. Moreover mutations in the NS4B have also shown to improve viral replication in cells, indicating its critical involvement in the viral replication (Blight *et al.*, 2000, Lohmann *et al.*, 2003). However by which manner the NS4B functions in replication is yet to be discovered.

The NS5A and the human vesicle membrane associated protein A (hVAP-A) in replication

The NS5B has also shown to be regulated by the NS5A by direct interaction (Shimakami *et al.*, 2004, Shirota *et al.*, 2002). The NS5A is a zinc-binding metallo-protein that forms dimers that bind the viral RNA (Huang *et al.*, 2005, Tellinghuisen *et al.*, 2004, Tellinghuisen *et al.*, 2005). It was shown to prefer the polypyrimidine (U and G) tract in the 3'NTR of the positive stranded RNA with high affinity, opening up the possibility for binding similar sequences in the 3'NTR of the negative strand and the 5'NTR as well.

A cellular protein shown necessary for the HCV replication is the human vesicle membrane associated protein A (hVAP-A). The hVAP-A normally interacts with proteins involved in membrane fusion such as the vesicle-associated membrane protein (VAMP) 1 and 2 and oxysterol binding proteins that are involved in oxysterol and cholesterol homeostasis (Weir *et*

al., 1998, Wyles *et al.*, 2002). This protein has also shown to interact with both NS5A and NS5B (Gao *et al.*, 2004, Tu *et al.*, 1999). As seen by siRNA against hVAP-A, reduced hVAP expression resulted in reduction of subgenomic replicon replication as well (Gao *et al.*, 2004). Furthermore, mutations in the NS5A causing disruption in the hVAP/NS5A interaction also caused replication inhibition (Evans *et al.*, 2004).

Moreover, the NS5A exists in at least two forms; phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms, regulated in *cis* by the proteins NS3, NS4A, and NS4B (Koch & Bartenschlager, 1999, Neddermann *et al.*, 1999). These two forms of NS5A were shown to bind differently to the hVAP-A; the phosphorylated form bound well to the hVAP, while the hyperphosphorylated form did not (Evans *et al.*, 2004). In parallel, studies with the subgenomic replicons has shown that mutations causing the loss of the hyperphosphorylated form results in much higher replication rates, however HCV viruses containing the same mutations are unable to produce infectious virions (Blight *et al.*, 2000, Bukh *et al.*, 2002). Perhaps the different degrees of phosphorylation signal transition from one phase of the viral life cycle to another, such as from the replication phase to the assembly phase (Evans *et al.*, 2004).

Viral evolution

The HCV is highly mutable due to the viral RdRp lacking proof-reading activity. It is a quickly mutating virus whose estimated evolution rate is $1\text{--}3 \times 10^3$ nucleotide substitutions per site per year, depending on the site (Fernandez *et al.*, 2004, Kato *et al.*, 2005a, Lu *et al.*, 2001, Major *et al.*, 1999). This makes it comparable to the evolutionary rate of the Human Immunodeficiency virus 1 (HIV-1) (Goudsmit *et al.*, 1991, Ina *et al.*, 1994, Smith *et al.*, 1997). Furthermore there are coding regions in the viral genome, such as the hypervariable regions (HVR) in the E2, that allow high mutability, presumably for the virus to escape the immune system (Bassett *et al.*, 1999, Booth *et al.*, 1998). It should be kept in mind that not all viral progeny are fit to propagate; the mutations caused by the viral replicase often result in unfit virions. Only those mutations that allow proper proliferation as well as immunoescape will be favored. This way, the high mutability results in the persistence of the virus in the infected individual as a collection of quasispecies (Bowen & Walker, 2005, Pawlotsky, 2006). Even within the same subtype, the virus may differ as much as 9 % in the over-all genome and up to 49 % in the HVR

regions (McAllister *et al.*, 1998). The constant production of new virus variants makes it very challenging for the host immune system recognize and clear the virus, enabling some variants to escape, persisting the infection.

The immune system

The infected individual's initial immune response is thought to be vital for the outcome of an HCV infection. For instance, an early and extensive increase in the expression of IFN- α/β response genes during the acute phase of the infection is associated with viral clearance (Bigger *et al.*, 2001). Accordingly this section will deal with the interaction between the host defense system contra the virus invasion.

The innate immune system

The immune system may be divided into two; the innate and the acquired immunity (Janeway & Travers, 1997). The innate immunity is the first line of defense once the physical barriers of the human body are breached. They consist of lymphocytes such as monocytes / macrophages and granulocytes, and natural killer (NK) cells, and nonlymphoid professional antigen presenting cells (APC) such as dendritic cells (Fig 8). These cells possess the capacity to recognize foreign antigens without initial priming. Macrophages and granulocytes recognize foreign antigens by their pattern-recognition receptors such as the Toll-like receptors that recognize e.g. double stranded RNA or bacterial glycoproteins (reviewed in (Kaisho & Akira, 2006). Upon activation, these cells engulf foreign

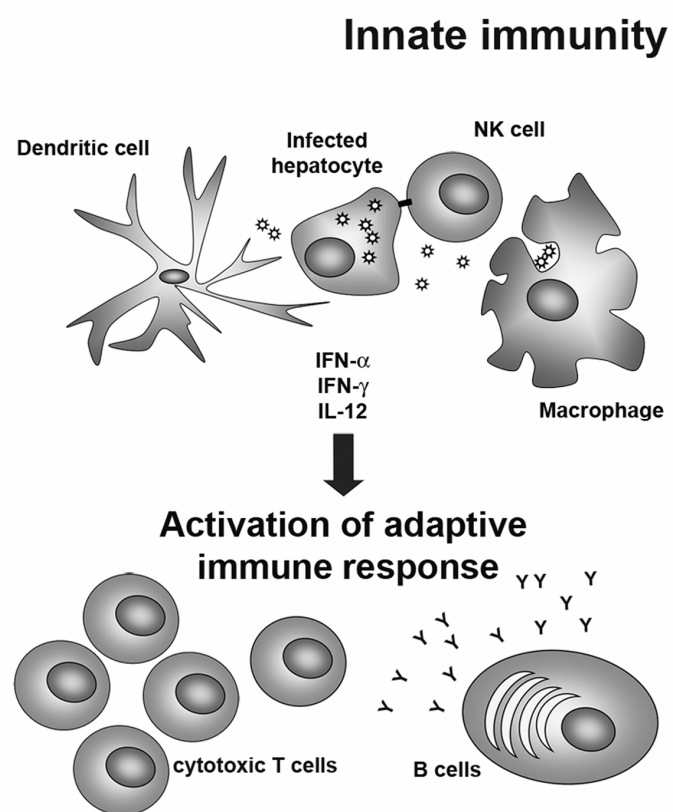


Figure 8. The innate immunity and the adaptive immunity

antigens/invaders and secrete cytokines such as the IFN- α or β . This results in the up-regulation of the immune response in the surrounding cells and infected cells as well, including up-regulation of their major histocompatibility complex I (MHC I) presentation of foreign antigens. As an immuno-evasive mechanism, many viruses and tumors down-regulate the cell's MHC I presentation. Therefore the NK cells recognize rather the “lack of self”; i.e. the NK cells are triggered by the lack of sufficient MHC I expression (Sentman *et al.*, 1995). Upon activation, these cells induce cytolysis or apoptosis in the target cells as well as being a potent producer of cytokines, especially the IFN- γ . The dendritic cells are mainly involved in the presentation of the foreign antigen to the rest of the immune system via MHC II and secreting cytokines such as interleukin 12 (IL-12) which is important for the development of the course of the acquired immune response (Maldonado-Lopez & Moser, 2001). Most importantly, all of the cells of the innate immune system are involved in attraction and activation of cells of the acquired immune system by secreting cytokines such as IL-12, IFN- α , and IFN- γ , and presenting the foreign antigens via MHC II (Fig 8).

The acquired immune system

The acquired immunity is mediated by CD4⁺ T helper cells (T_H), CD8⁺ cytotoxic T cells (T_C), and B cells (Fig 8). Upon infection via contact with APCs presenting the infecting agent's antigens, each cell that recognizes an antigen is selected from an existing pool of T and B cells and then clonally expanded. This way, the adaptive immunity is “tailored” to recognize specific antigens. Because of the

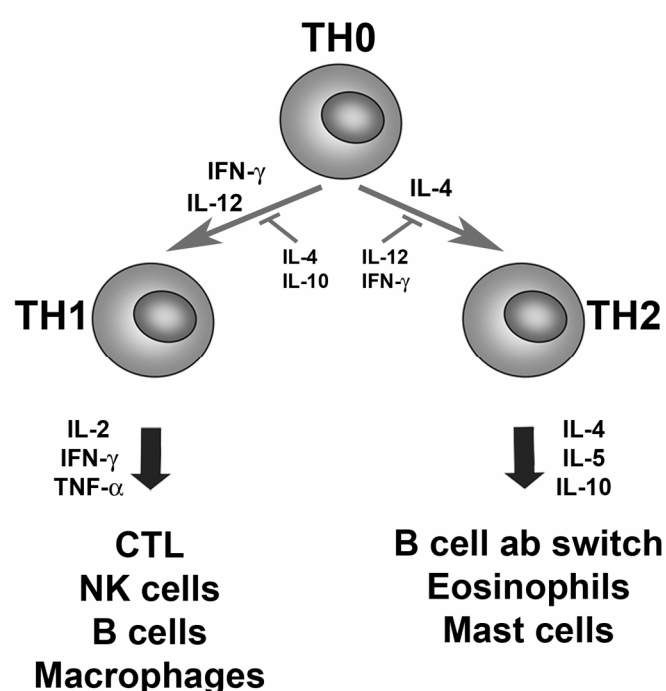


Figure 9. Cytokine regulation of the T_H1 and T_H2 immune response. Depending on the cytokines, the immature T_H0 cells are exposed to by the APCs, the T_H0 will mature into either T_H1 or T_H2 . Cytokines such as IFN- γ and IL-12 will induce T_H1 , while IL-4 will result in T_H2 (grey arrows). These inductions can be inhibited by the presence of IL-4 or 10 for T_H1 and IL-12 or IFN- γ for T_H2 (grey block arrows). The mature T_H1 and T_H2 then produce different sets of cytokines (black arrows); the T_H1 produces cytokines such as IL-2, IFN- γ , and TNF- α that stimulate the cell-mediated immune response while the T_H2 produces cytokines such as IL-4, 5, and 10 that triggers the humoral response.

constant selection pressure for specificity toward antigens, the efficiency of recognizing and clearing the infecting agent improves with time. Upon activation by the APCs, the CD4⁺ T_H develops to produce different sets of cytokines that regulates the balance between the humoral response and the cellular response of the acquired immune system. Depending on the cytokine profile secreted, the CD4⁺ T_H is divided into T_H1 and T_H2 (Fig 9) (D'Elis & Del Prete, 1998). The T_H1 producing predominantly IL-2, tumor necrosis factor α (TNF- α), and IFN- γ activates the CD8⁺ cytotoxic T cells and NK cells but also the B cells, thus the immune response is primarily cell-mediated. Contrarily, the T_H2 producing IL-4, IL-5, and IL-10 is responsible for humoral response maturation by stimulating B-cell proliferation and antibody class switch, and stimulating mast cell and eosinophil growth and differentiations (Janeway & Travers, 1997).

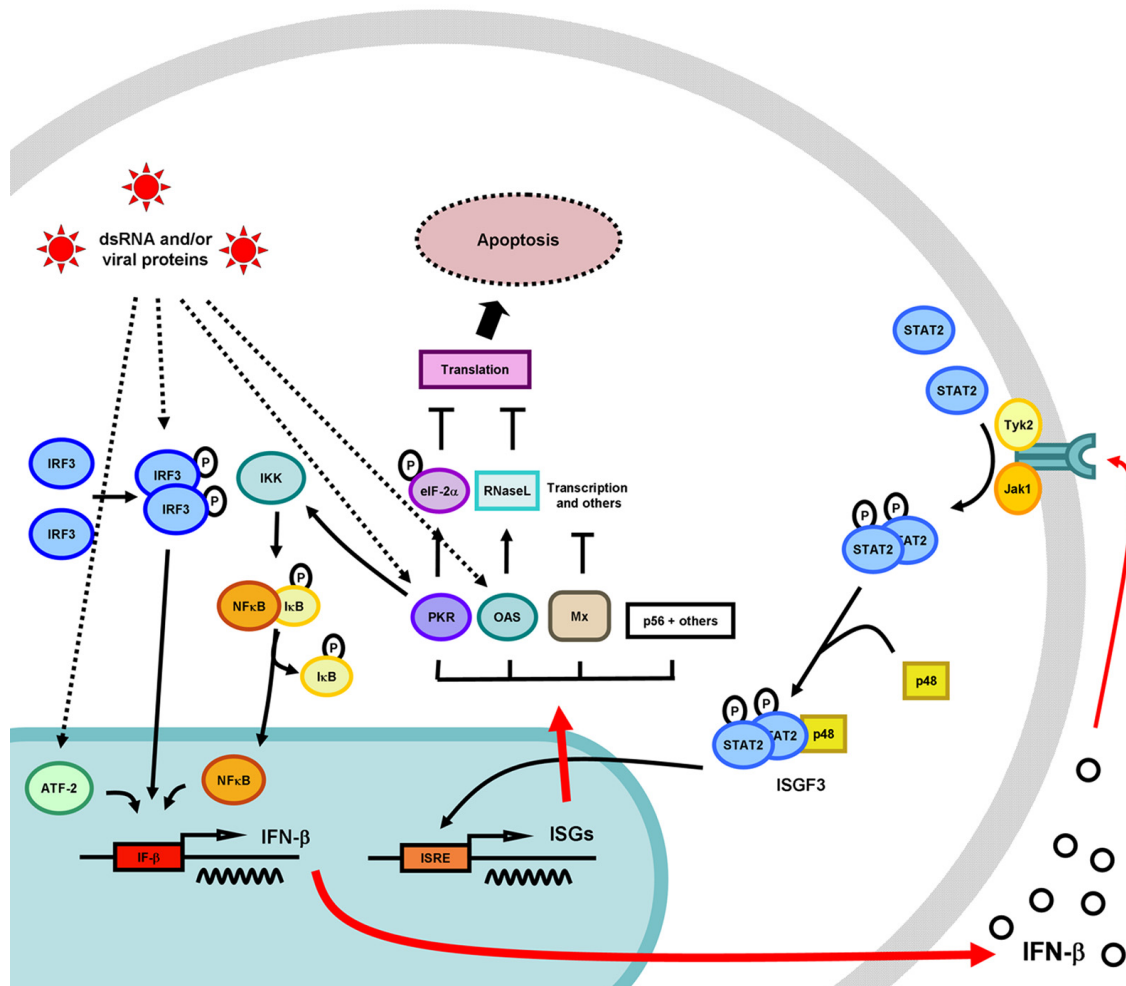


Figure 10. Mammalian cell's immune response to viral attack. The dotted arrows show the antiviral pathways that can be triggered by the presence of viral components in cells, such as viral proteins or RNA. Interestingly, HCV RNA did not trigger this pathway in subgenomic replicon systems (Abe *et al.*, 2005, Guo *et al.*, 2003, Zhu *et al.*, 2003a). The solid arrows show the antiviral pathways triggered by IFN.

The host immune system versus the virus

Triggering the innate immunity

The HCV infects mainly hepatocytes. Although it has also been found in other cell types such as blood monocytes and epithelial cells on small intestines, the replication of the virus has shown to occur mainly in hepatocytes (Crovetto *et al.*, 2000, Deforges *et al.*, 2004, Lerat *et al.*, 1998, Radkowski *et al.*, 2002). The infection in the hepatocytes causes a number of changes, including triggering genes responsible for the host defense such as the IFN response genes (Bigger *et al.*, 2001, Su *et al.*, 2002) (Fig 10). These include 2'-5' oligoadenylate synthetase (OAS), double-stranded (ds) RNA-dependent protein kinase (PKR), and Mx1 protein, as well as IFN-regulated transcriptional factor genes and genes that encode cytokines such as IFN- β that attract granulocytes and macrophages and stimulate the cytotoxicity of NK cells. This way, the innate immune system is triggered.

Recruiting the adaptive immunity

In the liver, the APCs consist of liver sinusoidal endothelial cells and resident macrophages (Kupffer cells). Upon infection, the T cells are recruited by the NK cells' IFN- γ secretion and the up-regulated chemokine expression (such as CXCL9 and CXCL10) on the APCs. Upon recruitment, the CD4⁺ and CD8⁺ T cells will recognize the HCV antigen presented by the MHC on the infected hepatocytes and APCs and respond with both cytotoxic (e.g. cytolytic T cell activation) and non-cytotoxic cytokine-mediated effector functions (e.g. humoral response) (Crispe, 2003). This is manifested in the liver by the concomitance of up-regulated genes involved in the antigen processing and MHC expression, and the presence of infiltrating CD4⁺ and CD8⁺ T cells (Bigger *et al.*, 2001, Su *et al.*, 2002). At this stage, the vigor and the quality of the T cell response have been correlated with viral clearance (Thimme *et al.*, 2001).

HCV escape from host defense

The first thing that comes to mind when speaking of HCV eluding the host immune system is the generation of quasispecies (Bowen & Walker, 2005, Pawlotsky, 2006). By rapid mutation of

especially the HVR regions of the envelope proteins, the HCV generates variants that evade recognition by the B and T lymphocytes mediating the specific immunity. Furthermore there are several other mechanisms by which the virus directly manipulate the host immune system; inhibiting NK cell's responses such as cytotoxicity, proliferation, and IFN- γ and TNF- α secretion by crosslinking the CD81 on the NK cell surface (Crotta *et al.*, 2001, Tseng & Klimpel, 2001); causing deficiency in peripheral-blood dendritic cells to lose their allostimulatory capacity by unknown mechanisms (Bain *et al.*, 2001); impairing IFN- γ production and proliferation by CD8⁺ T cells despite MHC binding, most likely by HCV nucleocapsid (core) proteins binding to complement (C1q) receptors on T cells (Urbani *et al.*, 2002); and viral block or diversion of HCV specific CD8⁺ T cell differentiation also by unknown viral effects (Lucas *et al.*, 2004).

Interferons

As mentioned before, the only HCV treatment to date is the combination therapy of peg-IFN- α 2a and RBV, where the sustained responders may range between 50-80 % (Hadziyannis *et al.*, 2004). This is actually a significant improvement from the previously used non-pegylated IFN- α , where the therapeutic outcome was at least 5-10 % lower (Craxi & Licata, 2003, Foster, 2003). This improvement may at least in part be ascribed to the longer systemic duration of the pegylated interferon. Also studies have shown that the earlier the onset of IFN treatment after the HCV infection initiation, the better was the outcome (Kamal *et al.*, 2006, Urbanek *et al.*, 2004, Wiegand *et al.*, 2006). HCV sensitivity to IFN has also been demonstrated with subgenomic replicons *in vitro* (Blight *et al.*, 2000, Chung *et al.*, 2001, Frese *et al.*, 2001, Frese *et al.*, 2002, Guo *et al.*, 2001). Other studies have shown that the effectiveness of IFN therapy in patients could be correlated to the degree of IFN- α / β receptor expression in their livers (Fukuda *et al.*, 1996). Together these show that interferons play a crucial role in the viral clearance.

Types of interferons

Interferons are grouped into two types: type I consists of numerous variants of IFN- α and one of β , and type II consists of IFN- γ (Levy, 2002). Many, if not all cells are capable of producing IFN- α and β , where the productions are triggered by the presence of viral components in cells.

Both IFN- α and β act on the same IFN receptor on cell surfaces. The IFN- γ is produced by the NK and T_H1 cells and is one of the most potent stimulators of the immune system. The IFN- γ triggers the acquired immunity's proliferation and cytotoxic responses as well as up-regulating the innate immune system (Billiau *et al.*, 1998). As seen in HCV infections, the initial response by the host to IFNs may be a key action that affects the course of the HCV infection (Bigger *et al.*, 2001).

Virus-triggered cell response

Antiviral actions in cells are normally triggered by double stranded RNA, an intermediate in the viral genome replication, and/or viral proteins (reviewed in Gale, 2003) (Fig 3). These may trigger the IFN- β production in cells by activating IFN regulatory factor 3 (IRF3), activating transcription factor 2 (ATF-2), or PKR. The produced IFN- β can activate both self and neighboring cells by binding to the type I IFN receptor on the cell surfaces (binds both IFN- α and β) trigger the Jak-STAT signaling via Jak1-Tyk2 (protein tyrosine kinase) proteins. These induce signal transducer and activator of transcription (STAT) phosphorylation and dimerization. The dimerized STAT then forms the IFN-stimulated gene factor 3 (ISGF3). The ISGF3 in turn translocates into the nucleus and initiates the transcription of the IFN-stimulated response elements (ISRE). The resulting IFN-stimulated genes (ISG) in turn activate PKR, OAS, and the Mx1. The PKR triggers the eIF-2 α and the OAS triggers the ribonuclease L (RNase L), both which result in the inhibition of translation in the cell, that may ultimately lead to apoptosis. The Mx inhibits cellular transcription. The PKR triggers also the I κ B kinase complex (IKK), activating the nuclear factor κ B (NF κ B) that initiates the transcription of IFN- β again.

HCV-triggered cell response and interferon

Studies with HCV subgenomic replicons in cell cultures have shown that the PKR, OAS, and Mx were not triggered by the replicating viral RNA per se (Abe *et al.*, 2005, Guo *et al.*, 2003, Zhu *et al.*, 2003a). In addition, down-regulation of proteins involved in antigen processing for MHC presentation was also observed (Abe *et al.*, 2005). However at least PKR and OAS antiviral actions were easily triggered via the Jak-STAT pathway by treating the cells with IFN (Frese *et al.*, 2001, Gale, 1997 #33, Guo *et al.*, 2004, Guo *et al.*, 2003, Wang *et al.*, 2003, Zhu *et*

al., 2003a) (Fig 10). Remarkably, despite activation of the antiviral responses in cells, the complete eradication of viral presence was not always guaranteed, at least in the context of type I interferons (Cheney *et al.*, 2002, Guo *et al.*, 2001).

Interferon treatment and viral clearance

The prognosis for the antiviral treatment outcome varies greatly between the infecting HCV genotypes, as seen in an extensive study including over thousand patients who had been treated with the combination therapy of peg-IFN- α 2a and RBV (Hadziyannis *et al.*, 2004). The number of patients that remained clear of the virus even 6 months after treatment for the HCV genotype 1 ranged between 29-52 %. While for the genotypes 2 and 3, the cure rate could be as high as 80-84 %. Furthermore the study also showed difference between the viral clearance depending on the initial viral load of the patient. For the genotype 1, clearance rates for those with high initial viral load ranged from 16-47 %, while the corresponding number for patients with low initial viral load was 41-65 %. Hence the outcome of the IFN treatment seems very much dependent on the infecting genotype as well as the host immune system. Together these data show that much remains to be revealed in the interaction between the virus and the host.

Model systems

Chimpanzees

The study of the HCV has been very difficult due to the lack of small *in vitro* culture systems. Until very recently the virus was only possible to propagate in human or chimpanzee hosts. The chimpanzees and humans are 98.8 % genetically identical (Ebersberger *et al.*, 2002). Sharing such high degree of identity, the chimpanzees have been, and still are invaluable tools for studying the viral progression in host organisms. Many studies of both humoral and cellular immune response on the viral progression have been done in chimpanzees, where the time and progression of the infection may be studied under controlled circumstances. However these are highly intelligent animals that risk extinction in the world, and are very costly to maintain. Furthermore, as similar to humans as they are, it should be kept in mind that chimpanzees still differ from us in the genetic expression profile: the all-over expression patterns of protein levels differ up to 31.4 % between humans and chimpanzees (Enard *et al.*, 2002). This difference may result in the slight differences observed in the viral disease progression between chimpanzees

and humans. For instance the necroinflammatory lesions are almost always mild in chimpanzees while the spectrum is wider in humans, ranging from mild inflammation to cirrhosis leading to liver failure (Walker, 1997). Another difference that should be noted is the difference in the humoral response; chimpanzees seem to have a more restricted antibody response against the virus than humans (Walker, 1997).

