Structural and Functional Analysis

of the Hepatitis C Virus

Non-Structural Protein 5A

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Summary

Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus that belongs to the family *Flaviviridae*. Its genome has a length of about 9600 nucleotides and encodes a polyprotein with a length of about 3000 amino acids. This polyprotein is cleaved by host cell and viral proteinases into 10 different products. One of these is NS5A, a highly phosphorylated protein that contains an amphipathic α-helix at the N-terminus responsible for membrane association of the protein. The three dimensional structure of NS5A has not yet been resolved and the role of this protein in the HCV life cycle is unknown. NS5A is implicated in counteracting the antiviral state that is induced by interferon-α. In addition, NS5A is assumed to be involved in RNA replication. This assumption is based on the facts that NS5A is part of the viral replicase complex and that many cell culture adaptive mutations localize to the center of NS5A. However, its exact mode of action in RNA replication is not known.

It was the overall aim of this PhD thesis to gain insight into the role of NS5A for RNA replication. At the time this PhD thesis started, HCV replication could only be monitored by using subgenomic HCV replicons, carrying the selectable marker *neo*. RNA replication was scored by determining the G418 transduction efficiency upon transfection of the human hepatoma cell line Huh-7. To unravel the role of NS5A for RNA replication, the replicon system had first to be improved to allow monitoring of HCV replication in a transient and robust assay. To achieve this goal, replication enhancing, cell culture adaptive mutations were identified by analyzing persistently high replicating HCV-RNAs present in a selected cell-clone. Two mutations in NS3 and one in NS5A could be identified enhancing replication by several orders of magnitude in a cooperative manner. Upon insertion of these mutations into a replicon carrying a luciferase reporter gene transient replication of HCV RNA could be monitored. Taking advantage of this improvement, we analyzed the structure and function of NS5A with respect to its role for RNA replication.

First, we studied the importance of NS5A phosphorylation for RNA replication. A large panel of mutations affecting potential phospho acceptor sites were designed and analyzed for their replication ability and phosphorylation status. We found that certain serine substitutions in the center of NS5A enhanced RNA replication concomitant with a reduction of NS5A hyperphosphorylation. The same effect was observed for adaptive mutations in NS4B arguing for the same mechanism of replication enhancement. However, substitution of more than one potential NS5A phosphorylation site in the same molecule abrogated RNA replication. These

results suggest that phosphorylation of NS5A is a key regulatory mechanism controlling RNA replication.

Second, we studied the impact of mutations in the N-terminal amphipathic α-helix of NS5A on membrane association, RNA replication and NS5A phosphorylation. Structural changes of the α-helix were found to disturb incorporation of NS5A into the HCV replicase complex, concomitant with a block of RNA replication. Furthermore, these mutations interfered with hyperphosphorylation of NS5A arguing that the amphipathic α-helix besides its function to recruit NS5A to membranes is also required to ensure proper folding and phosphorylation of NS5A, which in turn is required for RNA replication.

Third, we wanted to know whether components of the HCV replicase complex (RC) are strictly cis-acting or can be complemented in trans. To address this question, two alternative trans-complementation assays were established. We found that NS5A is the only non-structural protein that can be complemented in trans. However, mutations localized in the amphipathic α-helix that interfere with membrane association and proper intergration of NS5A into the RC could not be rescued. Thus, intergration of NS5A into the RC is a prerequisite for functional replacement and restoration of RNA replication.

In the last chapter of this thesis, we aimed at the identification of cellular phosphoproteins that interact with NS5A. In the course of this study, amphiphysin II, a ubiquitously expressed protein was identified as a novel NS5A interaction partner. Amphiphysin II is supposed to play an important role in clathrin-mediated endocytosis by interaction with clathrin, clathrin adaptor protein 2 and dynamin. The interaction sites were mapped to a proline rich domain at the C-terminus of NS5A and an SH3-domain in amphiphysin II. Although this protein-protein interaction was observed in RCs of replicon cell-clones, it was dispensable for RNA replication.

In summary, these data show that NS5A plays a very important role for RNA replication that is most likely regulated by its phosphorylation status. Based on most recent data, showing that adaptive mutations in NS5A enhance RNA replication, but reduce virion formation, we speculate that NS5A may be a key regulator of the switch from RNA replication to virus assembly. This switch may be regulated by differential phosphorylation of this important protein.