Small animal models

So far creating smaller animal models using small primates or different strains of mice for the study of HCV have achieved limited success, primarily due to the lack of robust HCV replication (Grakoui *et al.*, 2001, Guha *et al.*, 2005). However there is one mouse model that has shown some promise. By grafting immunodeficient SCID mice homozygous for urokinase-type plasminogen-activator under the albumin promoter (Alb-uPA) with human hepatocytes, up to 70 % of the mouse liver could be bestowed with human hepatocytes (Mercer *et al.*, 2001). The Alb-uPA gene causes overproduction of urokinase in the liver, ultimately resulting in accelerated death of endogenous hepatocytes. This way the transplanted human hepatocytes could compensate for that loss, resulting in the chimeric liver. These mice were able to become infected with the HCV virus, where viral titres could reach 3×10^4 to 3×10^6 copies/ml blood. However virus production seemed to generally cease after 15-17 weeks. Furthermore, the immunodeficiency in these mice and the grafting with human hepatocytes is extremely laborious and requires specialist competence. Together, it makes a costly and complicated model to use.

***In vitro* HCV models**

As mentioned above, there still are no satisfactory small and easy to handle animal models available to date. Fortunately during the course of this thesis, leaps and bounds of improvements have been made in small and easy to handle *in vitro* model systems for the HCV proliferation. These novel systems can now enable controlled biochemical and molecular studies of different aspects of the viral life cycle, as well as mass-screening in search of novel antiviral agents.

The HCV subgenomic replicons

Initially, *in vitro* HCV models were limited to infecting primary hepatocyte cultures or hepatoma cell lines with HCV isolates (Fournier *et al.*, 1998, Iacovacci *et al.*, 1997, Ikeda *et al.*,

1997, Lanford *et al.*, 1994, Seipp *et al.*, 1997, Shimizu *et al.*, 1992, Yoo *et al.*, 1995). These systems provided some measurable HCV replication, however viral replication in these cultures was extremely inefficient and dwindled rapidly. A major breakthrough was made in 1999 when Lohmann and colleagues successfully created a subgenomic HCV replicon that could self-replicate in the human hepatoma cell line Huh7 (Lohmann *et al.*, 1999). The replicon was based on the Con1 isolate (HCV genotype 1b) from a chronically infected patient, and consisted of the Neomycin resistance gene under the HCV IRES, and the proteins NS3 to NS5B of the Con1 flanked by the Encephalomyocarditis virus IRES (EIRES) and the HCV 3'NTR (Fig 11). This replicon was further improved by the discovery of a cell adaptive mutant with the change S2204I in the NS5A region that vastly improved the replication capacity in cell culture (Blight *et al.*, 2000). Since then replicons with other adaptive mutations (Blight *et al.*, 2000, Krieger *et al.*, 2001, Lohmann *et al.*, 2001, Yi & Lemon, 2004), derived from other HCV genotypes (1a and 2a) (Blight *et al.*, 2003, Kato *et al.*, 2003), both subgenomic and genomic (Blight *et al.*, 2002, Ikeda *et al.*, 2002, Pietschmann *et al.*, 2002), with different cell tropisms (Ali *et al.*, 2004, Date *et al.*, 2004, Kato *et al.*, 2005b, Zhu *et al.*, 2003b) have been generated making replicons a powerful tool. Gene expression profiling of cells treated with IFN supporting replicons and biopsies from infected patients treated with IFN showed remarkable similarities further validating this system (Ji *et al.*, 2003, Zhu *et al.*, 2003a). Despite lacking the ability to produce infectious viral particles, it has the advantage of having fully on-going HCV RNA replication similar to that of a chronic infection. And since no viral particles are produced, these are safe and easy to handle. These replicons enabled biochemical studies of the HCV replication that was previously impossible. In this thesis, the study of how mutations in the NS4B affect the HCV replication was based on using this very system (paper IV).

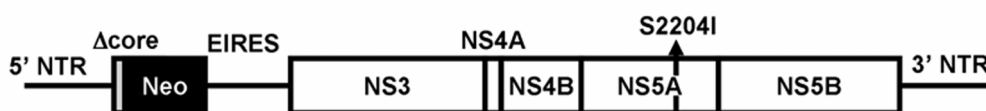


Figure 11. The HCV subgenomic replicon

HCV production in vitro

In the past year, another milestone of HCV research has been achieved. Finally, an *in vitro* cell culture system that enables robust production of infectious virions have been achieved by three independent groups (Lindenbach *et al.*, 2005, Wakita *et al.*, 2005, Zhong *et al.*, 2005) based on the same JFH isolate from a patient with fulminant HCV infection (HCV genotype 2a) (Kato *et*

al., 2003, Kato *et al.*, 2001). What is more, the chimera virus J6/JFH, both derived from genotype 2a isolates, created by Lindenbach and colleagues showed to be infectious in chimpanzees as well, i.e. transferable between the *in vivo* and *in vitro* systems (Lindenbach *et al.*, 2006). Since then *in vitro* production of infectious virions of the genotypes 1a as well as 1b has been reported (Murakami *et al.*, 2006, Yi *et al.*, 2006). Regrettably, these newer systems were not available at the time for our studies. It shall be very exciting to see what these systems will reveal in the future.

AIMS

The aim of this thesis was to shed some light on two aspects of the viral life cycle; the translation and the replication of the virus genome. In particular, the focus has been put on the HCV IRES mediated translation in relation to clinical outcome, and the role of the NS4B on the viral replication.

More specifically:

- Study the role of the IRES sequence in the translation initiation: how do differences in the IRES sequences affect the translation initiation, and are there any relations to clinical outcome (Paper I)?
- Biochemical studies of different NS4B variants recombinantly expressed in cells in hope of illuminating some of its functional properties (Paper II and III).
- Study the functional integrity of the NS4B on the HCV replication in the context of the viral replicon complex (Paper IV).

COMMENTS ON MATERIALS AND METHODS

This section describes and discusses the methods used in this thesis in general terms. A more detailed report on materials and methods used can be found in the original articles.

Cell lines

The cell lines used in these studies were HepG2, Hep3B, HEK293, Hela (all Paper I), Huh7 (Papers II to IV), T#9 (see description below; Paper IV), Cos7 (Papers II and III). All cell lines except Huh7 and T#9 were grown in Dulbecco's modified Eagle medium (DMEM) containing GlutaMAX I supplemented with 10 % foetal calf serum with 100 U penicillin and 100 µg streptomycin per ml media (all from Invitrogen). The Huh7 and T#9 were grown in DMEM with 10 % foetal calf serum only.

The “cured” cell line T#9

For the replicon survival trials (Paper IV), the cell lines Huh7 and T#9 were used. The Huh7 cell line was obtained from Japanese Collection of Research Bioresources. The T#9 is a so-called “cured” cell line, derived from our lineage of Huh7.

The Huh7 is a rather unstable cell line; clear variations can be observed between cell lines derived from the same parental cell. For instance, it has been seen that certain Huh7 clones display more favorable environments for the replication of the HCV subgenomic replicon than others (Blight *et al.*, 2002, Lohmann *et al.*, 2003, Murray *et al.*, 2003). Furthermore, Huh7 cell lines that support replicons for longer time periods seem to promote the selection of cells that favors replicon replication. By treating these cells with IFN- α , Blight and colleagues showed that cells harboring replicons could be “cured”, i.e. the replication of the replicon ceased (Blight *et al.*, 2002). When new replicons were reintroduced into these cured cell lines, they showed to

be superior for replicon establishment. Therefore, in order to create highly permissive cell lines for our replicon establishment efficiency studies, the T#9 cell line was accordingly created (Paper IV).

The T#9 cell line had been supporting S2204I replicon replication for 9 months prior to being treated (i.e. “cured”), by adding 1000 IU/ml IFN- γ (Roche) to the media for 5 weeks. The loss of replicon replication was verified by the loss of resistance to G418, the loss of expression of NS3 and NS5A detectable by Western blotting, and the loss of replicon RNA detectable by RT-PCR. In contrast to that reported by Blight and colleagues, the T#9 was not curable by IFN- α treatment. Although the IFN- α did suppress the replication, it could not completely abolish the replicons even under prolonged IFN- α treatment. This inability of the IFN- α to abolish replicon replication in cell cultures has also been observed by other groups (Cheney *et al.*, 2002, Guo *et al.*, 2001). However treatment with IFN- γ efficiently suppressed and abolished the replicon replication, as also reported by other groups (Cheney *et al.*, 2002, Frese *et al.*, 2002). Again, these discrepancies in replicon clearance by IFN between different research groups may be due to clonal differences in the Huh7 cell lines.

The above once more emphasizes the instability of the Huh7 derived cell lines (our own observations, (Lohmann *et al.*, 2003, Murray *et al.*, 2003). In order to keep the conditions between the different transfection rounds in the experiments of Paper IV as similar as possible, the cells were stored aliquoted in liquid nitrogen prior to use and discontinued after a maximum of 20 passages for Huh7 and 16 passages for T#9.

Dual luciferase assay

For the study of HCV IRES driven translations (Paper I), the dual luciferase assay was chosen. It is a simple way of determining the efficiency of the HCV IRES by using a bi-cistronic reporter system (Collier *et al.*, 1998). In the original construct pDL-T₇, the reporters consist of two different luciferase enzymes each driven by its own promoter; the Renilla luciferase under the T7 promoter and the Firefly luciferase under the HCV IRES to be tested. In our work the T7 promoter was exchanged with the cytomegalovirus (CMV) promoter, since the T7 required additional transfection with a construct carrying the T7 polymerase (Fig 12). Both reporter

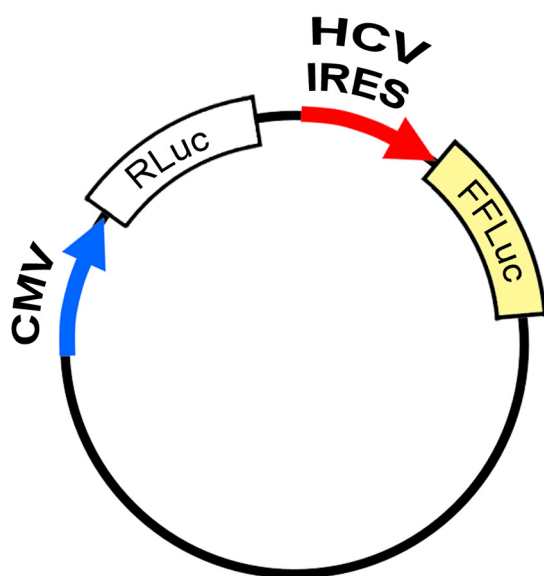


Figure 12. The bi-cistronic dual luciferase vector. The CMV promoter drives the internal control Renilla luciferase (R Luc), and the HCV IRES drives the Firefly luciferase (FF Luc).

luciferase activities are assayed in the same cell lysate aliquot in a sequential succession using the Dual-Luciferase® Reporter (DLR™) Assay System (Promega) and a luminometer: first the activity of the firefly luciferase reporter is measured and quenched; then the activity of the Renilla luciferase reporter is measured. Here, the Renilla luciferase activity functions as the internal control. This enables the comparison of different HCV IRES to each other by normalizing the HCV IRES driven Firefly luciferase activity against the CMV promoter driven Renilla luciferase activity. Both reporters yield linear assays with attomole ($< 10^8$) sensitivities with no endogenous activities from the host cells, and the range of measurement extends 7 logs. This gives the dual luciferase assay the

advantage of enabling different samples to be compared to each other despite great differences in transfection efficiencies.

Comparing the translation efficiencies of different IRES

The constructs containing the different HCV IRES were transfected into cells and allowed to develop for 48-72 hours in a time study in order to establishing the optimal expression time, where maximum luciferase activities were achieved after 48 hours. For the quantification of IRES mediated translation, the cells were harvested and the lysates were measured for luciferase activities as described above. The activity of the CMV driven Renilla luciferase activity was arbitrarily set at 100 %. The IRES driven translation efficiency was assayed by normalizing the Firefly luciferase activity against that of the Renilla luciferase.

Studying the effects of antiviral treatment on IRES-mediated translation

For studying the effects of antiviral treatment on IRES mediated translation, a dose-response curves were established on the IFN- α and RBV by treating the cells 24 hours post-transfection with 500, 1000, 1500, 2000, and 5000 IU/ml for IFN- α , or 50, 100, and 500 μ M for RBV,

where dose-dependent suppression of the translation could be seen as previously reported (Kato *et al.*, 2002a). However at the highest doses of both IFN and RBV, considerable amounts of cell death were observed, therefore optimal doses (non-lethal) for both substances were chosen for the ensuing combination experiments: 500 and 1000 IU/ml IFN- α were used together with 25 μ M RBV for the combination treatment. For the evaluation of the effects of the antiviral treatment on IRES mediated translation *in vitro*, the activity of the Firefly luciferase of dual luciferase vector from transfected but untreated cells was first normalized against its internal control, the Renilla luciferase activity. The resulting value was then set at 100 %. Subsequently, the normalized value (i.e. the ratio Firefly/Renilla luciferase activity values) of the IFN and RBV treated cells were reported in proportion to their untreated counterpart.

Statistical analysis of the IRES-mediated translation efficiencies

For our IRES mediated translation efficiency studies, a rather low number of IRES were used (15 for SR and 7 for NR). Thus to test the degree of statistical significance, the non-parametrical Mann-Whitney *U*-test was used.

Introducing mutations to NS4B DNA templates

In our work, numerous vectors and *in vitro* transcription templates were created (Papers I to IV). In instances of introducing single mutations to a DNA sequence or few mutations at the 5' or 3' end of a sequence, PCR amplification of a protein coding sequence with elongated primers carrying the wanted mutations may be used (Fig 13A). This is followed by standard restriction enzyme-based cloning into a DNA vector. Another alternative is the use of ready-to-go kits such as Quick-Change site-directed mutagenesis kit (Stratagene). There, two primers carrying the desired mutations which are reverse-complementary to each other are used to amplify the entire vector carrying the protein to be mutated (Fig 13B). A major advantage of this method is that the resulting PCR product may be directly amplified in *E. coli*, making the restriction enzyme-based cloning unnecessary (e.g. used to make the pNS3-4B/E210 vector in Paper II). However both of the above methods are limited by the size and location of the mutation to be introduced. The scope of the mutagenesis kits is also limited by the size of the vector carrying the gene to be mutated, under the principle “the larger the vector, the lower the success rate”. This is due to the limitations of the DNA polymerase's ability to amplify larger DNA fragments

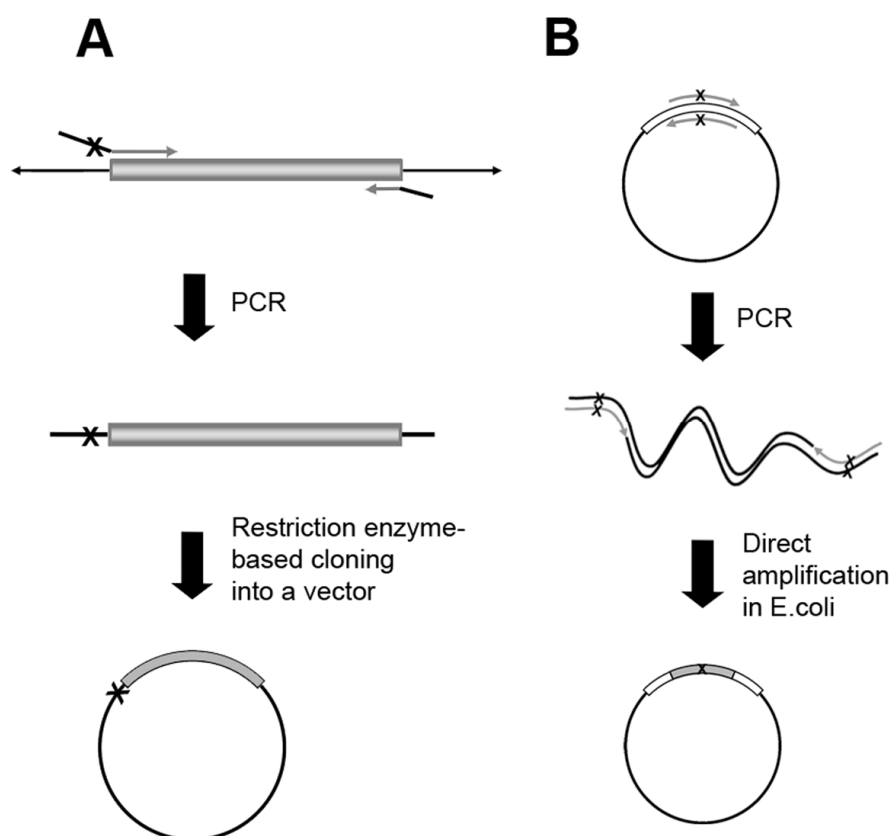


Figure 13. Introducing small mutations to a protein coding sequence. A. Standard PCR-based cloning. B. Quick-Change site-directed mutagenesis (Stratagene). X – changes introduced to the protein coding sequence.

as well as the limits to its proof-reading capacity. In most of our cases, larger changes had to be made, such as the introduction of mutations to perturb a putative AH in the N-terminus of the NS4B (Papers III and IV). Alternatively mutations had to be inserted in very large vectors, such as the replicon vectors with sizes over 11 kilobases (Paper IV). In these cases the assembly PCR technique was used (Ho *et al.*, 1989).

Assembly PCR

Assembly PCR is a very efficient method of manipulating large sections of DNA templates. An example is given in Figure 14. It is a two-step process. The mutation carrying primers are primers B and C, one being the reverse-complementary to the other. In the first step, the template with forward primer A and the reverse primer B, and the forward primer C and the reverse primer D is used for the PCR amplification. This gives rise to the products AB and CD. In the second step, equimolar amounts of AB and DC are used as templates with the primers A and D for the final PCR, giving rise to the assembled product.

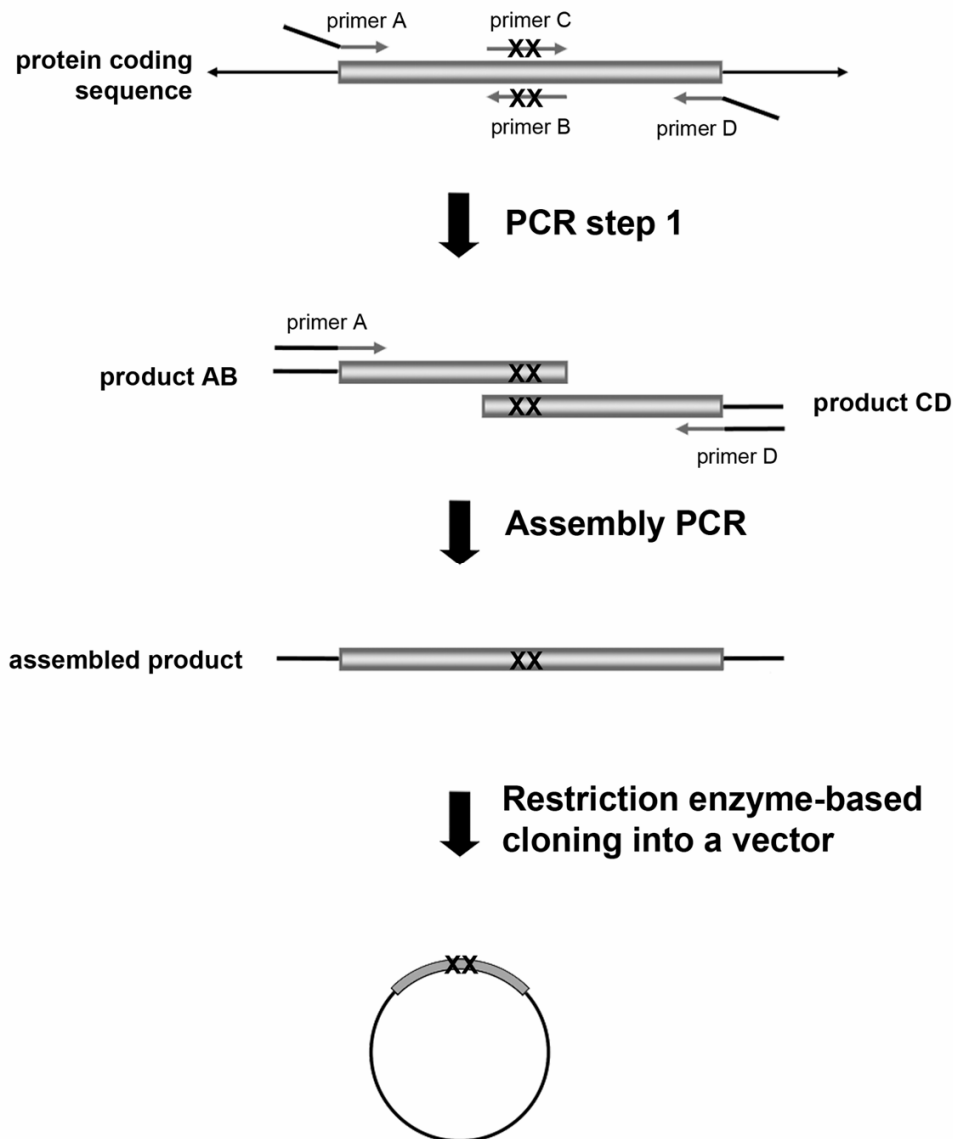


Figure 14. Assembly PCR. X – changes introduced to the protein coding sequence.

One negative aspect of the assembly PCR is the “many” PCR steps: the higher the number of PCR cycles, the more prone the product may be to unwanted changes. We however did not suffer markedly from the increase of unwanted mutations upon sequencing the finished constructs. Sequencing results showed that roughly ~30-100 % of the finished vectors were as designed, depending on the length and complexity of the assembled product.

Using this method, we have created several variants of NS4B mutant vectors (e.g. pNS3-NST4B in Paper II and pAHmut-EGFP, various p4B- and pAHmut NST-EGFP constructs in Paper III), as well as introduced mutations to the replicon vector (e.g. L1715R, P1716G, etc in Paper IV). The assembly PCR has also been used to create numerous templates for *in vitro*

transcription (e.g. various T7-4B NST templates for *in vitro* glycosylation scan and topology studies in Paper III).

***In vitro* transcription and *in vitro* translation**

In vitro transcription followed by *in vitro* translation of the resulting transcript is a quick and easy way to manipulate and test proteins. Briefly, the T7 promoter, the Kozak translation initiation sequence, and other desired changes are added to the DNA template by PCR (see the section “Introducing changes to NS4B DNA templates”), and the resulting T7 DNA template is used in the ensuing *in vitro* transcription by T7 RNA polymerase. The resulting RNA is then used in the *in vitro* translation using rabbit reticulocyte lysate. The rabbit reticulocyte lysates are crude extract containing all the macromolecular components required for translation of exogenous RNA such as 80S ribosomes, tRNAs, aminoacyl-tRNA synthetases, initiation, elongation and termination factors, etc. It is a rather insensitive system that requires neither 5' capping nor poly-adenylation of the RNA transcript. Proteins requiring membrane insertions, or simpler modifications such as glycosylations are expressed in the presence of canine pancreatic microsomal membranes, which are crude extracts of ER membranes. Using this system the creation from a DNA template to the finished protein may take less than a day! However it should be kept in mind that the capacity of *in vitro* translation system to translate, process, and modify resulting proteins is rather limited compared to the production in cells. There are also quantity and size limitations to the protein or proteins that can be translated *in vitro*, especially when polyproteins or multimeric complexes are concerned. In addition proteins requiring higher chaperone activity may be very difficult to produce despite the addition of microsomes.

The *in vitro* transcription reactions were performed using T7 polymerase (Invitrogen) according to the manufacturer's recommendations using DNA templates. The ensuing translations consisted each of 1 µl of the *in vitro* transcribed RNA, 9 µl nuclease-treated rabbit reticulocyte lysate, 40 µM amino acid mixture without L-Methionine, 40 units RNasinI (all from Promega), and 30 µCi L-[³⁵S]-Methionine (Perkin-Elmer). For the translations of NS4B in *in vitro* glycosylation studies (Papers II and III), the above reactions were supplemented with or without 1 µl Canine Pancreatic Microsomal Membranes. The reactions containing microsomes were further supplemented by either 0.4 mM of competitive glycosylation acceptor peptide (benzoyl-Asn-Leu-Thr-methylamide) dissolved in DMSO (total volume 0.2 µl) or 0.2 µl DMSO only.

This was to verify true glycosylation (see the below section “Glycosylation studies”). For the NS4B processing studies (Paper IV), both NS4A-NS4B and NS4B-NS5A translation reactions were supplemented with 1 µl microsomes. The NS3 or NS3-NS4A were however translated without microsomes or L-[³⁵S]-Methionine then added to the NS4A-NS4B and NS4B-NS5A reactions, respectively, for *in vitro* processing of the NS4B. The finished products were run on SDS-PAGE under reducing conditions. The gels were then fixed (10 % Acetic acid, 30 % methanol, and 60 % H₂O), dried (under vacuum pressure at 80 °C), and analyzed using Fuji BAS 1000 Phosphoimager and Image Gauge V3.45 software (Fuji Film Science Lab 99/2001).

Quantification of protein populations

In order to quantify a specific population of proteins in a sample of mixed proteins, the different populations of proteins were first separated by running the sample on SDS-PAGE gels. The proteins (made detectable by incorporation of radioactive amino acids (³⁵S) or binding of Horseradish peroxidase (HRP) conjugated antibodies) were quantified by measuring the intensities of unsaturated bands, using either film exposed to Enhanced chemiluminescence (ECL) development or image scanned by the Phosphoimager. Then the resulting bands were quantified by using the Image Gauge V3.45 software (Fuji Film Science Lab 99/2001). For instance, the populations of NS4B with lumenally located N-terminus (Paper II) were quantified by calculating the quotient between the intensity of the glycosylated band divided by the summed intensities of the glycosylated and unglycosylated bands. The processing efficiencies of the NS3-4B and NS4B-5A (Paper IV) were calculated as the quotient of densitometric band intensity of NS4B divided by the band intensity of either NS4A-B or NS4B-5A for NS4A-B or NS4B-5A processing, respectively.

Glycosylation studies

The most common glycosylation of a protein or a peptide in eukaryotes is the N-linked glycosylation (Kornfeld & Kornfeld, 1985). It occurs co-translationally in the lumen of the ER membrane by the oligosaccharyl transferase (OST). The enzyme recognizes the Asn-X-Thr/Ser sequence of the nascent polypeptide chain that has been translocated into the lumen, where X is

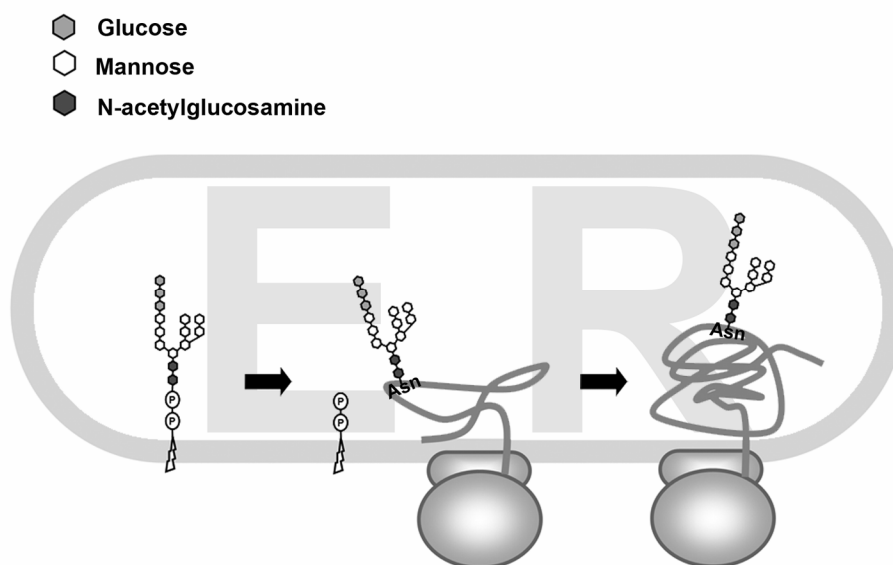


Figure 15. Glycosylation of nascent protein. The oligosaccharyl transferase (OST) is associated to the translocase complex on the luminal side of the ER membrane. It catalyzes the reaction between the Asn of the Asn-X-Ser/Thr sequence of the nascent polypeptide chain and the lipid-linked the tetradecasaccharide ($\text{GlcNAc}_2\text{-Man}_9\text{-Glc}_3$), as soon as a distance of 12-14 amino acid residues clears from the ER membrane.

any amino acid residue except Pro (Imperiali *et al.*, 1999) (Fig 15). The enzyme hydrolyzes the reaction between the tetradecasaccharide ($\text{GlcNAc}_2\text{-Man}_9\text{-Glc}_3$) to the Asn side chain. The glycosylation site needs 12-14 amino acid residues' distance from the ER membrane in order for the OST to be able to catalyze this reaction (Nilsson & von Heijne, 1993).

In our work, we have taken advantage of the glycosylation occurring exclusively inside the ER lumen to determine the membrane topology of the NS4B protein (Fig 15). The NS4B is an integral membrane protein located to the ER or the membranes derived from the ER (Egger *et al.*, 2002, Gretton *et al.*, 2005, Hugle *et al.*, 2001, Lundin *et al.*, 2003). By introducing the glycosylation site Asn-Ser-Thr (NST) to strategic locations of the protein, we could determine the membrane orientation of that particular site, depending on the outcome of the glycosylation results. The glycosylation of the NST site increasing the protein by 2 kDa would indicate luminal location of that site. However if the glycosylation outcome was positive, true glycosylation had to be verified, as false positives could arise due to ribosomal slippage, alternative translation start sites, etc.

Verifying true glycosylation *in vitro*

For the *in vitro* glycosylation studies, we used a competitive glycosylation acceptor peptide (benzoyl-Asn-Leu-Thr-methylamide) (see the above section “*In vitro* transcription and *in vitro* translation”). If it was true glycosylation, the glycosylation of the protein could be inhibited by adding the peptide (Fig 16A).

Verifying true glycosylation of proteins expressed in cells

For the glycosylation studies of proteins expressed in cells, the proteins were treated with PNGase F. PNGase F is an amidase that cleaves between the innermost GlcNAc and the Asn residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins (Fig 16B). It hydrolyzes almost all types of N-glycan chains from peptides or proteins. The NST sites introduced to different NS4B variants results in the N-linked glycosylation of the Asn. Accordingly this enzyme was used to verify true glycosylation of the NS4B proteins expressed in cells (Maley *et al.*, 1989).

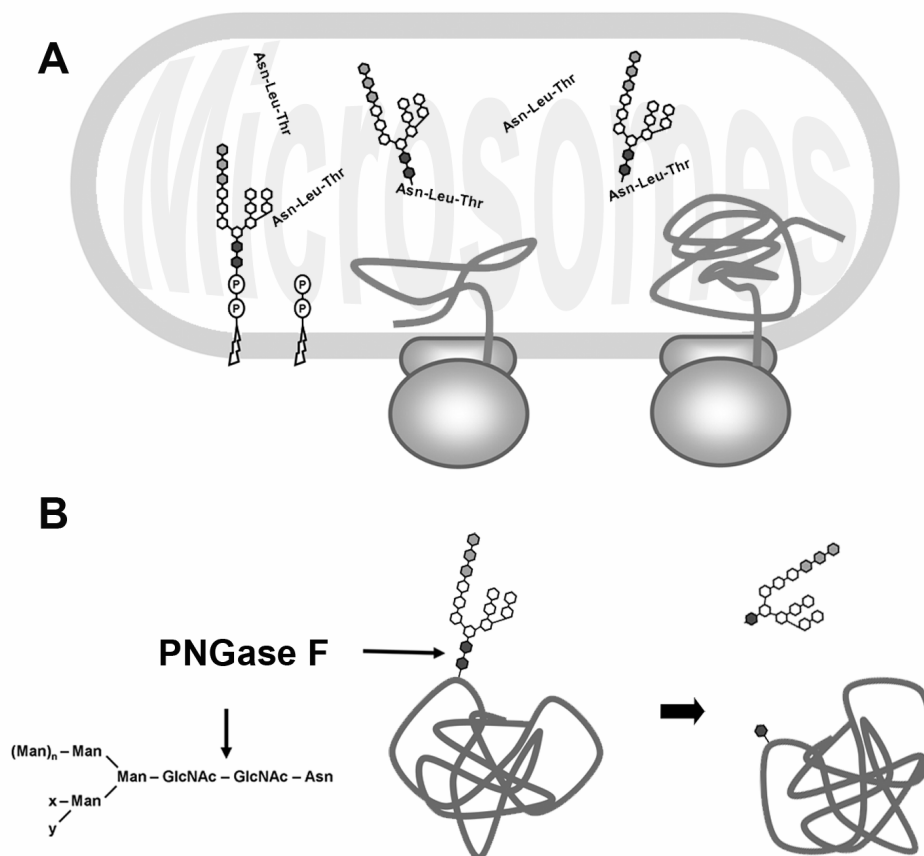


Figure 16. Glycosylation verification *in vitro* versus *in vivo*. A. In *in vitro* glycosylation verification, peptides that compete for the glycosylation is added to the translation reactions. This way they hinder the translated proteins from getting glycosylated. B. In *in vivo* glycosylation, the oligosaccharide moiety is removed by the treatment with PNGase F.

Briefly the cell lysates were divided into equal aliquots and denatured; one of the aliquots was then treated with PNGase F according to the manufacturer's recommendation. Both aliquots were analyzed in parallel by Western blotting to verify true glycosylation.

Immunoprecipitation studies

Immunoprecipitation is a commonly used tool for studying protein-protein interactions. There are many ways of initiating the immunoprecipitation assays. For instance there are various different ways of producing the proteins; e.g. by *in vitro* translation, by *E coli* expression, by expression in eukaryotic cells, etc. The process of letting the interaction occur between the two proteins to be studied may also vary; the proteins may simply be produced together for interaction, be produced by separate means then mixed together for interaction, and so on.

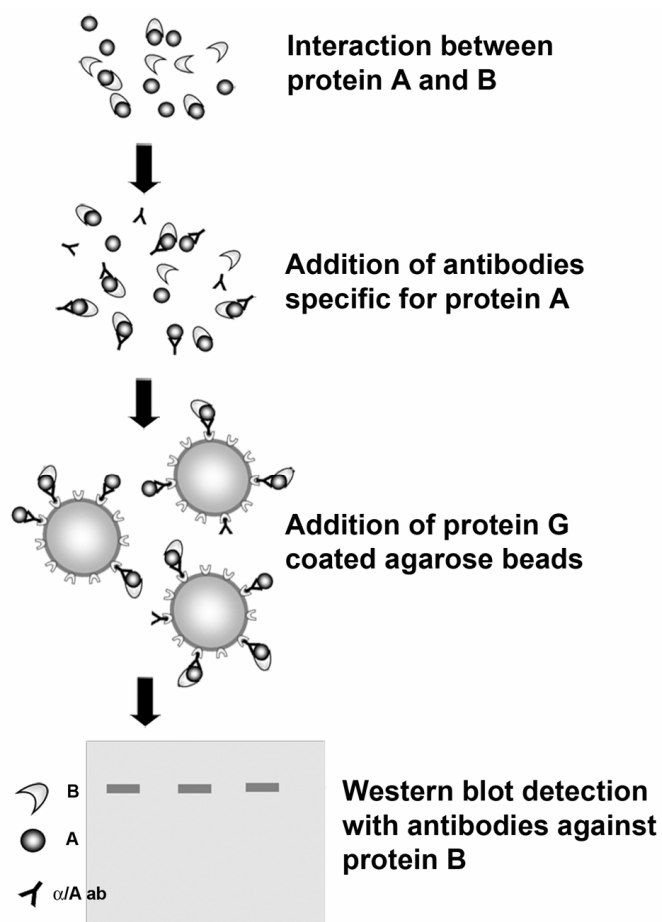


Figure 17. Schematic cartoon of immunoprecipitation

However the proteins are produced and allowed to interact with each other, they all arrive at the immunoprecipitation step with antibodies specific for one of the interacting proteins, ensued by the detection of the other protein, i.e. the co-precipitated protein, by Western-blotting (Fig 17). This way, interaction may be determined.

In our work we chose the co-expression method, where two constructs, each carrying one protein, were co-expressed in Cos7 cells. Differentially-tagged NS4B and NS4B mutants were used to study NS4B homo-oligomerization capacities (Paper III). The wild-type NS4B of the HCV genotype 1a was tagged at the N-terminus with Xpress (Invitrogen) and co-expressed with different variants of NS4B

(wild-type of genotype 1a and 1b, mutants with perturbed putative AH, various deletion mutants, etc) tagged with EGFP at the C-terminus. The cell lysates were first incubated with Anti-Xpress antibodies and then pulled down using Protein G PLUS-agarose suspension (Santa Cruz Biotechnology). The cell lysate from prior to the immunoprecipitation, the unbound proteins left after the removal of the agarose beads and the bound proteins, and the agarose beads with the bound proteins were all analyzed by Western blotting. There, the co-precipitated proteins were detected with anti-GFP antibodies and HRP conjugated secondary antibodies using the ECL Western blotting Analysis system (GE Healthcare).

Crosslinking proteins in intact cells

A way of stabilizing protein-protein interaction for the immunoprecipitation studies is by using crosslinkers (Wong & Wong, 1992). Crosslinkers strengthen the interaction between two proteins by covalent binding. They are often small bidirectional molecules with reactive groups at each end. The choice of the length between the reactive groups may vary depending on the interaction to be studied, the general rule of thumb being “the shorter the distance the closer the interaction”. The reactive groups at each end binds to various regions of the protein polypeptide chain, such as the primary amine groups (-NH₂), carboxyl (-CO₂H), sulphydryl (-SH), or hydroxyl groups (-OH). The crosslinker may be homobifunctional, i.e. both ends of the cross linker reacts with the same group, or heterobifunctional, i.e. each end reacts with different groups. By choosing either reversible or irreversible crosslinkers, linkage may be made either permanent or reversible. In reversible linkers, the arms of the molecule are held together by a disulphide bridge thus the crosslinking may be reversed by a simple reduction. Additionally depending on what proteins to crosslink, crosslinkers that may or may not permeate membranes may be chosen.

Disuccinimidyl suberate (DSS)

In our work, the irreversible crosslinker disuccinimidyl suberate (DSS) (Pierce) was chosen for the crosslinking of NS4B in live cells (Fig 18). DSS was chosen on the account of having a relatively short spacer arm length of 11.4 Å and reacting with primary amine groups, the most common in proteins compared to the

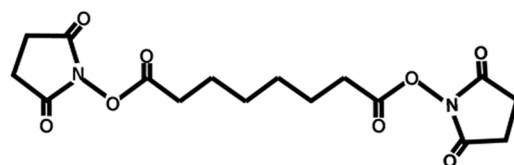


Figure 18. Disuccinimidyl suberate (DSS), (Pierce)

other groups. Moreover this crosslinker is membrane permeable, making it ideal for crosslinking membrane proteins such as the NS4B, while keeping the cellular membranes intact (Paper III). Since it is lipophilic, it is perfect for the intramembrane conjugations likely for NS4B proteins.

Briefly, 1 mM DSS solution was added to cells for 20 minutes at room temperature and quenched by adding Tris-HCl to a final concentration of 10 mM. The cells were then harvested and the lysates were used in ensuing immunoprecipitation assays.

Immunofluorescence studies

For the visualization of the membrane altering ability of various variants of NS4B, the proteins were fused to EGFP at the C-terminus and expressed in Cos7 cells (Paper II) and Huh7 cells (Papers II to IV). Since previous studies have shown that the EGFP alone do not localize to the ER nor induce membrane changes, it made a good tool for easy visualization of the NS4B proteins (Elazar *et al.*, 2004, Lundin *et al.*, 2003, Zheng *et al.*, 2005b).

As prolonged NS4B expressions resulting in higher NS4B concentrations were shown toxic for the cells (Fig 7), the cells were usually fixed after 18-24 hours and only in certain cases 48 hours post-transfection. The Huh7 cells expressing different variants of NS4B fused to EGFP were fixed for 10 minutes in 4 % formaldehyde prior to further treatments. The nuclei were stained for 3 minutes in 4',6-diamidino-2-phenylindole (DAPI) (Sigma) for easy detection (Papers II to IV). The lipid droplets were visualized by staining for 5 minutes in 0.6 % 1-([4-(Xylylazo)xylyl]azo)-2-naphthol (Oil Red O, Sigma) in 60 % isopropanol (Paper III). The cells were mounted with MOWIOL onto glass slides and analyzed by fluorescence microscopy and photographed using a Leica DFC300 FX camera and analyzed using Leica QWin V3 Software (Leica Microsystems Imaging Solutions Ltd).

Transfection and selection of HCV subgenomic replicons

In order to study the role of the NS4B in the HCV replication, the HCV subgenomic replicon was used (Paper IV). Various mutations were introduced to strategic locations of the NS4B in the subgenomic replicon, and the effects were evaluated by comparing the replicon establishment efficiencies of the different mutants. At the time we initiated our studies, the only *in vitro* HCV system available was the subgenomic replicon system. Of course the most recent

systems in which infectious virions may be produced may provide more accurate data concerning the NS4B's effects on the viral life cycle (Bartenschlager & Pietschmann, 2005, Lindenbach *et al.*, 2005, Murakami *et al.*, 2006, Wakita *et al.*, 2005, Yi *et al.*, 2006). However, microarray data has shown the subgenomic replicon system to have remarkable similarities with the viral replication *in vivo* (Bigger *et al.*, 2001, Su *et al.*, 2002, Zhu *et al.*, 2003a). Moreover it is a safe and easy system to handle.

For easy manipulation of the replicons, a DNA construct carrying the replicon coding sequence is used. This is in turn used for the *in vitro* transcription of the replicon RNA. The replicon RNA is then purified from the contaminating DNA and transfected into Huh7 cells by electroporation. Once inside the cell, the replicon RNA is directly used

HCV subgenomic replicon S2204I

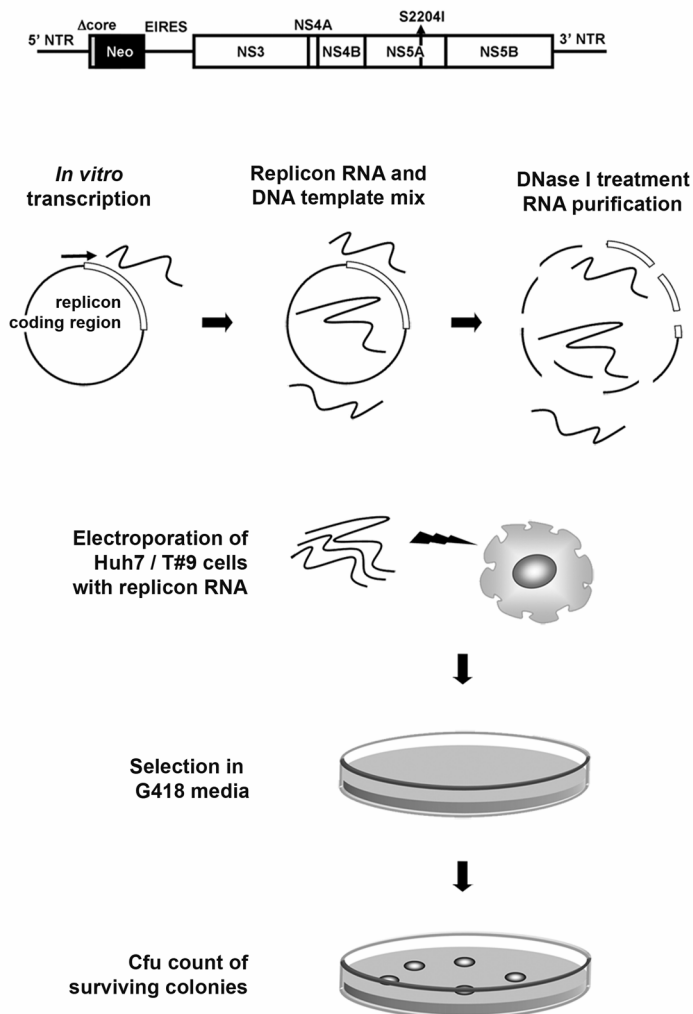


Figure 19. Establishing HCV subgenomic replicon in Huh7 or T#9 cells.

as template for the translation of the Neo and the NS3-5B proteins. The NS3-5B form the replication complex for the continued production of progeny replicon RNA. The Neo permits the cells' survival in G418 selection media only if on-going replication of the replicon RNA is present. Since no DNA is present, resistance due to selective integration of Neo gene into the host cell genome is not possible (Fig 19).

The plasmid vectors carrying the different replicon mutants, the original replicon S2204I, and the polymerase deficient replicon pol- were used as templates in *in vitro* transcriptions and DNaseI treated as described by Blight and colleagues (Blight *et al.*, 2000). The resulting RNA was purified to eliminate the remaining contaminating DNA debris using RNeasy® MinElute™ Cleanup Kit (Qiagen). One or 4 µg RNA and one mock sample with H₂O only were used to transfect $\sim 3 \times 10^5$ to 2×10^6 Huh7 or T#9 cells by electroporation. For estimation of replication establishment efficiency, the cells were subjected to selection media containing 1 mg/ml G418 (Invitrogen) 24-48 hours post-transfection and changed every 2–3 days. After 10-16 days the cells were fixed and stained with crystal violet (Sigma-Aldrich) and the resulting colonies were counted. For the replicon establishment efficiency comparisons, the colony forming unit (cfu) per µg RNA of the replicon mutants were normalized against that of S2204I and pol- set at 100 and 0 %, respectively. The given values are in percentage of colony calculations displayed as mean \pm standard error of mean. These results were based on calculations of over 10 transfection rounds.

RESULTS

The hepatitis C virus life cycle can be summarized in the following steps; viral entry into the host cell, translation of the viral RNA genome, processing of the viral proteins, the replication complex formation and MAF induction, replication of the viral genome, virion assembly, and exit from the host cell. Unfortunately much of these steps in the viral life cycle are shrouded in obscurities. This thesis attempts to add its share to the vast puzzle of HCV infection, focusing on two aspects of the viral life cycle; the translation and the replication of the HCV. Accordingly, the following results will be presented in the order befitting the HCV life cycle, starting with the study of viral IRES mediated translation followed by the study of the viral replication with focus on the NS4B protein.

The IRES-mediated translation and its correlations with antiviral treatment (Paper I)

In the first part of this thesis, the differences in the IRES elements derived from patients infected with the HCV genotype 3 and their impacts on translation was scrutinized *in vitro* cell culture systems (Paper I). As seen in patient studies, the outcome of the combination therapy of IFN- α and RBV may differ greatly depending on the infecting HCV genotype (Hadziyannis *et al.*, 2004). The molecular mechanisms behind these differences are yet to be defined. Previous studies have tried to correlate sequence variability of the IRES with IFN therapy outcome with conflicting results (Collier *et al.*, 1998, Saiz *et al.*, 1999, Yamamoto *et al.*, 1997).

A large number of patients chronically infected with the HCV genotype 3 attending the Aga Khan University Hospital clinics in Karachi were chosen for this trial. These patients were chosen on the basis of the demographic features of the sustain responder (SR) and non-responder (NR) groups being comparable; no difference was observed in age spread, gender, pretreatment serum ALT levels, and Histological Activity Index on liver biopsies. Two hundred

patient serum samples were collected and genotyped, revealing 72 % of the patients to be infected with the genotype 3. These patients were subjected to six months treatment with IFN- α and Ribavirin (RBV) and followed up for up to one year. The genotype 3 infected patients were then grouped into sustained responders (SR, 70 %) and non-responders (NR, 30 %), based on the absence/presence of HCV RNA in serum and normal/elevated ALT levels one year after the completed treatment.

Sequencing of the HCV isolated from the SR and NR groups revealed intriguing sequence differences in the 5'NTR between the two groups. Sequence alignment of the IRES regions of the SR and NR groups with a previously published genotype 3a isolate (#D17763) showed that most IRES of the SR group exhibited greater variations in the IRES sequence than the NR group (Sakamoto *et al.*, 1994). In the SR group insertions were seen mainly in stemloop I, and

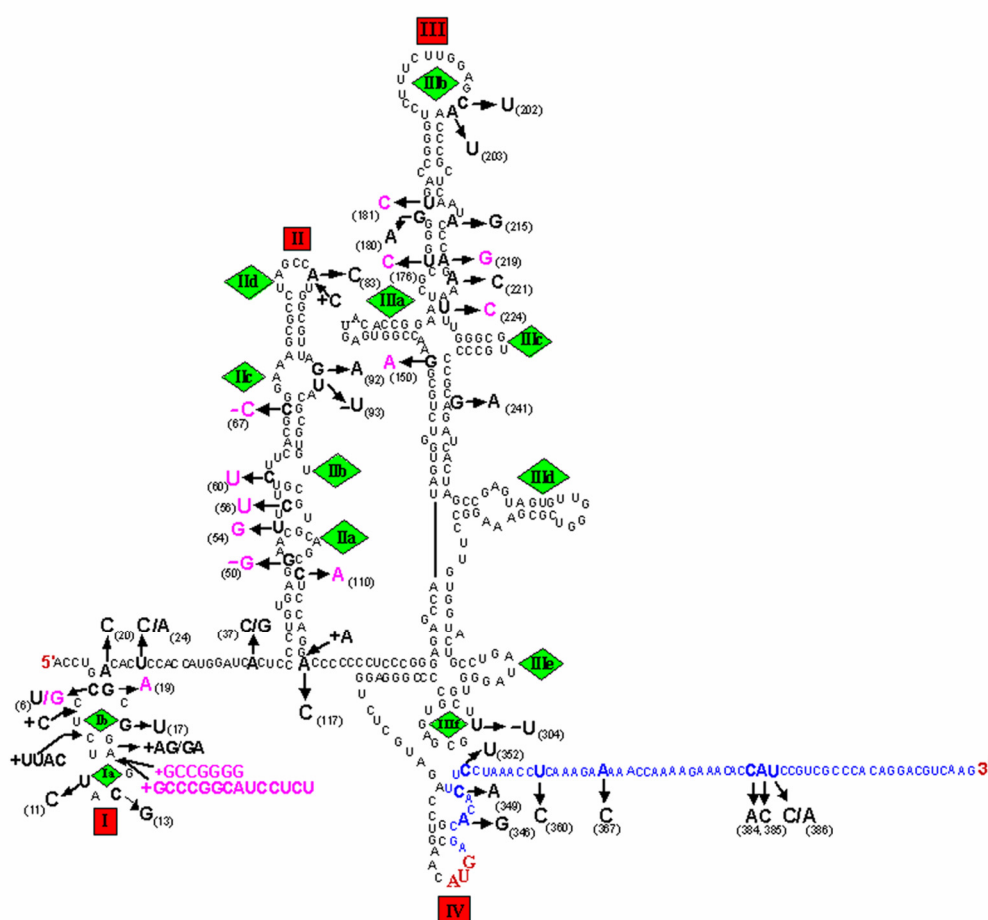


Figure 20. Differences in the IRES sequences of the SR group compared to a reference IRES sequence of the HCV genotype 3a (D17763) (Paper I). The arrows show the mutations. Bold letters– substitutions. Plus sign (+) – insertions. Minus sign (-) – deletions. Pink letters - putative change in the secondary structure. Red letters – AUG start codon. Blue letters - core coding sequence.

deletions in stemloop II, while substitutions were scattered all three loops (Fig 20). The IRES of the NR group displayed fewer differences than the SR sequences, located mainly to the stemloops I and III, of which most substitutions were concentrated to the stemloop III (Fig 21).

To study this further, 15 SR and 7 NR patients were chosen at random. The IRES nucleotide sequences 1-407 derived from the HCV in the pre-treatment samples of these patients were cloned into a bi-cistronic dual luciferase vector and the IRES mediated translation efficiencies were determined in a dual luciferase assay in HepG2, and Hep3B cell lines. Remarkably, of the different isolates, the IRES of the SR group showed significantly less efficient translation than those of the NR group ($29.7 \pm 13 \%$ and $69.4 \pm 22 \%$, respectively $p < 0.01$, Mann-Whitney U-test).

This prompted us to also examine the IRES from the SR and NR groups' responses to antiviral treatment with IFN- α and RBV *in vitro*. Seven IRES constructs from each group were

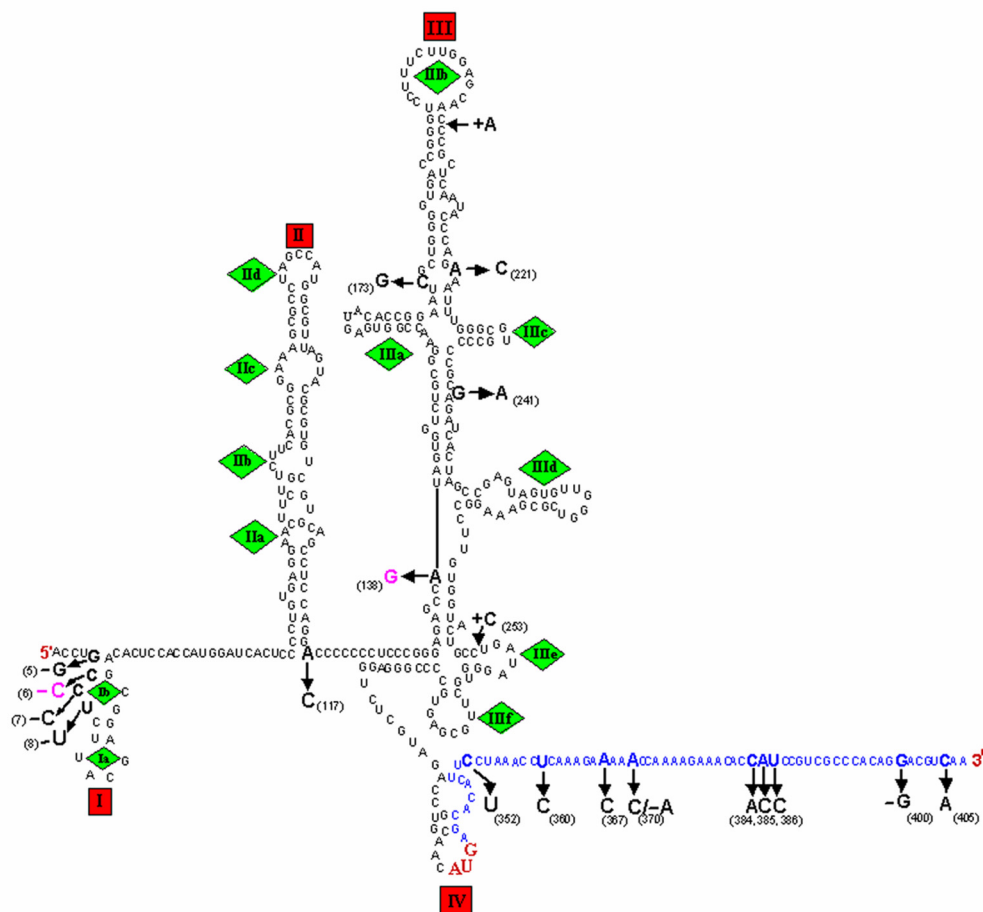


Figure 21. Differences in the IRES sequences of the NR group compared to a reference IRES sequence of the HCV genotype 3a (D17763) (Paper I). Likewise symbols have been used here as in Fig 20.

transfected into cells and treated with IFN- α and RBV (500 or 1000 IU/ml IFN- α and 25 μ M RBV) prior to examining the translation efficiency. Here, the translation efficiency of the IRES from the SR isolates showed greater reduction than those of the NR isolates upon antiviral treatment (67-68 % and 86-88 % of untreated cells for SR and NR, respectively, $P > 0.05$). Though the results were not statistically significant, indications were that even antiviral therapy *in vitro* may potentially be correlated to the *in vivo* results.

In this study significant correlations between the IRES translation efficiency and HCV genotype 3 treatment outcomes could be seen. The IRES elements are highly dependent on their tertiary structure for proper function. Disruption of this structure may result in less efficient recruitment of cellular factors necessary for translation (Collier *et al.*, 1998, Honda *et al.*, 1999, Kamoshita *et al.*, 1997, Lerat *et al.*, 2000, Pestova *et al.*, 1998, Saiz *et al.*, 1999). Therefore SR derived IRES having lower all-over translation activity than those of the NR group may be a result of a more disrupted IRES tertiary structure. As observed, the increased mismatch in the stemloop forming sequences of the SR derived IRES did not seem to have any complementary mutations to compensate for the mismatch.

The IFN- α and RBV treatment had greater impact on reducing translation efficiency of the SR derived IRES than those derived from the NR group. Once again the more disrupted tertiary structures of the IRES derived from the SR group may have made them more vulnerable to the adverse effects of the antiviral treatment than those of the NR group.

In conclusion, this study indicates that IRES mediated translation efficiency may be an important factor in the viral treatment outcome, albeit the *in vitro* assays need further optimizing, perhaps using even larger numbers of SR and NR derived IRES sequences.

Study of NS4B and its role in HCV replication

Continued elucidation of the topology of the NS4B (Paper II)

In this work we continued our study of the NS4B N-terminal translocation into the lumen *in vivo*. In the previous work by our group (Lundin *et al.*, 2003), the NS4B of the genotype 1a was suggested to initially have 4 transmembrane domains (TM1, TM2, TM3, and TM4) with both

N- and C-termini in the cytoplasm. However soon after the translation, the N-terminus was shown to translocate into the lumen, resulting in the fifth transmembrane domain (TMX) (Fig 22). This gave rise to the 5 transmembrane domain model of the NS4B protein, with the N-terminus in the lumen and the C-terminus in the cytoplasm. However this work was preliminary in the sense that the NS4B had been expressed alone and studied by *in vitro* translation only. In Paper II, the translocation of the N-terminus was studied by expressing the NS4B in cells, both by itself and in the context of the viral polyprotein. Furthermore the NS4B of all genotypes were studied.

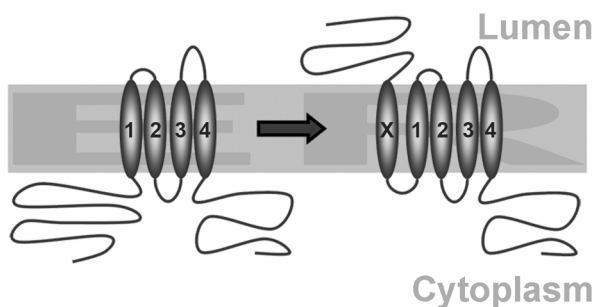


Figure 22. Dual topology of the NS4B. The putative transmembrane domains TMX, TM1, TM2, TM3, and TM4 are shown.

Firstly the amino acid residue positions 9-11 of the NS4B N-terminus was changed to the glycosylation sequence Asn-Ser-Thr (NST) in the NS4B-EGFP fusion protein and studied by fluorescence microscopy. This was to ensure that the introduced glycosylation motif did not affect the only known function of the NS4B; its membrane rearranging capacities (Egger *et al.*, 2002, Gretton *et al.*, 2005, Lundin *et al.*, 2003). Subsequently the same glycosylation site was introduced to the NS4B in the context of the NS3-4B, NS3-5A, and NS3-5B and tested for translocation. The glycosylated protein (NS4B band with 2 kDa shift) would indicate N-terminal translocation.

The results showed that a substantial portion of the NS4B N-terminal was indeed glycosylated, indicating lumenal location also when expressed in cells. Intriguingly the NS4B in the context of the NS3-5A and the NS3-5B had less efficient N-terminal translocation than that of the NS3-4B expression (Fig 23A). Quantification data showed that in NS3-4B, the NS4B population with lumenally located N-terminus was 61 % compared to 17 % and 16 % (mean values) for NS3-5A and NS3-5B, respectively (Fig 23B).

Next the N-terminal translocation of all the HCV genotypes was tested. The glycosylation site NST was introduced to the N-termini of the NS4Bs of the genotypes 1b, 2b, 3a, 4a, 5a and 6a

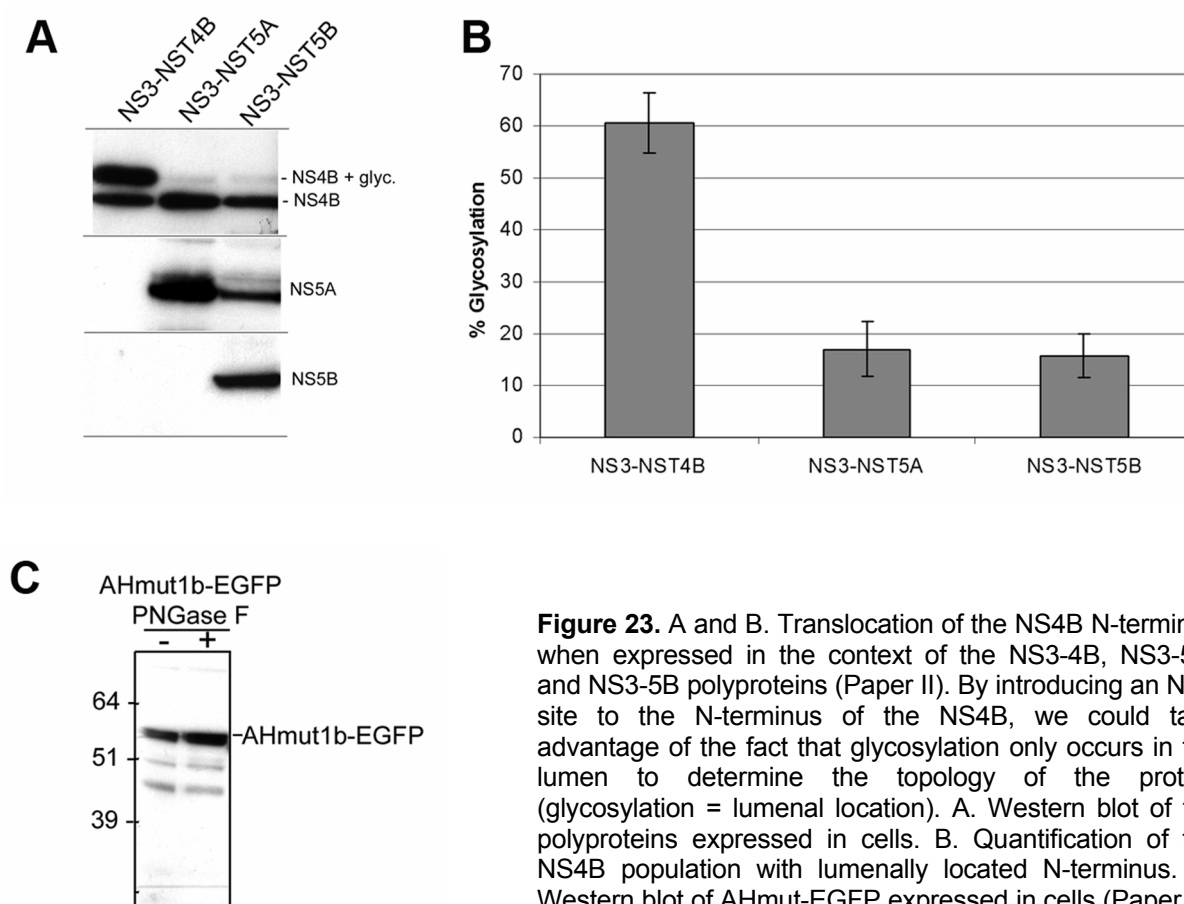


Figure 23. A and B. Translocation of the NS4B N-terminus when expressed in the context of the NS3-4B, NS3-5A, and NS3-5B polyproteins (Paper II). By introducing an NST site to the N-terminus of the NS4B, we could take advantage of the fact that glycosylation only occurs in the lumen to determine the topology of the protein (glycosylation = luminal location). A. Western blot of the polyproteins expressed in cells. B. Quantification of the NS4B population with lumenally located N-terminus. C. Western blot of AHmut-EGFP expressed in cells (Paper II). This AHmut also contains an NST site at the N-terminus but fails to become glycosylated.

for translation studies *in vitro*. In the presence of microsomes, all genotypes showed N-terminal glycosylation of a substantial population, comparable to that of the HCV 1a genotype used in previous studies. Hence the N-terminal translocation occurs indeed in all genotypes.

Recently Elazar and colleagues reported of an amphipatic helix (AH) located to the amino acid residues 6-29 of the NS4B (Elazar *et al.*, 2004). Perturbing this AH in the NS4B abolished MAF inductions. Furthermore introducing the same mutations in the subgenomic replicon resulted in the abolishment of replication as well. Due to the N-terminal location of the perturbing mutations, N-terminal translocation capacity of the same AH mutant was studied in Paper II by introducing the NST site to its N-terminus. Interestingly this AH mutant was not able to translocate its N-terminus into the ER lumen when expressed in cells, suggesting a correlation between the translocation and membrane rearrangements (Fig 23C).

As shown in this study, a substantial portion of the N-terminal domain translocated into the lumen even when NS4B was expressed in cells, either by itself or in the context of NS3-4B

polyprotein. Although when the NS4B was expressed in the context of NS3-5A or NS3-5B polyproteins, less efficient translocation was observed. We conclude that the NS5A may have a negative regulatory effect on the NS4B translocation; i.e. keeping the N-terminus in the cytoplasm. However it should be kept in mind that over 25 % of the NS4B population with the N-terminus in the lumen still remained despite the presence of the NS5A (61 % for NS3-4B compared to 17 % for NS3-5A and 16 % for NS3-5B). This suggests that a substantial population of the NS4B even in the context of the viral replication may have its N-terminus located to the lumen.

The NS4B of all HCV genotypes showed the capacity for N-terminal translocation, indicating that the dual location is a conserved function of the N-terminus. Such a conserved feature usually indicates an important function. This is further supported by the fact that the mutations perturbing the AH that rendered the NS4B unable to translocate its N-terminus, also disabled MAF induction as well as rendering the subgenomic replicon unable to replicate (Elazar *et al.*, 2004).

In conclusion the translocation of the NS4B N-terminus into the lumen is a conserved feature that may be negatively influenced by the NS5A. In addition, translocation and MAF induction may be interlinked.

Correlation between N-terminal translocation, oligomerization and membrane rearrangements of the NS4B (Paper III)

The most well known function of the NS4B to date is the capacity to induce membrane rearrangements, resulting in the membrane associated foci where viral replication is thought to take place (Egger *et al.*, 2002, El-Hage & Luo, 2003, Gosert *et al.*, 2003, Lundin *et al.*, 2003, Shi *et al.*, 2003, Waris *et al.*, 2004). A likely way of achieving membrane rearrangements is by protein-protein interactions. Though previous studies have shown the NS4B to interact with several other NS proteins such as the NS2, NS3, NS4A, and NS5A (Dimitrova *et al.*, 2003, Flajolet *et al.*, 2000, Lin *et al.*, 1997), only weak interaction with itself had been observed at the time when we started our investigations (Dimitrova *et al.*, 2003). As other NS proteins such as the NS2, NS3, NS5A, and NS5B have shown to function in oligomeric forms, the NS4B also operating in an oligomeric form was not unlikely (Levin & Patel, 1999, Lorenz *et al.*, 2006, Tellinghuisen *et al.*, 2005, Wang *et al.*, 2002). Another interesting aspect is that the NS4B

mutant that lacked the ability to induce MAF was also unable to translocate its N-terminus into the lumen (Paper II). In Paper III the role of oligomerization and N-terminal translocation in the context of MAF induction was studied.

To assess oligomerization, differentially tagged NS4B of the HCV genotype 1a was used; one with the Xpress tag in the N-terminus and the other with the EGFP protein fused to its C-terminus. The Xpress tag was used in the pull-down and the co-precipitated protein was analyzed by detection with anti-GFP antibodies in a Western blot. This way homo-oligomerization of the NS4B proteins could be examined (Fig 24). Investigations of NS4B with a glycosylation site introduced to its N-terminus (NST4B-EGFP) revealed that NS4B of both topologies were involved in the interaction. In addition treatment with an irreversible crosslinker DSS prior to the immunoprecipitation revealed that NS4B may exist even as trimers and possibly even larger complexes, although the majority was found in the monomeric form. Comparing NS4B treated with DTT to untreated NS4B revealed that the interaction may be via disulphide bridges.

Co-precipitation studies between the Xpress-tagged wild type NS4B and N- or C-terminal deletion mutants expressed in fusion with the EGFP (NS4B(92-261)-EGFP and NS4B(1-191)-EGFP, respectively) revealed that both termini were required for the interaction (Fig 24). Interestingly, neither of the deletion mutants was able to induce the stereotypical MAFs seen with the wild type NS4B (Fig 24).

Having established that the NS4B is indeed capable of homo-oligomerization, the correlation between the oligomerization and the N-terminal translocation was studied. However as a starting point, the identity of the TMX, the transmembrane domain formed after the translocation (Fig 22), had to be established. NST sites were introduced after every three amino acid residues of the N-terminus and tested first *in vitro* for approximation, then by cellular expression for the determination of the N-terminal end of the TMX. Based on these results in conjunction with our computer predictions, we propose the TMX to be approximately located to the amino acid residues 60-84 of the NS4B.

As shown in Paper II, a NS4B mutant with perturbed AH located to the amino acid residues 6-29 (AHmut) could not translocate its N-terminus into the lumen. In Paper III the very same mutant was tested for oligomerization against the wild type NS4B. In addition AHmut and wild

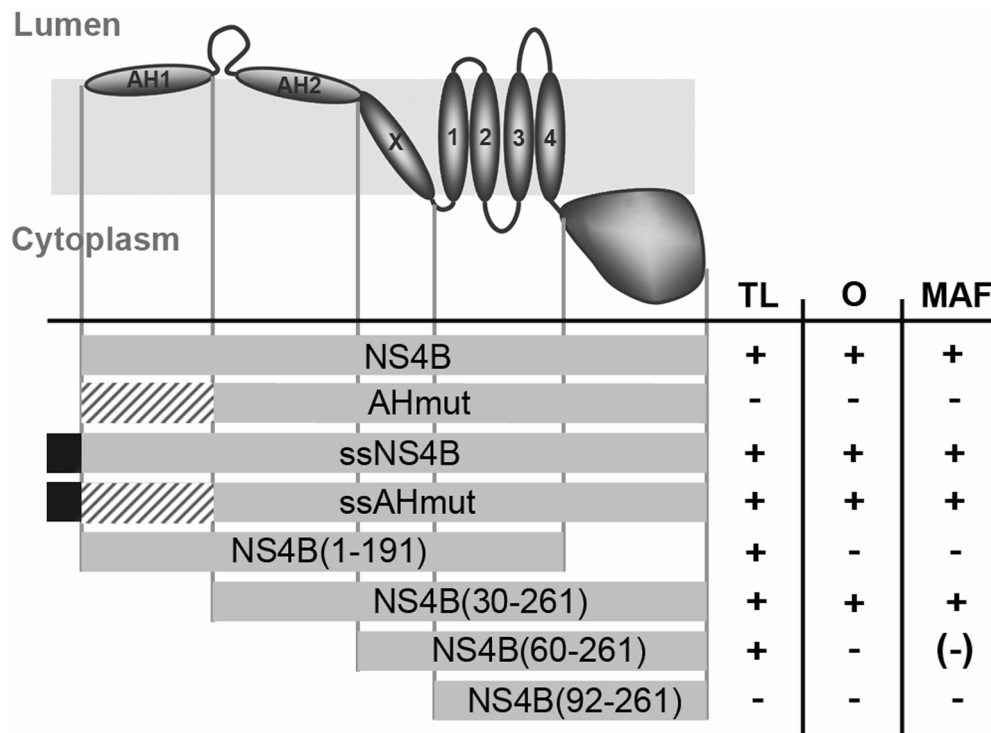


Figure 24. Summary of the translocation (TL), oligomerization (O), and MAF induction (MAF) results of the NS4B variants used in the Paper III. Above the list is a schematic cartoon of the different regions of the NS4B that were included in the assays. Striped domain – mutated region. Black domain – signal sequence.

type NS4B variants with an export signal sequence added to their N-termini were also tested in parallel (Kabat *et al.*, 1991). The signal sequence causes co-translational translocation of the N-termini of the entire NS4B or AHmut population and is cleaved off after entering the lumen.

The AH mutant was not able to interact with the wild type NS4B. However forcing the N-terminus into the lumen, as in the ssAHmut, restored the AHmut's oligomerization capacities (Fig 24). In addition, immunofluorescence studies with the signal sequence mutants revealed that not only did the oligomerization capacities return, but also the MAF induction. Together, these data strongly indicated a correlation between N-terminal translocation, oligomerization, and MAF induction.

Interestingly the immunofluorescence studies with the signal sequence protein variants also revealed that the survival rate of the ssNS4B was much lower than that of the ssAHmut or the wild type NS4B. Topology verification of the signal sequence protein variants revealed that although all of ssNS4B were correctly folded with their N-termini in the lumen, almost half of the ssAHmut population was most likely malformed. Possibly the large population of malformed

ssAHmut alleviated the would-be detrimental effect of the entire population having lumenally positioned N-terminus. Furthermore previous translocation studies have shown that only a small population of the wild type NS4B translocates its N-termini into the lumen due to negative influence by the NS5A (Paper II). Together these data indicate an advantage for not having too much of the NS4B population with lumenally located N-terminus.

In order to map what parts of the N-terminus that may be involved in the above three functions, the N-terminus was divided into three domains; AH1 in the first 30 amino acid residues of the protein (Elazar *et al.*, 2004); AH2, a second putative AH recently proposed to the amino acid residues 40-69 (Castet *et al.*, 2004); and the TMX, proposed to the amino acid residues 60-84 (Fig 24). Three deletion mutants were accordingly tested for oligomerization, translocation, and MAF induction; NS4B(30-261) lacking AH1; NS4B(60-261), lacking both AH1 and AH2; and NS4B(92-261) lacking AH1, AH2, and TMX as negative control for the above three functions. Interestingly the NS4B(30-261) was shown to translocate its N-terminus, oligomerize to the wild type NS4B, and induce MAFs, showing that the first AH was not necessary for these functions (Fig 24). In contrast the NS4B(60-261) revealed that despite the N-terminus translocated into the lumen, the protein could not oligomerize and lost most of the MAF inducing capacity (Fig 24). Together these results indicate that the AH2 may be involved in both oligomerization and MAF induction.

In Paper III, we showed that the NS4B formed homo-oligomers when expressed in cells. While this manuscript was being written, Yu and colleagues reported of homo-oligomerization of the NS4B via palmitoylation of the C-terminus (Yu *et al.*, 2006). However considering *i)* the presence of three C-terminal Cysteins, among which one is fully conserved, *ii)* the close vicinity of these to the ER membrane, and *iii)* the reducibility when treated with DTT, we propose an alternative explanation; that the oligomerization via the C-terminus is due to disulphide bridge/s rather than due to palmitoylation. Albeit disulphide bridge formations are rare in the cytosol, it does occur, especially in very close vicinity of the ER membrane (Schouten *et al.*, 2002).

The domain responsible for both the oligomerization and the MAF induction is most likely the AH2. This notion is supported by the NS4B(30-261) mutant lacking the AH1 still being able to translocate, oligomerize, and induce MAFs while the NS4B(60-261) mutant lacking both AH1 and AH2 not being able to oligomerize or induce MAFs (Fig 24). What is more, it seems that the AH2 should be located on the luminal side of the ER membrane for the MAF induction to

occur. This is supported by the fact that mutations in the AH1 (AHmut) obstructing all three events could be reversed by forcing the defective AH1 into the lumen by a signal sequence (Fig 24). Perhaps the mutated AH1 on the cytoplasmic side disturbs the conformation/function of the AH2 and/or the TMX, thus inhibiting oligomerization, translocation, and MAF induction. Yet by forcing luminal location with a signal sequence, the AH2 and TMX may achieve correct conformation as a result of TMX integration into the membrane, thus returning the three events.

However translocation may not necessarily be correlated with the oligomerization. The immunoprecipitation study with NST4B-EGFP showed that the majority of the precipitated NS4B population had the N-terminus cytoplasmically located (see Paper III Fig 2c, lanes 3 and 4). This shows that interaction of the N-terminus is most likely between NS4Bs with their N-termini on the same side of the ER membrane or that the location of the interacting N-termini does not matter. Conversely it is unlikely between NS4Bs where one has cytoplasmically located and the other lumenally located N-terminus. In the latter case co-precipitation studies should have yielded equal populations of glycosylated and unglycosylated NS4Bs, which was not the case. However oligomerization may indeed be correlated to MAF induction; as seen with the C-terminus deletion mutant (NS4B(1-191)), it could neither oligomerize nor induce MAFs, despite being able to translocate its N-terminus (see Paper III, Fig3D, lanes 1 and 2).

In conclusion we propose the following; the translocation of the N-terminus may be necessary on the luminal side to induce the membrane curvature and the oligomerization may provide the local high concentration of the NS4B that are necessary to form the MAFs. The dual topology of the NS4B may be a way to regulate the induction of MAFs as too efficient induction may be detrimental for the cells.

Effects of point-mutations in the NS4B of the HCV subgenomic replicon replication (Paper IV)

The NS4B has shown to be indispensable in the context of the viral replication in HCV subgenomic replicon as well as replicons and *in vitro* cultures of both pestiviruses and flaviviruses (Appel *et al.*, 2005, Grassmann *et al.*, 2001, Khromykh *et al.*, 2000, Li & McNally, 2001, Qu *et al.*, 2001, Tong & Malcolm, 2006). It has shown necessary in *cis* in the viral polyprotein in order to maintain the viral replication (Grassmann *et al.*, 2001, Khromykh *et al.*, 2000, Tong & Malcolm, 2006). Furthermore single mutations in the NS4B has shown to

mediate alterations in cytopathy of both the BVDV and West Nile virus, changes in cellular tropisms of both Dengue and HCV, as well as mediate IFN resistance in the *Flaviviridae* (Hanley et al., 2003, Lohmann et al., 2003, Lundin et al., 2003, Munoz-Jordan et al., 2003, Namba et al., 2004, Qu et al., 2001, Sumpter et al., 2004, Wicker et al., 2006, Zhu et al., 2003b). These effects exerted by the NS4B are presumably by protein-protein interaction, however the obsolete numbers of interacting cellular proteins reported in current literature is rather enigmatic. Hence in the final part of this thesis we studied the effects of different regions of the NS4B on the HCV replication by using the HCV subgenomic replicon (Blight *et al.*, 2000).

Fourteen point-mutations were separately introduced to the bi-cistronic subgenomic HCV 1b RNA replicon with the adaptive mutation S2204I at strategic positions based on a previous topology model of the NS4B (Blight *et al.*, 2000, Lundin *et al.*, 2003) (Fig 25). The mutations were introduced to the regions at the N- and C-terminal ends and the loop regions between the transmembrane domains. The introduced mutations at the N- and C-terminus were chosen on the basis of being non-conserved and close to the N- and C-terminal ends without disrupting the P6, P4, P1, and P1' positions around the sites crucial for the NS4B processing from the polyprotein (Bartenschlager *et al.*, 1995, Kim *et al.*, 2000).

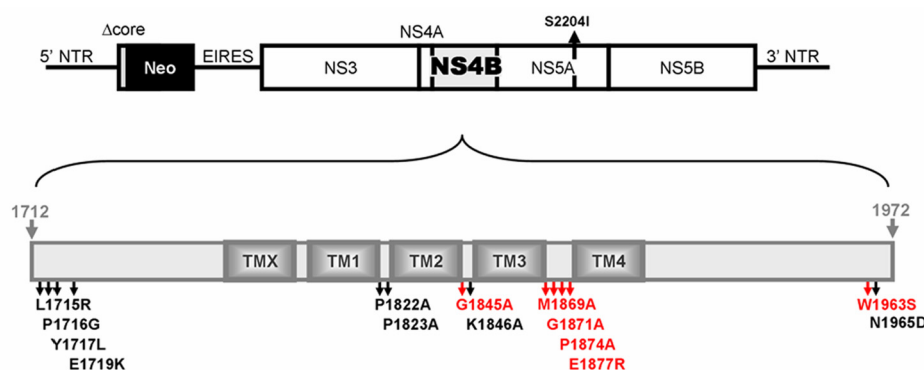


Figure 25. The 14 point mutations introduced to strategic regions of the NS4B in the subgenomic replicon (Paper IV). Red letters – changes of conserved amino acid residues.

The introduced mutations resulted in the changes of the following amino acid residues; L1715R, P1716G, Y1717L, and E1719K changes in the N-terminus of a highly variable region, and W1963S and N1965D in the C-terminus, of which the W1963S change was that of a conserved residue (Fig 25). The changes in the loop regions were; P1822A and P1823A in the lumenally located TM1-TM2 loop; G1845A and K1846A in the cytoplasmically located TM2-

TM3 loop; M1869A, G1871A, P1874A, and F1877R in the lumenally located TM3-TM4 loop (Fig 25). Of these, the changes G1845A of the TM2-TM3 loop and all the changes made in the TM3-TM4 loop were conserved throughout the HCV genotypes.

The replicons containing these mutations were introduced to the Huh7 and T#9 cells, and the impact of the mutations on the replication establishment efficiency was evaluated by comparing the efficacies of the mutants to those of the parental S2204I and the polymerase deficient pol-replicons (negative control). This was done by calculating the cfu/ μ g replicon RNA (used for the transfection) and presenting it as a percentage (including the standard error of mean) of those of S2204I and pol- replicons set at 100 % and 0 %, respectively.

Most of the introduced mutations gave varying degrees of negative effect on the replicon establishment (Fig 26). The ranking order of the mutants from the most efficient to the least in the N- and C-termini were E1719K (66.8 ± 18.9 %), P1716G (28.2 ± 10.1 %), and Y1717L (4.2 ± 1.8 %) all in the N-terminus, and N1965D (3.3 ± 2.1 %) in the C-terminus. Interestingly only the W1963S change of a conserved amino acid residue in the C-terminus gave total abolishment of replication (0.0 ± 0.1 %). What is more, the L1715R change of a non-conserved amino acid residue closest to the N-terminal end gave increased replicon establishment efficiency (207 ± 77.6 %).

S2204I \rightarrow 100 %

L1715R \rightarrow **$210 \pm 77\%$**

P1716G \rightarrow $28 \pm 10\%$

Y1717L \rightarrow $4.2 \pm 1.8\%$

E1719K \rightarrow $67 \pm 19\%$

P1822A \rightarrow $88 \pm 13\%$

P1823A \rightarrow $31 \pm 4.8\%$

G1845A \rightarrow $0.6 \pm 0.1\%$

K1846A \rightarrow $60 \pm 8.6\%$

M1869A \rightarrow $0.3 \pm 0.1\%$

G1871A \rightarrow $52 \pm 5.4\%$

P1874A \rightarrow $16 \pm 2.1\%$

E1877R \rightarrow $0.3 \pm 0.1\%$

W1963S \rightarrow **$0.0 \pm 0.1\%$**

N1965D \rightarrow $3.3 \pm 2.1\%$

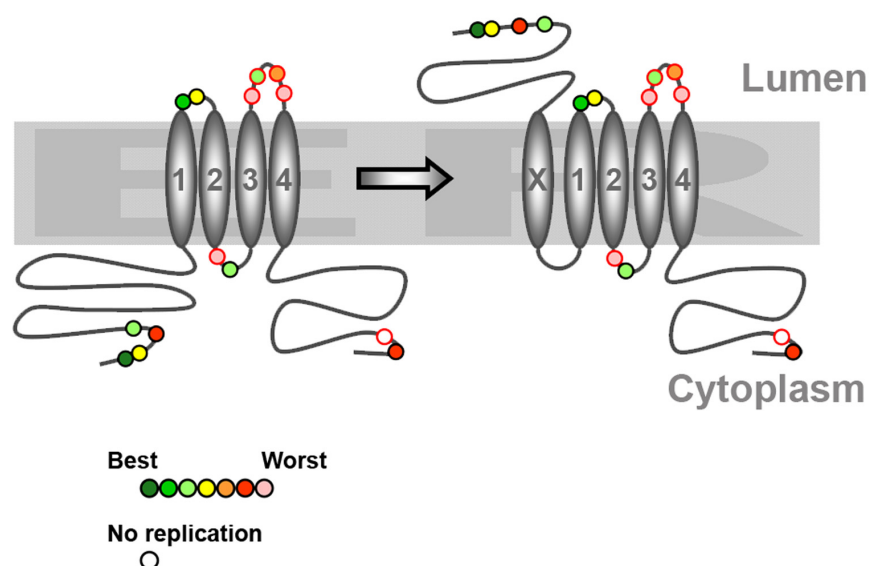


Figure 26. The replicon establishment efficiencies of the 14 replicon mutants (Paper IV). Red letters/circles – changes of conserved amino acid residues. The highest (L1715R) and the lowest (W1963S) replicon establishment efficiency values are shown in blue.

The following ranking is in the order most efficient to the least efficient of the inter-transmembrane loop mutations; P1822A (88.0 ± 12.5 %), K1846A (59.8 ± 8.6 %), G1871A (52.4 ± 5.4 %), P1823A (31.2 ± 4.8 %), P1874A (16.0 ± 2.1 %), G1845A (0.6 ± 0.1 %), and M1869A and E1877R at the bottom (0.3 ± 0.1 % for both). However none of the changes in the loop regions, even of the fully conserved amino acid residues, resulted in complete elimination of replicon establishment, such as seen with the W1963S of the C-terminus. Interestingly, the M1869A and E1877R changes of the conserved residues in the luminal loop between the TM3 and TM4 resulted in the near abolishment of the replicon establishment. This is especially remarkable in the light of that the replication complex presumably resides on the cytosolic side of the ER membrane (Wölk *et al.*, 2000).

Despite having introduced the N- and C-terminal mutations in sites thought not to affect the viral polyprotein processing, we still had to ensure that this was truly the case. Hence the processing of the NS4B in these mutants (in the sites between NS4A-4B and NS4B-5A) was studied *in vitro*. No differences could be observed in the processing of the NS4B derived from the different mutant replicons and the parental S2204I replicon.

Remarkably once established in cells, all replicon mutants gave similar variations in cell growth rate in selection medium, replicon RNA copy number, and amount of NS proteins produced irrespective of their initial establishment efficiency. This could be due to reversion or compensatory mutations occurring once the replicons manage to establish in cells. In order to see if such was the case, the NS4B regions of several of the least efficient replicon mutants extracted from single colony-derived cell lines were sequenced. These revealed that no such reversion or compensatory mutations had taken place, at least not within the NS4B region. Although in order to exclude compensatory mutations in other regions of the replicon, further sequencing is necessary.

As reported by Elazar and colleagues, mutations introduced to the N-terminus resulted in the dissolution of the MAF induction as well as replication of the subgenomic replicon (Elazar *et al.*, 2004). Hence the membrane rearranging capacities of all of the above mutants were tested. The NS4Bs of all the mutant replicons and the parental S2204I replicon were expressed in fusion with the EGFP and transfected into Huh7 cells for immunofluorescence studies. The NS4Bs from all of the above mutants could induce MAFs. This indicated that mechanisms other than the perturbation of the membrane altering ability were disrupted by the introduced mutants.

Additionally the intact MAF induction also suggests that the proper topology of these NS4B mutants were maintained.

Mutations in the NS4B were shown to have a strong impact on the replicon establishment in cell culture. Reduction of replicon establishment due to changes in the TM1-TM2 and TM2-TM3 loops may possibly be ascribed to minor perturbations in the structural integrity of the NS4B protein as these loops are predicted to be very tight turns (Lundin *et al.*, 2003). However the effects of the changes introduced to the N- and C-terminal ends are presumably due to alterations in protein-protein interactions. As their location suggests, the termini of a protein sequence are likely to be at sites exposed for interaction with other factors.

The results obtained from mutations introduced to the lumenally located TM3-TM4 loop are intriguing. This loop is predicted to be larger and protrude deeper into the lumen than that of the TM1-TM2. In addition the replication complex is thought to reside on the cytoplasmic side (Wölk *et al.*, 2000). This raises the notion that NS4B could be affecting the replication by recruiting lumenal factors.

Another intriguing set of mutations is in the TM2-TM3 loop in the cytoplasm; the G1845A and the adjoining K1846A. This is especially interesting as Einav and colleagues have suggested these two residues to be a part of a conserved GTP binding motif (Einav *et al.*, 2004). While the G1845A almost abolishing replication (0.6 ± 0.1 %) may support that notion, the adjoining K1846A being rather well-tolerated (59.8 ± 8.6) does not. Moreover Lohmann and colleagues have reported of an adaptive mutant replicon with the K1846T change (Lohmann *et al.*, 2003). Together we believe that although this site being most likely involved in protein-protein interaction, it may have other functions than binding GTP.

In conclusion, Paper IV shows the importance of NS4B integrity, where even non-conserved amino acid residues have strong impact on the viral replication. Moreover, even mutations in the loop regions protruding into the lumen showed to strongly affect the replication, implying interactions with factors beyond the replication complex. This indicates that elucidating interacting partners for NS4B may offer important insights on the replication process.

DISCUSSION

In this thesis aspects of the translation and the replication of the viral life cycle have been investigated. In the study of the viral translation, the focus was put on the role of the translation mediated by the HCV IRES *in vitro* and how it may correlate to the therapeutic outcome *in vivo*. The investigations of the viral replication have been approached two ways; by using a collection of recombinantly expressed NS4B for deciphering its topology and function, and by using the HCV subgenomic replicon for scrutinizing the NS4B in an on-going HCV replication.

For the IRES mediated translation studies, statistically significant differences in translation efficiencies *in vitro* could be seen between the SR and NR groups. The IRES derived from the SR group showed significantly less efficient translation than that of the NR group. This is in contrast to previous studies where no significant difference between the SR and the NR groups was seen (Saiz *et al.*, 1999). One possible explanation could be the differences in the IRES segments used in our study compared to that by Saiz and colleagues; we studied the translation efficiency of the entire IRES region (nucleotides 1-407) while the IRES studied by Saiz and colleagues lacked the first 40 nucleotides (nucleotides 40-407). The nucleotides 1-43 contain a *cis*-acting RNA element that regulates HCV RNA replication and translation (Luo *et al.*, 2003). Hence the difference in the presence or absence of the first 40 nucleotides may have affected the outcome of the studies by our group and that of Saiz and colleagues (Saiz *et al.*, 1999).

Our results suggest that IRES translation efficiency may be an important factor influencing the clinical outcome. It would be very interesting to repeat this study with IRES derived from other genotypes to see whether similar correlation may be observed, especially for the HCV genotype 1 which is the most frequent (60 % of all HCV infections in the world) yet has the worst prognosis for the therapeutic treatment outcome (Hadziyannis *et al.*, 2004). A robust universal prediction model of the therapeutic outcome of the HCV in patients is yet to be revealed. Perhaps in the future, our work could result in a universal prediction model of the therapeutic outcome. IFN therapy is not only costly, it is also very demanding on the patient. Ideally, by

sequencing the IRES regions of the infecting HCV, perhaps a more efficient treatment regime may be tailored for the patient.

At the time when our studies of the NS4B's role in the viral replication were initiated, the only established function of the NS4B was to induce membrane arrangements in which replication of the viral RNA was thought to occur (Egger *et al.*, 2002, El-Hage & Luo, 2003, Gosert *et al.*, 2003, Gretton *et al.*, 2005, Lundin *et al.*, 2003, Shi *et al.*, 2003, Waris *et al.*, 2004). However it was soon also shown to be an integral part of the viral replication complex, necessary in *cis* for the replication to transpire (Appel *et al.*, 2005, Tong & Malcolm, 2006). Therefore, an important part of the study of NS4B proteins role in the HCV replication was the extended studies of the topology of this elusive protein. This was in hope of achieving indications of the protein's function in the viral life cycle based on its molecular characteristics.

Paper II substantiated the N-terminal translocation of the NS4B even when it was expressed in the context of the viral polyprotein in cells. Moreover *in vitro* translation studies showed that this feature was conserved in all HCV genotypes. Such conserved features generally indicate important functions. We interpreted these results as further substantiating our findings that a fraction of the NS4B population must translocate the N-terminus into the lumen even in the context of the viral proliferation. Indeed, studies using a collection of NS4B mutants indicated that the N-terminal translocation may be one of the main occurrence necessary for the MAF induction. In fact these experiments showed that the amino acid residues 30-60 containing the putative AH2 were most likely responsible for both the oligomerization and MAF induction (Paper III).

I believe that it is the AH2 on the luminal side of the ER membrane that enables the MAF induction. As seen in other proteins, the AH is a recurring motif for inducing membrane curvature (Ford *et al.*, 2002, Henzler Wildman *et al.*, 2003, Peter *et al.*, 2004, Richnau *et al.*, 2004). Perceivably, the lumenally positioned AH2 acts as a wedge in the membrane inducing positive curvature into the ER lumen. This would result in the enclosure of the replicon complexes in spherules and explain the resistance of the replicon complex and nascent RNA to both RNases and proteases (Aizaki *et al.*, 2004, El-Hage & Luo, 2003, Quinkert *et al.*, 2005).

However N-terminal translocation alone was not enough to induce MAFs: Another likely way for the NS4B to achieve membrane rearrangements would be by protein-protein interaction

either with itself or with other viral and/or cellular factors. Attempts have been made to find cellular interactants of the NS4B, by us as well as other groups, by means of immunoprecipitation studies or yeast two-hybrid assays, using the NS4B as bait (our unpublished data, (Flajolet *et al.*, 2000, Tong *et al.*, 2002). Disappointingly little has been substantiated. Yet the effects exerted by the NS4B on the MAF induction, and even other cellular effects such as the inhibition of protein synthesis of the host cell, IL-8 trans-activation, must surely be due to some sort of interaction with cellular factors (Florese *et al.*, 2002, Kadoya *et al.*, 2005, Kato *et al.*, 2002b, Tong *et al.*, 2002).

While interacting cellular factors are yet to be identified, our oligomerization studies showed that the NS4B does indeed interact with itself. Both its N- and C-terminal domains were necessary, for oligomerization, and it was essential for the formation of MAFs (Paper III). The oligomerization perhaps increases the local concentrations of the NS4Bs necessary for MAF induction. Could the NS4Bs in these oligomeric forms be involved in the formation of the replicon complexes as well? All the other NS proteins have shown to require at least dimerization for the proper function (Levin & Patel, 1999, Lorenz *et al.*, 2006, Tellinghuisen *et al.*, 2005, Wang *et al.*, 2002). Quinkert and colleagues have also shown that less than 5 % of the NS proteins produced in a cell was estimated to actually be involved in replication of the viral RNA, with 1:1 stoichiometry between the NS proteins (Quinkert *et al.*, 2005). Our oligomerization data showed that only a small minority of the NS4B was in oligomeric form (Paper III). This may support the notion that only the oligomeric forms of the NS4B may be involved in MAF induction and that the very same are also involved in the formation of the replicon complexes.

Interestingly the study with the NS4B mutants revealed another phenomenon; that too much MAF induction may be detrimental for the cell survival. This is especially interesting in the light of all positive stranded RNA viruses seeming to require rearrangement of cellular membranes for the viral replication to occur (Ahlquist *et al.*, 2003, Salonen *et al.*, 2005). In Paper II we noticed that the N-terminal translocation was negatively regulated by the NS5A. Moreover in on-going replicon replication, MAFs are kept at a rather low constant level; distinct MAFs visible by standard immunofluorescence are only seen in cells supporting efficient replicon replication (El-Hage & Luo, 2003, Gosert *et al.*, 2003, Pietschmann *et al.*, 2001). Although a prerequisite for the viral replication, too much MAF induction seems to be detrimental for the host cell, thus vital to regulate. Also in Kunjin viruses, replication efficiency

could be correlated to the degree of MAF induction (Mackenzie *et al.*, 2001). Although in the Kunjin virus, the membrane induction seems to be regulated by differential processing of the MAF-inducing protein in the Kunjin virus, the NS4A. Here, the NS4A processing results in two isoforms; by either retaining or removing the C-terminal domain 2K of the NS4A (Roosendaal *et al.*, 2006, Westaway *et al.*, 1997a). It is the NS4A retaining the 2K that induces the spherules in the cytoplasmic membranes, while the NS4A lacking the 2K relocates to the Golgi to recruit the membranes used within the replication complex. As observed, MAF induction seems to be a result of the balance between the two NS4A populations (Roosendaal *et al.*, 2006).

Remarkably, the NS4B's role in the HCV replication does not seem to end with merely inducing MAFs. In Paper IV, 14 point-mutations were introduced to strategic positions of the NS4B protein in the context of the subgenomic replicon based on our 5TM topology model (Paper IV). Despite all the mutants maintaining the ability to rearrange membranes, the replicon replication establishment ranged from increased to strongly reduced efficiency. Some of the negative effects of the alterations in the NS4B may be ascribed to minor disruption in the intramolecular integrity of the NS4B. However the sites of many of the mutations, especially in the N- and C-termini and in the lumenally located TM3-TM4 loop, are more likely involved in intermolecular interactions; *i)* the N- and C-termini mutations are at the ends of their respective domains thus potentially superficially exposed (Fig 26); *ii)* according to our model, the TM3-TM4 loop is longer than the other inter-transmembrane domain loops, therefore likely to protrude deeper into the lumen (Fig 26). What is even more appealing with these mutants, they ignite the possibility of intermolecular interaction, not only with other viral factors, but also with cellular factors, especially when considering the dual topology of the N-terminus and the TM3-TM4 loop's luminal protrusion (N.b.! the replication complex is on the cytoplasmic side of the ER membrane (Wölk *et al.*, 2000)). As seen in studies by other groups, the NS4B has shown to physically interact with NS2, NS3, NS4A and NS5A (Dimitrova *et al.*, 2003, Flajolet *et al.*, 2000, Lin *et al.*, 1997). As for the cellular effects such as altering of cellular tropism, conferring IFN resistance, inhibiting protein synthesis of the host cell, and trans-activating IL-8 must surely be due to some sort of interaction with cellular factors (Florese *et al.*, 2002, Kadoya *et al.*, 2005, Kato *et al.*, 2002b, Lohmann *et al.*, 2001, Sumpter *et al.*, 2004, Tong *et al.*, 2002, Zheng *et al.*, 2005a, Zhu *et al.*, 2003b).

Studies with different Huh7 derived cell lines have shown that individual cells differ in their capacity to support replicons, most likely due to differential expression of cell factors promoting

viral replication (Blight *et al.*, 2002, Lohmann *et al.*, 2003, Murray *et al.*, 2003). Selection of such permissible cells that compensate the detrimental effects of the mutations may also have been the case for our replicon mutants: Once the replicon mutants established in cells, all gave similar variations in cell growth rate in selection medium, replicon RNA copy number, and amount of NS proteins produced irrespective of their initial establishment efficiency. This is despite no reversion or compensatory mutations were observed within the NS4B region (though compensatory mutations outside of the NS4B region cannot be excluded at this time). Together they support the notion that there indeed could be direct interactions between the NS4B and cellular factors. However the replication complex comprises merely 5 % of all NS proteins produced during viral proliferation in cells, hence finding the cellular factors interacting with the NS4B in the context of viral replication provides a big challenge (Quinkert *et al.*, 2005).

Why the dual topology? The NS4B's two topologies may have two different functions in the viral life cycle. Ergo lumenally located N-terminus of the NS4B induces the MAFs and the cytoplasmically located N-terminus could enable other function of the protein, among others affecting the replication. An example of such protein with dual topology is the integral membrane protein, large surface protein (L) of the hepatitis B virus (HBV). The luminal location of the protein's N-terminus functions as the viral receptor while its cytoplasmic location may act as the matrix protein, interacting with the nucleocapsid (Bruss & Vieluf, 1995). This gives the two different topologies of the L protein distinctly separate functions in the viral life cycle. Such could also be the case with the two topologies of the HCV NS4B. For instance one of the great mysteries of HCV life cycle is how the newly synthesized viral RNA is encapsidated by the core: while the viral RNA is compartmentalized in the MAFs, the core is located to the membranes around the lipid droplets (Barba *et al.*, 1997, McLauchlan *et al.*, 2002, Rouille *et al.*, 2006). Intriguingly our computer modulation studies have shown that the N-terminus has an AH-loop-AH structure very similar to that of the core lipid membrane association domain (Paper III) (Boulant *et al.*, 2006). That this NS4B structure indeed is capable of associating with the lipid droplets was seen when only the first 93 amino acid residues of the NS4B was expressed in cells (Paper III). Perhaps the cytoplasmically located N-terminus of the NS4B could act as the bridge between lipid droplets and the replicon complex, thus also between the core and the newly synthesized viral RNA.

SUMMARY

In this thesis, two aspects of the HCV viral life cycle were studied; the IRES mediated translation and the viral replication with focus on the NS4B protein. The first section showed that studying the correlation between the IRES sequence and the therapeutic outcome may serve as a prediction model for the therapeutic outcome of clinical treatment of HCV genotype 3. In the future this may be extended to other HCV genotypes, hopefully resulting in a robust prediction model.

The study of the NS4B's role in the viral life cycle showed that oligomerization and balancing the N-terminal translocation may be the key to forming the MAFs, a prerequisite for the viral RNA replication to occur. What is more, direct involvement by NS4B in viral RNA replication beyond MAF induction is also implied by the impacts on replication observed for mutations in even seemingly non-conserved amino acid residues, and more surprisingly in the lumen. Our findings show that elucidating interacting partners of the NS4B on both sides of the ER membrane may offer important insight on the viral replication. Together these results emphasize the pivotal roles the elusive NS4B has in the viral life cycle, and deciphering those may be a key to novel therapeutic strategies.

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REFERENCES

- Abe, K., Ikeda, M., Dansako, H., Naka, K., Shimotohno, K. & Kato, N. (2005). cDNA microarray analysis to compare HCV subgenomic replicon cells with their cured cells. *Virus Res* **107**, 73-81.
- Ahlquist, P., Noueiry, A. O., Lee, W. M., Kushner, D. B. & Dye, B. T. (2003). Host factors in positive-strand RNA virus genome replication. *J Virol* **77**, 8181-8186.
- Aizaki, H., Lee, K. J., Sung, V. M., Ishiko, H. & Lai, M. M. (2004). Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* **324**, 450-461.
- Ali, S., Pellerin, C., Lamarre, D. & Kukolj, G. (2004). Hepatitis C virus subgenomic replicons in the human embryonic kidney 293 cell line. *J Virol* **78**, 491-501.
- Alter, H. J., Purcell, R. H., Holland, P. V. & Popper, H. (1978). Transmissible agent in non-A, non-B hepatitis. *Lancet* **1**, 459-463.
- Appel, N., Herian, U. & Bartenschlager, R. (2005). Efficient rescue of hepatitis C virus RNA replication by trans-complementation with nonstructural protein 5A. *J Virol* **79**, 896-909.
- Bain, C., Fatmi, A., Zoulim, F., Zarski, J.-P., Trepo, C. & Inchauspe, G. (2001). Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* **120**, 512-524.
- Barba, G., Harper, F., Harada, T., Kohara, M., Goulinet, S., Matsuura, Y., Eder, G., Schaff, Z., Chapman, M. J., Miyamura, T. & Brechot, C. (1997). Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc Natl Acad Sci U S A* **94**, 1200-1205.
- Bartenschlager, R., Ahlborn-Laake, L., Yasargil, K., Mous, J. & Jacobsen, H. (1995). Substrate determinants for cleavage in cis and in trans by the hepatitis C virus NS3 proteinase. *J Virol* **69**, 198-205.
- Bartenschlager, R. & Pietschmann, T. (2005). Efficient hepatitis C virus cell culture system: what a difference the host cell makes. *Proc Natl Acad Sci U S A* **102**, 9739-9740.
- Bartosch, B. & Cosset, F. L. (2006). Cell entry of hepatitis C virus. *Virology* **348**, 1-12.
- Bartosch, B., Verney, G., Dreux, M., Donot, P., Morice, Y., Penin, F., Pawlotsky, J.-M., Lavillette, D. & Cosset, F.-L. (2005). An Interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. *J Virol* **79**, 8217-8229.
- Bartosch, B., Vitelli, A., Granier, C., Goujon, C., Dubuisson, J., Pascale, S., Scarselli, E., Cortese, R., Nicosia, A. & Cosset, F.-L. (2003). Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J Biol Chem* **278**, 41624-41630.
- Bassett, S. E., Thomas, D. L., Brasky, K. M. & Lanford, R. E. (1999). Viral persistence, antibody to E1 and E2, and hypervariable region 1 sequence stability in hepatitis C virus-inoculated chimpanzees. *J Virol* **73**, 1118-1126.

- Baumann, O. & Walz, B. (2001). Endoplasmic reticulum of animal cells and its organization into structural and functional domains. In *Int Rev Cytol*, pp. 149-214. Edited by K. Jeon. Knoxville: Academic Press, University of Tennessee, Knoxville, U.S.A.
- Beales, L. P., Holzenburg, A. & Rowlands, D. J. (2003). Viral internal ribosome entry site structures segregate into two distinct morphologies. *J Virol* **77**, 6574-6579.
- Beales, L. P., Rowlands, D. J. & Holzenburg, A. (2001). The internal ribosome entry site (IRES) of hepatitis C virus visualized by electron microscopy. *RNA* **7**, 661-670.
- Behrens, S. E., Tomei, L. & De Francesco, R. (1996). Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J* **15**, 12-22.
- Bigger, C. B., Brasky, K. M. & Lanford, R. E. (2001). DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. *J Virol* **75**, 7059-7066.
- Billiau, A., Heremans, H., Vermeire, K. & Matthys, P. (1998). Immunomodulatory properties of interferon-gamma: An update. *Ann NY Acad Sci* **856**, 22-32.
- Bishop, W. R. & Bell, R. M. (1988). Assembly of phospholipids into cellular membranes: Biosynthesis, transmembrane movement and intracellular translocation. *Annu Rev Cell Biol* **4**, 579-606.
- Blanchard, E., Belouzard, S., Goueslain, L., Wakita, T., Dubuisson, J., Wychowski, C. & Rouille, Y. (2006). Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J Virol* **80**, 6964-6972.
- Blight, K. J., Kolykhalov, A. A. & Rice, C. M. (2000). Efficient initiation of HCV RNA replication in cell culture. *Science* **290**, 1972-1974.
- Blight, K. J., McKeating, J. A., Marcotrigiano, J. & Rice, C. M. (2003). Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. *J Virol* **77**, 3181-3190.
- Blight, K. J., McKeating, J. A. & Rice, C. M. (2002). Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* **76**, 13001-13014.
- Booth, J. C., Kumar, U., Webster, D., Monjardino, J. & Thomas, H. C. (1998). Comparison of the rate of sequence variation in the hypervariable region of E2/NS1 region of hepatitis C virus in normal and hypogammaglobulinemic patients. *Hepatology* **27**, 223-227.
- Boulant, S., Montserret, R., Hope, R. G., Ratniner, M., Targett-Adams, P., Laverne, J.-P., Penin, F. & McLauchlan, J. (2006). Structural determinants that target the hepatitis C virus core protein to lipid droplets. *J Biol Chem* **281**, 22236-22247.
- Bowen, D. G. & Walker, C. M. (2005). The origin of quasispecies: cause or consequence of chronic hepatitis C viral infection? *J Hepatol* **42**, 408-417.
- Bressanelli, S., Stiasny, K., Allison, S. L., Stura, E. A., Duquerroy, S., Lescar, J., Heinz, F. X. & Rey, F. A. (2004). Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. *EMBO J* **23**, 728-738.
- Bruss, V. & Vieluf, K. (1995). Functions of the internal pre-S domain of the large surface protein in hepatitis B virus particle morphogenesis. *J Virol* **69**, 6652-6657.
- Bukh, J., Pietschmann, T., Lohmann, V., Krieger, N., Faulk, K., Engle, R. E., Govindarajan, S., Shapiro, M., St Claire, M. & Bartenschlager, R. (2002). Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. *Proc Natl Acad Sci USA* **99**, 14416-14421.
- Carrere-Kremer, S., Montpellier, C., Lorenzo, L., Brulin, B., Cocquerel, L., Belouzard, S., Penin, F. & Dubuisson, J. (2004). Regulation of hepatitis C virus polyprotein processing by signal peptidase involves structural determinants at the p7 sequence junctions. *J Biol Chem* **279**, 41384-41392.

- Castet, V., Arora, N., Blum, H. E., Penin, F. & Moradpour, D. (2004). Identification of a novel determinant for membrane association in HCV nonstructural protein 4B. In *11th International Symposium on HCV & Related Viruses*, pp. P-94. Heidelberg.
- Cheney, I. W., Lai, V. C., Zhong, W., Brodhag, T., Dempsey, S., Lim, C., Hong, Z., Lau, J. Y. & Tam, R. C. (2002). Comparative analysis of anti-hepatitis C virus activity and gene expression mediated by alpha, beta, and gamma interferons. *J Virol* **76**, 11148-11154.
- Choo, Q. L., Han, J., Weiner, A. J., Overby, L. R., Bradley, D. W., Kuo, G. & Houghton, M. (1991a). Hepatitis C virus is a distant relative of the flaviviruses and pestiviruses. In *Viral Hepatitis C, D, and E*, pp. 47-52. Edited by T. Shikata, H. Purcell & T. Uchida: Elsevier Science Publishers B. V.
- Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359-362.
- Choo, Q. L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, R., Barr, P. J. & et al. (1991b). Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci U S A* **88**, 2451-2455.
- Choo, Q. L., Weiner, A. J., Overby, L. R., Kuo, G., Houghton, M. & Bradley, D. W. (1990). Hepatitis C virus: the major causative agent of viral non-A, non-B hepatitis. *Br Med Bull* **46**, 423-441.
- Chung, R. T., He, W., Saquib, A., Contreras, A. M., Xavier, R. J., Chawla, A., Wang, T. C. & Schmidt, E. V. (2001). Hepatitis C virus replication is directly inhibited by IFN-alpha in a full-length binary expression system. *Proc Natl Acad Sci U S A* **98**, 9847-9852.
- Clarke, A. & Kulasegaram, R. (2006). Hepatitis C transmission -- where are we now? *Int J STD AIDS* **17**, 74-80.
- Cocquerel, L., Op de Beeck, A., Lambot, M., Roussel, J., Delgrange, D., Pillez, A., Wychowski, C., Penin, F. & Dubuisson, J. (2002). Topological changes in the transmembrane domains of hepatitis C virus envelope glycoproteins. *EMBO J* **21**, 2893-2902.
- Collier, A. J., Tang, S. & Elliott, R. M. (1998). Translation efficiencies of the 5' untranslated region from representatives of the six major genotypes of hepatitis C virus using a novel bicistronic reporter assay system. *J Gen Virol* **79**, 2359-2366.
- Craxi, A. & Licata, A. (2003). Clinical trial results of peginterferons in combination with ribavirin. *Semin Liver Dis* **23 Suppl 1**, 35-46.
- Crispe, I. N. (2003). Hepatic T cells and liver tolerance. *Nat Rev Immunol* **3**, 51-62.
- Crotta, S., Stilla, A., Wack, A., D'Andrea, A., Nuti, S., D'Oro, U., Mosca, M., Filliponi, F., Brunetto, R. M., Bonino, F., Abrignani, S. & Valiante, N. M. (2001). Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. *J Exp Med* **195**, 35-42.
- Crovatto, M., Pozzato, G., Zorat, F., Pussini, E., Nascimben, F., Baracetti, S., Grando, M. G., Mazzaro, C., Reitano, M., Modolo, M. L., Martelli, P., Spada, A. & Santini, G. (2000). Peripheral blood neutrophils from hepatitis C virus-infected patients are replication sites of the virus. *Haematologica* **85**, 356-361.
- D'Elios, M. & Del Prete, G. (1998). Th1/Th2 balance in human disease. *Transplant Proc* **30**, 2373-2377.
- Date, T., Kato, T., Miyamoto, M., Zhao, Z., Yasui, K., Mizokami, M. & Wakita, T. (2004). Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells. *J Biol Chem* **279**, 22371-22376.
- de Martin, E. P., Novick, P. & Ferro-Novick, S. (2005). The organization, structure, and inheritance of the ER in higher and lower eukaryotes. *Biochem Cell Biol* **83**, 752-761.
- Deforges, S., Evlashev, A., Perret, M., Sodoyer, M., Pouzol, S., Scoazec, J.-Y., Bonnaud, B., Diaz, O., Paranhos-Baccala, G., Lotteau, V. & Andre, P. (2004). Expression of hepatitis C virus proteins in epithelial intestinal cells in vivo. *J Gen Virol* **85**, 2515-2523.

- Dimitrova, M., Imbert, I., Kieny, M. P. & Schuster, C. (2003). Protein-protein interactions between hepatitis C virus nonstructural proteins. *J Virol* **77**, 5401-5414.
- Dubuisson, J., Duvet, S., Meunier, J.-C., Op De Beeck, A., Cacan, R., Wychowski, C. & Cocquerel, L. (2000). Glycosylation of the Hepatitis C Virus Envelope Protein E1 Is Dependent on the Presence of a Downstream Sequence on the Viral Polyprotein. *J Biol Chem* **275**, 30605-30609.
- Duvet, S., Cocquerel, L., Pillez, A., Cacan, R., Verbert, A., Moradpour, D., Wychowski, C. & Dubuisson, J. (1998). Hepatitis C virus glycoprotein complex localization in the endoplasmic reticulum involves a determinant for retention and not retrieval. *J Biol Chem* **273**, 32088-32095.
- Ebersberger, I., Metzler, D., Schwarz, C. & Paabo, S. (2002). Genomewide comparison of DNA sequences between humans and chimpanzees. *Am J Hum Genet* **70**, 1490-1497.
- Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H. E., Moradpour, D. & Bienz, K. (2002). Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* **76**, 5974-5984.
- Einav, S., Elazar, M., Danieli, T. & Glenn, J. S. (2004). A nucleotide binding motif in hepatitis C virus (HCV) NS4B mediates HCV RNA replication. *J Virol* **78**, 11288-11295.
- Eisenhaber, B., Maurer-Stroh, S., Novatchkova, M., Schneider, G. & Eisenhaber, F. (2003). Enzymes and auxiliary factors for GPI lipid anchor biosynthesis and post-translational transfer to proteins. *Bioessays* **25**, 367-385.
- El-Hage, N. & Luo, G. (2003). Replication of hepatitis C virus RNA occurs in a membrane-bound replication complex containing nonstructural viral proteins and RNA. *J Gen Virol* **84**, 2761-2769.
- Elazar, M., Cheong, K. H., Liu, P., Greenberg, H. B., Rice, C. M. & Glenn, J. S. (2003). Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. *J Virol* **77**, 6055-6061.
- Elazar, M., Liu, P., Rice, C. M. & Glenn, J. S. (2004). An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. *J Virol* **78**, 11393-11400.
- Enard, W., Khaitovich, P., Klose, J., Zollner, S., Heissig, F., Giavalisco, P., Nieselt-Struwe, K., Muchmore, E., Varki, A., Ravid, R., Doxiadis, G. M., Bontrop, R. E. & Paabo, S. (2002). Intra- and interspecific variation in primate gene expression patterns. *Science* **296**, 340-343.
- Evans, M. J., Rice, C. M. & Goff, S. P. (2004). Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc Natl Acad Sci U S A* **101**, 13038-13043.
- Falck-Ytter, Y., Kale, H., Mullen, K. D., Sarbah, S. A., Sorescu, L. & McCullough, A. J. (2002). Surprisingly small effect of antiviral treatment in patients with hepatitis C. *Ann Intern Med* **136**, 288-292.
- Fan, Z., Yang, Q. R., Twu, J. S. & Sherker, A. H. (1999). Specific in vitro association between the hepatitis C viral genome and core protein. *J Med Virol* **59**, 131-134.
- Farci, P., Alter, H. J., Shimoda, A., Govindarajan, S., Cheung, L. C., Melpolder, J. C., Sacher, R. A., Shih, J. W. & Purcell, R. H. (1996). Hepatitis C virus-associated fulminant hepatic failure. *N Engl J Med* **335**, 631-634.
- Feinstone, S. M., Kapikian, A. Z., Purcell, R. H., Alter, H. J. & Holland, P. V. (1975). Transfusion-associated hepatitis not due to viral hepatitis type A or B. *N Engl J Med* **292**, 767-770.
- Fernandez, J., Taylor, D., Morhardt, D. R., Mihalik, K., Puig, M., Rice, C. M., Feinstone, S. M. & Major, M. E. (2004). Long-term persistence of infection in chimpanzees inoculated with an infectious hepatitis C virus clone is associated with a decrease in the viral amino acid substitution rate and low levels of heterogeneity. *J Virol* **78**, 9782-9789.

- Flajolet, M., Rotondo, G., Daviet, L., Bergametti, F., Inchauspe, G., Tiollais, P., Transy, C. & Legrain, P. (2000). A genomic approach of the hepatitis C virus generates a protein interaction map. *Gene* **242**, 369-379.
- Florese, R. H., Nagano-Fujii, M., Iwanaga, Y., Hidajat, R. & Hotta, H. (2002). Inhibition of protein synthesis by the nonstructural proteins NS4A and NS4B of hepatitis C virus. *Virus Res* **90**, 119-131.
- Ford, M. G. J., Mills, I. G., Peter, B. J., Vallis, Y., Praefcke, G. J. K., Evans, P. R. & McMahon, H. T. (2002). Curvature of clathrin-coated pits driven by epsin. *Nature* **419**, 361-366.
- Foster, G. R. (2003). Pegylated interferon with ribavirin therapy for chronic infection with the hepatitis C virus. *Expert Opin Pharmacother* **4**, 685-691.
- Fournier, C., Sureau, C., Coste, J., Ducos, J., Pageaux, G., Larrey, D., Domergue, J. & Maurel, P. (1998). In vitro infection of adult normal human hepatocytes in primary culture by hepatitis C virus. *J Gen Virol* **79**, 2367-2374.
- Frese, M., Pietschmann, T., Moradpour, D., Haller, O. & Bartenschlager, R. (2001). Interferon-alpha inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway. *J Gen Virol* **82**, 723-733.
- Frese, M., Schwarzle, V., Barth, K., Krieger, N., Lohmann, V., Mihm, S., Haller, O. & Bartenschlager, R. (2002). Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. *Hepatology* **35**, 694-703.
- Friebe, P. & Bartenschlager, R. (2002). Genetic Analysis of Sequences in the 3' Nontranslated Region of Hepatitis C Virus That Are Important for RNA Replication. *J Virol* **76**, 5326-5338.
- Friebe, P., Boudet, J., Simorre, J. P. & Bartenschlager, R. (2005). Kissing-loop interaction in the 3' end of the hepatitis C virus genome essential for RNA replication. *J Virol* **79**, 380-392.
- Fukuda, R., Ishimura, N., Ishihara, S., Tokuda, A., Satoh, S., Sakai, S., Akagi, S., Watanabe, M. & Fukumoto, S. (1996). Expression of interferon-alpha receptor mRNA in the liver in chronic liver diseases associated with hepatitis C virus: relation to effectiveness of interferon therapy. *J Gastroenterol* **31**, 806-811.
- Gao, L., Aizaki, H., He, J. W. & Lai, M. M. (2004). Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. *J Virol* **78**, 3480-3488.
- Gonzalez, M. E. & Carrasco, L. (2003). Viroporins. *FEBS Lett* **552**, 28-34.
- Gosert, R., Egger, D., Lohmann, V., Bartenschlager, R., Blum, H. E., Bienz, K. & Moradpour, D. (2003). Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. *J Virol* **77**, 5487-5492.
- Goudsmit, J., Back, N. K. & Nara, P. L. (1991). Genomic diversity and antigenic variation of HIV-1: links between pathogenesis, epidemiology and vaccine development. *FASEB J* **5**, 2427-2436.
- Grakoui, A., Hanson, H. L. & Rice, C. M. (2001). Bad time for Bonzo? Experimental models of hepatitis C virus infection, replication, and pathogenesis. *Hepatology* **33**, 489-495.
- Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M. & Rice, C. M. (1993a). A second hepatitis C virus-encoded proteinase. *Proc Natl Acad Sci U S A* **90**, 10583-10587.
- Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M. & Rice, C. M. (1993b). Expression and identification of hepatitis C virus polyprotein cleavage products. *J Virol* **67**, 1385-1395.
- Grassmann, C. W., Isken, O., Tautz, N. & Behrens, S. E. (2001). Genetic analysis of the pestivirus nonstructural coding region: defects in the NSSA unit can be complemented in trans. *J Virol* **75**, 7791-7802.
- Gretton, S. N., Taylor, A. I. & McLauchlan, J. (2005). Mobility of the hepatitis C virus NS4B protein on the endoplasmic reticulum membrane and membrane-associated foci. *J Gen Virol* **86**, 1415-1421.

- Griffin, S. D., Beales, L. P., Clarke, D. S., Worsfold, O., Evans, S. D., Jaeger, J., Harris, M. P. & Rowlands, D. J. (2003). The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett* **535**, 34-38.
- Guha, C., Lee, S.-W., Chowdhury, N. R. & Chowdhury, J. R. (2005). Cell culture models and animal models of viral hepatitis. Part II: Hepatitis C. *Lab Anim (NY)* **34**, 39-47.
- Guo, J. T., Bichko, V. V. & Seeger, C. (2001). Effect of alpha interferon on the hepatitis C virus replicon. *J Virol* **75**, 8516-8523.
- Guo, J. T., Sohn, J. A., Zhu, Q. & Seeger, C. (2004). Mechanism of the interferon alpha response against hepatitis C virus replicons. *Virology* **325**, 71-81.
- Guo, J. T., Zhu, Q. & Seeger, C. (2003). Cytopathic and noncytopathic interferon responses in cells expressing hepatitis C virus subgenomic replicons. *J Virol* **77**, 10769-10779.
- Gwack, Y., Kim, D. W., Han, J. H. & Choe, J. (1996). Characterization of RNA binding activity and RNA helicase activity of the hepatitis C virus NS3 protein. *Biochem Biophys Res Commun* **225**, 654-659.
- Hadziyannis, S. J., Sette Jr., H., Morgan, T. R., Balan, V., Diago, M., Marcellin, P., Ramadori, G., Bodenheimer Jr., H., Bernstein, D., Rizzetto, M., Zeuzem, S., Pockros, P. J., Lin, A., Ackrill, A. M. & for the, P. I. S. G. (2004). Peginterferon- α 2a and ribavirin combination therapy in chronic hepatitis C: A randomized study of treatment duration and ribavirin dose. *Ann Intern Med* **140**, 346-355.
- Hahn, B., Han, D. S., Back, S. H., Song, O. K., Cho, M. J., Kim, C. J., Shimotohno, K. & Jang, S. K. (1995). NS3-4A of hepatitis C virus is a chymotrypsin-like protease. *J Virol* **69**, 2534-2539.
- Hanley, K. A., Manlucu, L. R., Gilmore, L. E., Blaney, J. E., Jr., Hanson, C. T., Murphy, B. R. & Whitehead, S. S. (2003). A trade-off in replication in mosquito versus mammalian systems conferred by a point mutation in the NS4B protein of dengue virus type 4. *Virology* **312**, 222-232.
- Harada, T., Tautz, N. & Thiel, H.-J. (2000). E2-p7 region of the bovine viral diarrhea virus polyprotein: Processing and functional studies. *J Virol* **74**, 9498-9506.
- He, L. F., Alling, D., Popkin, T., Shapiro, M., Alter, H. J. & Purcell, R. H. (1987). Determining the size of non-A, non-B hepatitis virus by filtration. *J Infect Dis* **156**, 636-640.
- Hellen, C. U. & Pestova, T. V. (1999). Translation of hepatitis C virus RNA. *J Viral Hepat* **6**, 79-87.
- Helms, J. B. & Zurzolo, C. (2004). Lipids as targeting signals: lipid rafts and intracellular trafficking. *Traffic* **5**, 247-254.
- Henzler Wildman, K. A., Lee, D. K. & Ramamoorthy, A. (2003). Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37. *Biochemistry* **42**, 6545-6558.
- Hibbs, R. G., Corwin, A. L., Hassan, N. F., Kamel, M., Darwish, M., Edelman, R., Constantine, N. T., Rao, M. R., Khalifa, A. S., Mokhtar, S. & et al. (1993). The epidemiology of antibody to hepatitis C in Egypt. *J Infect Dis* **168**, 789-790.
- Hijikata, M., Mizushima, H., Tanji, Y., Komoda, Y., Hirowatari, Y., Akagi, T., Kato, N., Kimura, K. & Shimotohno, K. (1993). Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. *Proc Natl Acad Sci U S A* **90**, 10773-10777.
- Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51-59.
- Honda, M., Beard, M. R., Ping, L. H. & Lemon, S. M. (1999). A phylogenetically conserved stem-loop structure at the 5' border of the internal ribosome entry site of hepatitis C virus is required for cap-independent viral translation. *J Virol* **73**, 1165-1174.

- Hong, Z., Ferrari, E., Wright-Minogue, J., Chase, R., Risano, C., Seelig, G., Lee, C. G. & Kwong, A. D. (1996). Enzymatic characterization of hepatitis C virus NS3/4A complexes expressed in mammalian cells by using the herpes simplex virus amplicon system. *J Virol* **70**, 4261-4268.
- Hope, R. G. & McLauchlan, J. (2000). Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. *J Gen Virol* **81**, 1913-1925.
- Houghton, M. (1996). Hepatitis C Viruses. In *Fields Virology*, 3rd edn, pp. 1035-1058. Edited by D. M. K. B.N. Fields, and P.M. Howley. Philadelphia: Lippincott-Raven Publishers.
- Hsu, M., Zhang, J., Flint, M., Logvinoff, C., Cheng-Mayer, C., Rice, C. M. & McKeating, J. A. (2003). Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc Natl Acad Sci U S A* **100**, 7271-7276.
- Huang, L., Hwang, J., Sharma, S. D., Hargittai, M. R., Chen, Y., Arnold, J. J., Raney, K. D. & Cameron, C. E. (2005). Hepatitis C Virus Nonstructural Protein 5A (NS5A) Is an RNA-binding Protein. *J Biol Chem* **280**, 36417-36428.
- Hugle, T., Fehrman, F., Bieck, E., Kohara, M., Krausslich, H. G., Rice, C. M., Blum, H. E. & Moradpour, D. (2001). The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. *Virology* **284**, 70-81.
- Iacovacci, S., Manzin, A., Barca, S., Sargiacomo, M., Serafino, A., Valli, M. B., Macioce, G., Hassan, H. J., Ponzetto, A., Clementi, M., Peschle, C. & Carloni, G. (1997). Molecular characterization and dynamics of hepatitis C virus replication in human fetal hepatocytes infected in vitro. *Hepatology* **26**, 1328-1337.
- Ikeda, M., Kato, N., Mizutani, T., Sugiyama, K., Tanaka, K. & Shimotohno, K. (1997). Analysis of the cell tropism of HCV by using in vitro HCV-infected human lymphocytes and hepatocytes. *J Hepatol* **27**, 445-454.
- Ikeda, M., Yi, M., Li, K. & Lemon, S. M. (2002). Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. *J Virol* **76**, 2997-3006.
- Imperiali, B., O'Connor, S. E., Hendrickson, T. & Kellenberger, C. (1999). Chemistry and biology of asparagine-linked glycosylation. *Pure Appl Chem* **71**, 777-787.
- Ina, Y., Mizokami, M., Ohba, K. & Gojobori, T. (1994). Reduction of synonymous substitutions in the core protein gene of hepatitis C virus. *J Mol Evol* **38**, 50-56.
- Isherwood, B. J. & Patel, A. H. (2005). Analysis of the processing and transmembrane topology of the E2p7 protein of hepatitis C virus. *J Gen Virol* **86**, 667-676.
- Ishido, S., Fujita, T. & Hotta, H. (1998). Complex formation of NS5B with NS3 and NS4A proteins of hepatitis C virus. *Biochem Biophys Res Commun* **244**, 35-40.
- Ivashkina, N., Wolk, B., Lohmann, V., Bartenschlager, R., Blum, H. E., Penin, F. & Moradpour, D. (2002). The hepatitis C virus RNA-dependent RNA polymerase membrane insertion sequence is a transmembrane segment. *J Virol* **76**, 13088-13093.
- Janeway, C. A. & Travers, P. (1997). Immunobiology, 3 edn. Edited by E. Hunt, G. Montier & H. Richardson. London, New York: Current Biology Ltd./Garland Publishing Inc.
- Jang, S. K. (2006). Internal initiation: IRES elements of picornaviruses and hepatitis c virus. *Virus Res* **119**, 2-15.
- Jang, S. K., Krausslich, H. G., Nicklin, M. J., Duke, G. M., Palmenberg, A. C. & Wimmer, E. (1988). A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J Virol* **62**, 2636-2643.

- Ji, X., Cheung, R., Cooper, S., Li, Q., Greenberg, H. B. & He, X. (2003). Interferon alfa regulated gene expression in patients initiating interferon treatment for chronic hepatitis C. *Hepatology* **37**, 610-621.
- Johannes, G., Carter, M. S., Eisen, M. B., Brown, P. O. & Sarnow, P. (1999). Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray. *Proc Natl Acad Sci U S A* **96**, 13118-13123.
- Johnson, A. E. & van Waes, M. A. (1999). The translocon: a dynamic gateway at the ER membrane. *Annu Rev Cell Dev Biol* **15**, 799-842.
- Kaariainen, L. & Soderlund, H. (1978). Structure and replication of alpha-viruses. *Curr Top Microbiol Immunol* **82**, 15-69.
- Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, k. s. & Foeller, C. (1991). Sequences of proteins of immunological interest, 5 edn. Bethesda: National Institutes of Health Publications.
- Kadoya, H., Nagano-Fujii, M., Deng, L., Nakazono, N. & Hotta, H. (2005). Nonstructural proteins 4A and 4B of hepatitis C virus transactivate the interleukin 8 promoter. *Microbiol Immunol* **49**, 265-273.
- Kaisho, T. & Akira, S. (2006). Toll-like receptor function and signaling. *J Allergy Clin Immunol* **117**, 979-987.
- Kaito, M., Watanabe, S., Tsukiyama-Kohara, K., Yamaguchi, K., Kobayashi, Y., Konishi, M., Yokoi, M., Ishida, S., Suzuki, S. & Kohara, M. (1994). Hepatitis C virus particle detected by immunoelectron microscopic study. *J Gen Virol* **75** (Pt 7), 1755-1760.
- Kamal, S. M., Fouly, A. E., Kamel, R. R., Hockenjos, B., Al Tawil, A., Khalifa, K. E., He, Q., Koziel, M. J., El Naggat, K. M., Rasenack, J. & Afdhal, N. H. (2006). Peginterferon alfa-2b therapy in acute hepatitis C: impact of onset of therapy on sustained virologic response. *Gastroenterology* **130**, 632-638.
- Kamoshita, N., Tsukiyama-Kohara, K., Kohara, M. & Nomoto, A. (1997). Genetic Analysis of Internal Ribosomal Entry Site on Hepatitis C Virus RNA: Implication for Involvement of the Highly Ordered Structure and Cell Type-Specific Transacting Factors. *Virology* **233**, 9-18.
- Kanai, A., Tanabe, K. & Kohara, M. (1995). Poly(U) binding activity of hepatitis C virus NS3 protein, a putative RNA helicase. *FEBS Lett* **376**, 221-224.
- Kapadia, S. B. & Chisari, F. V. (2005). Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc Natl Acad Sci U S A* **102**, 2561-2566.
- Kato, J., Kato, N., Moriyama, M., Goto, T., Taniguchi, H., Shiratori, Y. & Omata, M. (2002a). Interferons specifically suppress the translation from the internal ribosome entry site of hepatitis C virus through a double-stranded RNA-activated protein kinase-independent pathway. *J Infect Dis* **186**, 155-163.
- Kato, J., Kato, N., Yoshida, H., Ono-Nita, S. K., Shiratori, Y. & Omata, M. (2002b). Hepatitis C virus NS4A and NS4B proteins suppress translation in vivo. *J Med Virol* **66**, 187-199.
- Kato, N., Nakamura, T., Dansako, H., Namba, K., Abe, K., Nozaki, A., Naka, K., Ikeda, M. & Shimotohno, K. (2005a). Genetic variation and dynamics of hepatitis C virus replicons in long-term cell culture. *J Gen Virol* **86**, 645-656.
- Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M. & Wakita, T. (2003). Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* **125**, 1808-1817.
- Kato, T., Date, T., Miyamoto, M., Zhao, Z., Mizokami, M. & Wakita, T. (2005b). Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon. *J Virol* **79**, 592-596.
- Kato, T., Furusaka, A., Miyamoto, M., Date, T., Yasui, K., Hiramoto, J., Nagayama, K., Tanaka, T. & Wakita, T. (2001). Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. *J Med Virol* **64**, 334-339.

- Khromykh, A. A., Sedlak, P. L. & Westaway, E. G. (2000). Cis- and trans-acting elements in flavivirus RNA replication. *J Virol* **74**, 3253-3263.
- Kiiver, K., Merits, A., Ustav, M. & Zusinaite, E. (2006). Complex formation between hepatitis C virus NS2 and NS3 proteins. *Virus Res* **117**, 264-272.
- Kim, D. W., Gwack, Y., Han, J. H. & Choe, J. (1995). C-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase activity. *Biochem Biophys Res Commun* **215**, 160-166.
- Kim, J. L., Morgenstern, K. A., Griffith, J. P., Dwyer, M. D., Thomson, J. A., Murcko, M. A., Lin, C. & Caron, P. R. (1998). Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Structure* **6**, 89-100.
- Kim, J. L., Morgenstern, K. A., Lin, C., Fox, T., Dwyer, M. D., Landro, J. A., Chambers, S. P., Markland, W., Lepre, C. A. & O'Malley, E. T. (1996). Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* **87**, 343-355.
- Kim, S. Y., Park, K. W., Lee, Y. J., Back, S. H., Goo, J. H., Park, O. K., Jang, S. K. & Park, W. J. (2000). In vivo determination of substrate specificity of hepatitis C virus NS3 protease: genetic assay for site-specific proteolysis. *Anal Biochem* **284**, 42-48.
- Koch, J. O. & Bartenschlager, R. (1999). Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A, and NS4B. *J Virol* **73**, 7138-7146.
- Kornfeld, R. & Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem* **54**, 631-664.
- Krieger, N., Lohmann, V. & Bartenschlager, R. (2001). Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. *J Virol* **75**, 4614-4624.
- Kunkel, M., Lorinczi, M., Rijnbrand, R., Lemon, S. M. & Watowich, S. J. (2001). Self-assembly of nucleocapsid-like particles from recombinant hepatitis C virus core protein. *J Virol* **75**, 2119-2129.
- Lagging, L. M., Meyer, K., Owens, R. J. & Ray, R. (1998). Functional role of hepatitis C virus chimeric glycoproteins in the infectivity of pseudotyped virus. *J Virol* **72**, 3539-3546.
- Lanford, R. E., Sureau, C., Jacob, J. R., White, R. & Fuerst, T. R. (1994). Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. *Virology* **202**, 606-614.
- Lerat, H., Rumin, S., Habersetzer, F., Berby, F., Trabaud, M.-A., Trepo, C. & Inchauspe, G. (1998). In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype. *Blood* **91**, 3841-3849.
- Lerat, H., Shimizu, Y. K. & Lemon, S. M. (2000). Cell type-specific enhancement of hepatitis C virus internal ribosome entry site-directed translation due to 5' nontranslated region substitutions selected during passage of virus in lymphoblastoid cells. *J Virol* **74**, 7024-7031.
- Lesburg, C. A., Cable, M. B., Ferrari, E., Hong, Z., Mannarino, A. F. & Weber, P. C. (1999). Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat Struct Mol Biol* **6**, 937-943.
- Levin, M. K. & Patel, S. S. (1999). The helicase from hepatitis C virus is active as an oligomer. *J Biol Chem* **274**, 31839-31846.
- Levy, D. E. (2002). Whence Interferon? Variety in the production of interferon in response to viral infection. *J Exp Med* **195**, 15F-18.
- Li, Y. & McNally, J. (2001). Characterization of RNA synthesis and translation of bovine viral diarrhea virus (BVDV). *Virus Genes* **23**, 149-155.

- Lin, C., Lindenbach, B. D., Pragai, B. M., McCourt, D. W. & Rice, C. M. (1994). Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini. *J Virol* **68**, 5063-5073.
- Lin, C. & Rice, C. M. (1995). The hepatitis C virus NS3 serine proteinase and NS4A cofactor: establishment of a cell-free trans-processing assay. *Proc Natl Acad Sci U S A* **92**, 7622-7626.
- Lin, C., Wu, J. W., Hsiao, K. & Su, M. S. (1997). The hepatitis C virus NS4A protein: interactions with the NS4B and NS5A proteins. *J Virol* **71**, 6465-6471.
- Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R., McKeating, J. A. & Rice, C. M. (2005). Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623-626.
- Lindenbach, B. D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A. J., McKeating, J. A., Lanford, R. E., Feinstone, S. M., Major, M. E., Leroux-Roels, G. & Rice, C. M. (2006). Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. *Proc Natl Acad Sci U S A* **103**, 3805-3809.
- Lohmann, V., Hoffmann, S., Herian, U., Penin, F. & Bartenschlager, R. (2003). Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. *J Virol* **77**, 3007-3019.
- Lohmann, V., Korner, F., Dobierzewska, A. & Bartenschlager, R. (2001). Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J Virol* **75**, 1437-1449.
- Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110-113.
- Lorenz, I. C., Marcotrigiano, J., Dentzer, T. G. & Rice, C. M. (2006). Structure of the catalytic domain of the hepatitis C virus NS2-3 protease. *Nature* **Epub ahead of print**, July 23.
- Love, R. A., Parge, H. E., Wickersham, J. A., Hostomsky, Z., Habuka, N., Moomaw, E. W., Adachi, T. & Hostomska, Z. (1996). The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. *Cell* **87**, 331-342.
- Lu, L., Nakano, T., Orito, E., Mizokami, M. & Robertson, B. H. (2001). Evaluation of accumulation of hepatitis C virus mutations in a chronically infected chimpanzee: comparison of the core, E1, HVR1, and NS5b regions. *J Virol* **75**, 3004-3009.
- Lucas, M., Vargas-Cuero, A. L., Lauer, G. M., Barnes, E., Willberg, C. B., Semmo, N., Walker, B. D., Phillips, R. & Klenerman, P. (2004). Pervasive influence of hepatitis C virus on the phenotype of antiviral CD8⁺ T cells. *J Immunol* **172**, 1744-1753.
- Lundin, M., Monné, M., Widell, A., von Heijne, A. & Persson, M. A. (2003). Topology of the membrane-associated hepatitis C virus protein NS4B. *J Virol* **77**, 5428-5438.
- Luo, G., Xin, S. & Cai, Z. (2003). Role of the 5'-proximal stem-loop structure of the 5' untranslated region in replication and translation of hepatitis C virus RNA. *J Virol* **77**, 3312-3318.
- Mackenzie, J. (2005). Wrapping Things up about Virus RNA Replication. *Traffic* **6**, 967-977.
- Mackenzie, J. M., Jones, M. K. & Young, P. R. (1996). Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. *Virology* **220**, 232-240.
- Mackenzie, J. M., Khromykh, A. A. & Westaway, E. G. (2001). Stable expression of noncytopathic Kunjin replicons simulates both ultrastructural and biochemical characteristics observed during replication of Kunjin virus. *Virology* **279**, 161-172.
- Mackenzie, J. M. & Westaway, E. G. (2001). Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. *J Virol* **75**, 10787-10799.

- Major, M. E. & Feinstone, S. M. (1997). The molecular virology of hepatitis C. *Hepatology* **25**, 1527-1538.
- Major, M. E., Mihalik, K., Fernandez, J., Seidman, J., Kleiner, D., Kolykhalov, A. A., Rice, C. M. & Feinstone, S. M. (1999). Long-term follow-up of chimpanzees inoculated with the first infectious clone for hepatitis C virus. *J Virol* **73**, 3317-3325.
- Major, M. E., Rehermann, B. & Feinstone, S. M. (2001). Hepatitis C viruses. In *Fields Virology*, Fourth edn, pp. 1127-1161. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.
- Maldonado-Lopez, R. & Moser, M. (2001). Dendritic cell subsets and the regulation of Th1/Th2 responses. *Semin Immunol* **13**, 275-282.
- Maley, F., Trimble, R. B., Tarentino, A. L. & Plummer, J. T. H. (1989). Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. *Anal Biochem* **180**, 195-204.
- Manabe, S., Fuke, I., Tanishita, O., Kaji, C., Gomi, Y., Yoshida, S., Mori, C., Takamizawa, A., Yosida, I. & Okayama, H. (1994). Production of nonstructural proteins of hepatitis C virus requires a putative viral protease encoded by NS3. *Virology* **198**, 636-644.
- Marin, M. G., Bresciani, S., Puoti, M., Rodella, A., Gussago, A., Ravaggi, A., Pizzocolo, G., Albertini, A. & Cariani, E. (1994). Clinical significance of serum hepatitis C virus (HCV) RNA as marker of HCV infection. *J Clin Microbiol* **32**, 3008-3012.
- Martinez-Salas, E., Ramos, R., Lafuente, E. & Lopez de Quinto, S. (2001). Functional interactions in internal translation initiation directed by viral and cellular IRES elements. *J Gen Virol* **82**, 973-984.
- Matsuura, Y., Tani, H., Suzuki, K., Kimura-Someya, T., Suzuki, R., Aizaki, H., Ishii, K., Moriishi, K., Robison, C. S., Whitt, M. A. & Miyamura, T. (2001). Characterization of pseudotype VSV possessing HCV envelope proteins. *Virology* **286**, 263-275.
- Matto, M., Rice, C. M., Aroeti, B. & Glenn, J. S. (2004). Hepatitis C virus core protein associates with detergent-resistant membranes distinct from classical plasma membrane rafts. *J Virol* **78**, 12047-12053.
- McAllister, J., Casino, C., Davidson, F., Power, J., Lawlor, E., Yap, P. L., Simmonds, P. & Smith, D. B. (1998). Long-term evolution of the hypervariable region of hepatitis C virus in a common-source-infected cohort. *J Virol* **72**, 4893-4905.
- McLauchlan, J., Lemberg, M. K., Hope, G. & Martoglio, B. (2002). Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J* **21**, 3980-3988.
- Mercer, D. F., Schiller, D. E., Elliott, J. F., Douglas, D. N., Hao, C., Rinfret, A., Addison, W. R., Fischer, K. P., Churchill, T. A., Lakey, J. R., Tyrrell, D. L. & Kneteman, N. M. (2001). Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* **7**, 927-933.
- Merrick, W. C. (2004). Cap-dependent and cap-independent translation in eukaryotic systems. *Gene* **332**, 1-11.
- Meyer, K., Basu, A. & Ray, R. (2000). Functional features of hepatitis C virus glycoproteins for pseudotype virus entry into mammalian cells. *Virology* **276**, 214-226.
- Miller, R. H. & Purcell, R. H. (1990). Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. *Proc Natl Acad Sci U S A* **87**, 2057-2061.
- Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature* **427**, 313-319.
- Mottola, G., Cardinali, G., Ceccacci, A., Trozzi, C., Bartholomew, L., Torrisi, M. R., Pedrazzini, E., Bonatti, S. & Migliaccio, G. (2002). Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. *Virology* **293**, 31-43.

- Munoz-Jordan, J. L., Sanchez-Burgos, G. G., Laurent-Rolle, M. & Garcia-Sastre, A. (2003). Inhibition of interferon signaling by dengue virus. *Proc Natl Acad Sci U S A* **100**, 14333-14338.
- Murakami, K., Ishii, K., Ishihara, Y., Yoshizaki, S., Tanaka, K., Gotoh, Y., Aizaki, H., Kohara, M., Yoshioka, H., Mori, Y., Manabe, N., Shoji, I., Sata, T., Bartenschlager, R., Matsuura, Y., Miyamura, T. & Suzuki, T. (2006). Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. *Virology* **351**, 381-392.
- Murray, E. M., Grobler, J. A., Markel, E. J., Pagnoni, M. F., Paonessa, G., Simon, A. J. & Flores, O. A. (2003). Persistent replication of hepatitis C virus replicons expressing the beta-lactamase reporter in subpopulations of highly permissive Huh7 cells. *J Virol* **77**, 2928-2935.
- Namba, K., Naka, K., Dansako, H., Nozaki, A., Ikeda, M., Shiratori, Y., Shimotohno, K. & Kato, N. (2004). Establishment of hepatitis C virus replicon cell lines possessing interferon-resistant phenotype. *Biochem Biophys Res Commun* **323**, 299-309.
- Neddermann, P., Clementi, A. & De Francesco, R. (1999). Hyperphosphorylation of the hepatitis C virus NS5A protein requires an active NS3 protease, NS4A, NS4B, and NS5A encoded on the same polyprotein. *J Virol* **73**, 9984-9991.
- Nilsson, I. M. & von Heijne, G. (1993). Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. *J Biol Chem* **268**, 5798-5801.
- Ohba, K., Mizokami, M., Lau, J. Y., Orito, E., Ikeo, K. & Gojobori, T. (1996). Evolutionary relationship of hepatitis C, pesti-, flavi-, plantviruses, and newly discovered GB hepatitis agents. *FEBS Lett* **378**, 232-234.
- Op De Beeck, A., Montserret, R., Duvet, S., Cocquerel, L., Cacan, R., Barberot, B., Le Maire, M., Penin, F. & Dubuisson, J. (2000). The transmembrane domains of hepatitis C virus envelope glycoproteins E1 and E2 play a major role in heterodimerization. *J Biol Chem* **275**, 31428-31437.
- Patel, J., Patel, A. H. & McLauchlan, J. (2001). The transmembrane domain of the hepatitis C virus E2 glycoprotein is required for correct folding of the E1 glycoprotein and native complex formation. *Virology* **279**, 58-68.
- Pawlotsky, J. M. (2006). Hepatitis C virus population dynamics during infection. *Curr Top Microbiol Immunol* **299**, 261-284.
- Pavlovic, D., Neville, D. C., Argaud, O., Blumberg, B., Dwek, R. A., Fischer, W. B. & Zitzmann, N. (2003). The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. *Proc Natl Acad Sci U S A* **100**, 6104-6108.
- Pelletier, J. & Sonenberg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334**, 320-325.
- Pestova, T. V., Shatsky, I. N., Fletcher, S. P., Jackson, R. J. & Hellen, C. U. T. (1998). A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. *Genes Dev* **12**, 67-83.
- Peter, B. J., Kent, H. M., Mills, I. G., Vallis, Y., Butler, P. J. G., Evans, P. R. & McMahon, H. T. (2004). BAR domains as sensors of membrane curvature: The amphiphysin BAR structure. *Science* **303**, 495-499.
- Piccininni, S., Varaklioti, A., Nardelli, M., Dave, B., Raney, K. D. & McCarthy, J. E. (2002). Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. *J Biol Chem* **277**, 45670-45679.
- Pieroni, L., Santolini, E., Fipaldini, C., Pacini, L., Migliaccio, G. & La Monica, N. (1997). In vitro study of the NS2-3 protease of hepatitis C virus. *J Virol* **71**, 6373-6380.
- Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., Strand, D. & Bartenschlager, R. (2002). Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J Virol* **76**, 4008-4021.

- Pietschmann, T., Lohmann, V., Rutter, G., Kurpanek, K. & Bartenschlager, R. (2001). Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. *J Virol* **75**, 1252-1264.
- Pozzan, T., Rizzuto, R., Volpe, P. & Meldolesi, J. (1994). Molecular and cellular physiology of intracellular calcium stores. *Physiol Rev* **74**, 595-636.
- Qu, L., McMullan, L. K. & Rice, C. M. (2001). Isolation and characterization of noncytopathic pestivirus mutants reveals a role for nonstructural protein NS4B in viral cytopathogenicity. *J Virol* **75**, 10651-10662.
- Quinkert, D., Bartenschlager, R. & Lohmann, V. (2005). Quantitative analysis of the hepatitis C virus replication complex. *J Virol* **79**, 13594-13605.
- Radkowski, M., Wilkinson, J., Nowicki, M., Adair, D., Vargas, H., Ingui, C., Rakela, J. & Laskus, T. (2002). Search for hepatitis C virus negative-strand RNA sequences and analysis of viral sequences in the central nervous system: Evidence of replication. *J Virol* **76**, 600-608.
- Rajendran, L. & Simons, K. (2005). Lipid rafts and membrane dynamics. *J Cell Sci* **118**, 1099-1102.
- Reynolds, J. E., Kaminski, A., Kettinen, H. J., Grace, K., Clarke, B. E., Carroll, A. R., Rowlands, D. J. & Jackson, R. J. (1995). Unique features of internal initiation of hepatitis C virus RNA translation. *EMBO J* **14**, 6010-6020.
- Richnau, N., Fransson, A., Farsad, K. & Aspenstrom, P. (2004). RICH-1 has a BIN/Amphiphysin/Rvsp domain responsible for binding to membrane lipids and tubulation of liposomes. *Biochem Biophys Res Commun* **320**, 1034-1042.
- Roosendaal, J., Westaway, E. G., Khromykh, A. & Mackenzie, J. M. (2006). Regulated cleavages at the West Nile virus NS4A-2K-NS4B junctions play a major role in rearranging cytoplasmic membranes and Golgi trafficking of the NS4A protein. *J Virol* **80**, 4623-4632.
- Rouille, Y., Helle, F., Delgrange, D., Roingeard, P., Voisset, C., Blanchard, E., Belouzard, S., McKeating, J., Patel, A. H., Maertens, G., Wakita, T., Wychowski, C. & Dubuisson, J. (2006). Subcellular localization of hepatitis C virus structural proteins in a cell culture system that efficiently replicates the virus. *J Virol* **80**, 2832-2841.
- Sagan, S. M., Rouleau, Y., Leggiadro, C., Supekova, L., Schultz, P. G., Su, A. I. & Pezacki, J. P. (2006). The influence of cholesterol and lipid metabolism on host cell structure and hepatitis C virus replication. *Biochem Cell Biol* **84**, 67-79.
- Saiz, J. C., Lopez de Quinto, S., Ibarrola, N., Lopez-Labrador, F. X., Sanchez-Tapias, J. M., Rodes, J. & Martinez-Salas, E. (1999). Internal initiation of translation efficiency in different hepatitis C genotypes isolated from interferon treated patients. *Arch Virol* **144**, 215-229.
- Sakai, A., Claire, M. S., Faulk, K., Govindarajan, S., Emerson, S. U., Purcell, R. H. & Bukh, J. (2003). The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. *Proc Natl Acad Sci USA* **100**, 11646-51.
- Sakamoto, M., Akahane, Y., Tsuda, F., Tanaka, T., Woodfield, D. G. & Okamoto, H. (1994). Entire nucleotide sequence and characterization of a hepatitis C virus of genotype V/3a. *J Gen Virol* **75**, 1761-1768.
- Salemi, M. & Vandamme, A. M. (2002). Hepatitis C virus evolutionary patterns studied through analysis of full-genome sequences. *J Mol Evol* **54**, 62-70.
- Salonen, A., Ahola, T. & Kaariainen, L. (2005). Viral RNA replication in association with cellular membranes. *Curr Top Microbiol Immunol* **285**, 139-173.
- Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R. M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R. & Vitelli, A. (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* **21**, 5017-5025.

- Schlauder, G. G., Leverenz, G. J., Mattsson, L., Weiland, O. & Mushahwar, I. K. (1992). Detection of hepatitis C viral RNA by the polymerase chain reaction in serum of patients with post-transfusion non-A, non-B hepatitis. *J Virol Methods* **37**, 189-199.
- Schmidt-Mende, J., Bieck, E., Hugle, T., Penin, F., Rice, C. M., Blum, H. E. & Moradpour, D. (2001). Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem* **276**, 44052-44063.
- Schouten, A., Roosien, J., Bakker, J. & Schots, A. (2002). Formation of disulfide bridges by a single-chain Fv antibody in the reducing ectopic environment of the plant cytosol. *J Biol Chem* **277**, 19339-19345.
- Seipp, S., Mueller, H. M., Pfaff, E., Stremmel, W., Theilmann, L. & Goeser, T. (1997). Establishment of persistent hepatitis C virus infection and replication in vitro. *J Gen Virol* **78**, 2467-2476.
- Selby, M. J., Glazer, E., Masiarz, F. & Houghton, M. (1994). Complex processing and protein:protein interactions in the E2:NS2 region of HCV. *Virology* **204**, 114-122.
- Sentman, C. L., Olsson, M. Y. & Karre, K. (1995). Missing self recognition by natural killer cells in MHC class I transgenic mice. A ’receptor calibration’ model for how effector cells adapt to self. *Semin Immunol* **7**, 109-119.
- Shi, S. T., Lee, K. J., Aizaki, H., Hwang, S. B. & Lai, M. M. (2003). Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. *J Virol* **77**, 4160-4168.
- Shi, S. T., Polyak, S. J., Tu, H., Taylor, D. R., Gretch, D. R. & Lai, M. M. (2002). Hepatitis C virus NS5A colocalizes with the core protein on lipid droplets and interacts with apolipoproteins. *Virology* **292**, 198-210.
- Shimakami, T., Hijikata, M., Luo, H., Ma, Y. Y., Kaneko, S., Shimotohno, K. & Murakami, S. (2004). Effect of interaction between hepatitis C virus NS5A and NS5B on hepatitis C virus RNA replication with the hepatitis C virus replicon. *J Virol* **78**, 2738-2748.
- Shimizu, Y. K., Iwamoto, A., Hijikata, M., Purcell, R. H. & Yoshikura, H. (1992). Evidence for in vitro replication of hepatitis C virus genome in a human T-cell line. *Proc Natl Acad Sci U S A* **89**, 5477-5481.
- Shindo, M., Di Bisceglie, A. M., Biswas, R., Mihalik, K. & Feinstone, S. M. (1992). Hepatitis C virus replication during acute infection in the chimpanzee. *J Infect Dis* **166**, 424-427.
- Shirota, Y., Luo, H., Qin, W., Kaneko, S., Yamashita, T., Kobayashi, K. & Murakami, S. (2002). Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. *J Biol Chem* **277**, 11149-11155.
- Simmonds, P. (1999). Viral heterogeneity of the hepatitis C virus. *J Hepatol* **31 Suppl 1**, 54-60.
- Simons, K. & Toomre, D. (2000). Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1**, 31-39.
- Smith, D. B., Pathirana, S., Davidson, F., Lawlor, E., Power, J., Yap, P. L. & Simmonds, P. (1997). The origin of hepatitis C virus genotypes. *J Gen Virol* **78**, 321-328.
- Soccio, R. E. & Breslow, J. L. (2004). Intracellular cholesterol transport. *Arterioscler Thromb Vasc Biol* **24**, 1150-1160.
- Stoneley, M. & Willis, A. E. (2004). Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression. *Oncogene* **23**, 3200-3207.
- Su, A. I., Pezacki, J. P., Wodicka, L., Brideau, A. D., Supekova, L., Thimme, R., Wieland, S., Bukh, J., Purcell, R. H., Schultz, P. G. & Chisari, F. V. (2002). Genomic analysis of the host response to hepatitis C virus infection. *Proc Natl Acad Sci U S A* **99**, 15669-15674.
- Sumpter, R. J., Wang, C., Foy, E., Loo, Y. M. & Jr., G. M. (2004). Viral evolution and interferon resistance of hepatitis C virus RNA replication in a cell culture model. *J Virol* **78**, 11591-11604.

- Suzich, J. A., Tamura, J. K., Palmer-Hill, F., Warrener, P., Grakoui, A., Rice, C. M., Feinstone, S. M. & Collett, M. S. (1993). Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with the related pestivirus and flavivirus enzymes. *J Virol* **67**, 6152-6158.
- Svitkin, Y. V., Pause, A., Lopez-Lastra, M., Perreault, S. & Sonenberg, N. (2005). Complete translation of the hepatitis C virus genome in vitro: membranes play a critical role in the maturation of all virus proteins except for NS3. *J Virol* **79**, 6868-6881.
- Tanaka, T., Kato, N., Cho, M. J., Sugiyama, K. & Shimotohno, K. (1996). Structure of the 3' terminus of the hepatitis C virus genome. *J Virol* **70**, 3307-3312.
- Tanaka, Y., Shimoike, T., Ishii, K., Suzuki, R., Suzuki, T., Ushijima, H., Matsuura, Y. & Miyamura, T. (2000). Selective binding of hepatitis C virus core protein to synthetic oligonucleotides corresponding to the 5' untranslated region of the viral genome. *Virology* **270**, 229-236.
- Tellinghuisen, T. L., Marcotrigiano, J., Gorbalenya, A. E. & Rice, C. M. (2004). The NS5A protein of hepatitis C virus is a zinc metalloprotein. *J Biol Chem* **279**, 48576-48587.
- Tellinghuisen, T. L., Marcotrigiano, J. & Rice, C. M. (2005). Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature* **435**, 374-379.
- Thibeault, D., Maurice, R., Pilote, L., Lamarre, D. & Pause, A. (2001). In vitro characterization of a purified NS2/3 protease variant of hepatitis C virus. *J Biol Chem* **276**, 46678-46684.
- Thimme, R., Oldach, D., Chang, K.-M., Steiger, C., Ray, S. C. & Chisari, F. V. (2001). Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* **194**, 1395-1406.
- Tomei, L., Failla, C., Santolini, E., De Francesco, R. & La Monica, N. (1993). NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J Virol* **67**, 4017-4026.
- Tong, W. Y., Nagano-Fujii, M., Hidajat, R., Deng, L., Takigawa, Y. & Hotta, H. (2002). Physical interaction between hepatitis C virus NS4B protein and CREB-RP/ATF6beta. *Biochem Biophys Res Commun* **299**, 366-372.
- Tong, X. & Malcolm, B. A. (2006). Trans-complementation of HCV replication by non-structural protein 5A. *Virus Res* **115**, 122-130.
- Tseng, C.-T. K. & Klimpel, G. R. (2001). Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. *J Exp Med* **195**, 43-50.
- Tsukiyama-Kohara, K., Iizuka, N., Kohara, M. & Nomoto, A. (1992). Internal ribosome entry site within hepatitis C virus RNA. *J Virol* **66**, 1476-1483.
- Tu, H., Gao, L., Shi, S. T., Taylor, D. R., Yang, T., Mircheff, A. K., Wen, Y., Gorbalenya, A. E., Hwang, S. B. & Lai, M. M. C. (1999). Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. *Virology* **263**, 30-41.
- Urbanek, P., Tesar, V., Prochazkova-Francisci, E., Lachmanova, J., Marecek, Z. & Svobodnik, A. (2004). Treatment of early diagnosed HCV infection in hemodialyzed patients with interferon-alpha. Treatment of hepatitis C. *Blood Purif* **22**, 344-350.
- Urbani, S., Boni, C., Missale, G., Elia, G., Cavallo, C., Massari, M., Raimondo, G. & Ferrari, C. (2002). Virus-specific CD8+ lymphocytes share the same effector-memory phenotype but exhibit functional differences in acute hepatitis B and C. *J Virol* **76**, 12423-12434.
- Vagner, S., Galy, B. & Pyronnet, S. (2001). Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. *EMBO Rep* **2**, 893-898.

- Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G., Mizokami, M., Bartenschlager, R. & Liang, T. J. (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**, 791-796.
- Walker, C. M. (1997). Comparative features of hepatitis C virus infection in humans and chimpanzees. *Springer Semin Immunopathol* **19**, 85-98.
- Wang, C., Pflugheber, J., Sumpter, R., Jr., Sodora, D. L., Hui, D., Sen, G. C. & Gale, M., Jr. (2003). Alpha interferon induces distinct translational control programs to suppress hepatitis C virus RNA replication. *J Virol* **77**, 3898-3912.
- Wang, Q. M., Hockman, M. A., Staschke, K., Johnson, R. B., Case, K. A., Lu, J., Parsons, S., Zhang, F., Rathnachalam, R., Kirkegaard, K. & Colacino, J. M. (2002). Oligomerization and cooperative RNA synthesis activity of hepatitis C virus RNA-dependent RNA polymerase. *J Virol* **76**, 3865-3872.
- Wang, T. H., Rijnbrand, R. C. & Lemon, S. M. (2000). Core protein-coding sequence, but not core protein, modulates the efficiency of cap-independent translation directed by the internal ribosome entry site of hepatitis C virus. *J Virol* **74**, 11347-11358.
- Waris, G., Sarker, S. & Siddiqui, A. (2004). Two-step affinity purification of the hepatitis C virus ribonucleoprotein complex. *RNA* **10**, 321-329.
- Weir, M. L., Klip, A. & Trimble, W. S. (1998). Identification of a human homologue of the vesicle-associated membrane protein (VAMP)-associated protein of 33 kDa (VAP-33): a broadly expressed protein that binds to VAMP. *Biochem J* **333** (Pt 2), 247-251.
- Wellnitz, S., Klumpp, B., Barth, H., Ito, S., Depla, E., Dubuisson, J., Blum, H. E. & Baumert, T. F. (2002). Binding of hepatitis C virus-like particles derived from infectious clone H77C to defined human cell lines. *J Virol* **76**, 1181-1193.
- Westaway, E. G., Khromykh, A. A., Kenney, M. T., Mackenzie, J. M. & Jones, M. K. (1997a). Proteins C and NS4B of the flavivirus Kunjin translocate independently into the nucleus. *Virology* **234**, 31-41.
- Westaway, E. G., Khromykh, A. A. & Mackenzie, J. M. (1999). Nascent flavivirus RNA colocalized in situ with double-stranded RNA in stable replication complexes. *Virology* **258**, 108-117.
- Westaway, E. G., Mackenzie, J. M., Kenney, M. T., Jones, M. K. & Khromykh, A. A. (1997b). Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. *J Virol* **71**, 6650-6661.
- WHO (1999). Weekly epidemiological record: World Health Organization.
- Wicker, J. A., Whiteman, M. C., Beasley, D. W. C., Davis, C. T., Zhang, S., Schneider, B. S., Higgs, S., Kinney, R. M. & Barrett, A. D. T. (2006). A single amino acid substitution in the central portion of the West Nile virus NS4B protein confers a highly attenuated phenotype in mice. *Virology* **349**, 245-253.
- Wiegand, J., Buggisch, P., Boecher, W., Zeuzem, S., Gelbmann, C. M., Berg, T., Kauffmann, W., Kallinowski, B., Cornberg, M., Jaeckel, E., Wedemeyer, H. & Manns, M. P. (2006). Early monotherapy with pegylated interferon alpha-2b for acute hepatitis C infection: the HEP-NET acute-HCV-II study. *Hepatology* **43**, 250-256.
- Wong, S. S. & Wong, L.-J. C. (1992). Chemical crosslinking and the stabilization of proteins and enzymes. *Enzyme Microb Technol* **14**, 866-874.
- Wyles, J. P., McMaster, C. R. & Ridgway, N. D. (2002). Vesicle-associated membrane protein-associated protein-A (VAP-A) interacts with the oxysterol-binding protein to modify export from the endoplasmic reticulum. *J Biol Chem* **277**, 29908-29918.
- Wölk, B., Sansonno, D., Krausslich, H.-G., Dammacco, F., Rice, C. M., Blum, H. E. & Moradpour, D. (2000). Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *J Virol* **74**, 2293-2304.

- Xu, Z., Choi, J., Yen, T. S., Lu, W., Strohecker, A., Govindarajan, S., Chien, D., Selby, M. J. & Ou, J. (2001). Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. *EMBO J* **20**, 3840-3848.
- Yamaga, A. K. & Ou, J.-h. (2002). Membrane topology of the hepatitis C virus NS2 protein. *J Biol Chem* **277**, 33228-33234.
- Yamamoto, C., Enomoto, N., Kurosaki, M., Yu, S., Tazawa, J., Izumi, N., Marumo, F. & Sato, C. (1997). Nucleotide sequence variations in the internal ribosome entry site of hepatitis C virus-1b: No association with efficacy of interferon therapy or serum HCV-RNA levels. *Hepatology* **26**, 1616-1620.
- Yi, M. & Lemon, S. M. (2003). Structure-function analysis of the 3' stem-loop of hepatitis C virus genomic RNA and its role in viral RNA replication. *RNA* **9**, 331-345.
- Yi, M. & Lemon, S. M. (2004). Adaptive mutations producing efficient replication of genotype 1a hepatitis C virus RNA in normal Huh7 cells. *J Virol* **78**, 7904-7915.
- Yi, M., Villanueva, R. A., Thomas, D. L., Wakita, T. & Lemon, S. M. (2006). Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc Natl Acad Sci U S A* **103**, 2310-2315.
- Yoo, B. J., Selby, M. J., Choe, J., Suh, B. S., Choi, S. H., Joh, J. S., Nuovo, G. J., Lee, H. S., Houghton, M. & Han, J. H. (1995). Transfection of a differentiated human hepatoma cell line (Huh7) with in vitro-transcribed hepatitis C virus (HCV) RNA and establishment of a long-term culture persistently infected with HCV. *J Virol* **69**, 32-38.
- Yu, G. Y., Lee, K. J., Gao, L. & Lai, M. M. (2006). Palmitoylation and Polymerization of Hepatitis C Virus NS4B Protein. *J Virol* **80**, 6013-6023.
- Zheng, Y., Gao, B., Ye, L., Kong, L., Jing, W., Yang, X., Wu, Z. & Ye, L. (2005a). Hepatitis C virus non-structural protein NS4B can modulate an unfolded protein response. *J Microbiol* **43**, 529-536.
- Zheng, Y., Ye, L. B., Liu, J., Jing, W., Timani, K. A., Yang, X. J., Yang, F., Wang, W., Gao, B. & Wu, Z. H. (2005b). Gene expression profiles of HeLa Cells impacted by hepatitis C virus non-structural protein NS4B. *J Biochem Mol Biol* **38**, 151-160.
- Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & Chisari, F. V. (2005). Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A* **102**, 9294-9299.
- Zhu, H., Zhao, H., Collins, C. D., Eckenrode, S. E., Run, Q., McIndoe, R. A., Crawford, J. M., Nelson, D. R., She, J. X. & Liu, C. (2003a). Gene expression associated with interferon alfa antiviral activity in an HCV replicon cell line. *Hepatology* **37**, 1180-1188.
- Zhu, Q., Guo, J. T. & Seeger, C. (2003b). Replication of hepatitis C virus subgenomes in nonhepatic epithelial and mouse hepatoma cells. *J Virol* **77**, 9204-9210.