1. Introduction

1.1 History of Hepatitis Viruses

Inflammation and necrosis of liver tissue, a condition termed hepatitis (hepar=liver) can have multiple causes. Besides bacterial pathogens like Leptospira, viruses are well known to cause severe liver diseases. Hepatitis is often accompanied by the occurrence of jaundice that was reported as early as in the 8th century AD. However, it was not until the 20th century that epidemiological studies and human volunteer experiments performed during and after World War II confirmed a viral etiology of this disease. Based on the epidemiological differences and clinical entities, in the 1940s viral hepatitis became separated into two distinct diseases termed *infectious hepatitis* and *homologous serum hepatitis*. Later, the terms *hepatitis A* (HA) and *hepatitis B* (HB), respectively, were applied, and the infectious agents associated with these diseases were named *hepatitis A virus* (HAV) and *hepatitis B virus* (HBV). In 1965 discovery of the Australia (Au) antigen was the beginning of a major breakthrough in viral hepatitis research. This Au antigen, found in a serum sample from an Australian aborigine reacted specifically with an antibody in the serum of an American hemophilia patient. Three years later in 1968 researchers demonstrated that the Au antigen was found specifically in the serum of hepatitis B patients. The Au antigen is now designated the hepatitis B surface antigen, or HbsAg. HBV is the prototype virus for the family of *Hepadnaviridae*. It is transmitted by parenteral and sexual routes. The majority of patients recover from their infection, however 2 to 10% of infected patients progress to chronic hepatitis. In 1973 HAV was identified as the causative agent of an infectious viral hepatitis. HAV represents the sole member of the genus Hepatovirus that belongs to the family *Picornaviridae*. The principal mode of transmission is by the fecal-oral route. Contamination of water and food by feces containing HAV therefore often results in community-wide outbreaks. In the mid-1970s another viral agent causing hepatitis was identified. The gastroenterologist Mario Rizzetto detected a previously unrecognized nuclear antigen in hepatocytes of patients suffering from chronic hepatitis B. The antigen was initially believed to be a previously unrecognized HBV-specific antigen, since it was never found in patients infected with non-B hepatitis. However transmission experiments in 1980 demonstrated that the so-called delta antigen (nucleocapsid protein) was a component of a transmissible pathogen called Hepatitis D virus (HDV) that required co-infection with HBV for virion formation and infection. For this reason, HDV virus represents a "defective virus" or virusoid. It

is able to replicate on its own, but the envelope protein, which is required for assembly, is provided by HBV.

In the early 1970s diagnostic tests were developed that allowed identification of all patients infected by HBV. Surprisingly, using these assays, it was found that most patients with transfusion-associated hepatitis did not have hepatitis B, nor did they suffer from an HAV infection. The term *non-A, non-B hepatitis* (NANBH) was introduced for these patients and the search began for the causative agent. During the ensuing 15 years, it was shown that NANBH was frequently transmitted by blood transfusion from presumably chronic carriers, resulting in chronic infections that could progress to liver fibrosis, cirrhosis and hepatocellular carcinoma. Several viral characteristics were also described using the chimpanzee as a model system for the detection of infectivity. There was evidence that the pathogen was a small (<50nm), enveloped virus. However it was not until 1989 that a group of scientists at Chiron Corporation identified the causative agent of NANBH. They used plasma from a chronically infected chimpanzee to generate an expression library that was screened by using sera from chronic NANBH patients. By using this approach, they were able to identify a virus that carried a single-stranded RNA molecule of about 10,000 nucleotides in length, containing one continuous long open reading frame (ORF) (15). The virus was designated Hepatitis C virus (HCV) and since its genomic organization most closely resembled the one of flaviviruses, it was classified as the distinct genus *Hepacivirus* within the family of *Flaviviridae*. First diagnostic assays for HCV demonstrated that at least 80% of patients with posttransfusion NANBH had developed antibodies against HCV (78).

Another insidious agent causing enterically transmitted non-A, non-B (ENANB) hepatitis was identified in 1990. The agent has been renamed hepatitis E virus (HEV). It is a small non-enveloped positive strand RNA virus that has been classified to the family "Hepatitis E-like viruses". Although most cases of viral hepatitis are caused by HAV, HBV, HCV, HDV or HEV, liver infections not due to these viruses occur. Efforts to identify additional hepatitis agents revealed 3 novel viruses designated GB virus-A (GBV-A), GBV-B (102) and GBV-C (31, 82, 85) (the latter also called Hepatitis G virus). These viruses have in common an enveloped particle, harboring a positive-sense RNA genome that carries one long ORF. GBVs have been categorized within the *Flaviviridae*, although they remain unclassified at the genus level. Nevertheless, it is now clear that these viruses do not cause liver disease. The following Table gives an overview about the known hepatitis viruses and the most important characteristics.

Table 1: Classification and characteristics of human hepatitis viruses (modified according to Bradley, 1990)

Virus	A	E	B	D	C	G
Virus family	Picorna	Hepatitis E-like viruses	Hepadna	Virusoid	Flavi	Flavi
Size (nm)	28	34	45	36	60	<100
Genome (kb)	+ RNA[a]	+ RNA	(DNA)[b]	- RNA[c]	+ RNA	+RNA
Size of genome	7,8	7,5	3,2	1,7	9,6	9,5
Envelope	-	-	+	+	+	+
Transmission	fecal-oral	fecal-oral	parenteral	parenteral	parenteral	parenteral
Persistence	-	-	+	+	+	+
fulminant hepatitis	rare	pregnant women	rare	often	rare	no
chronic hepatitis	never	never	often	very often	very often	no
onkogen	-	-	+	+	+	?
Therapy	-	-	α–Ifn*; ddI*	α–Ifn	α–Ifn	-
Vaccine	inactivated virus	-	rek. HBsAg	-	-	-
Diagnosis	Ag/Ab/NA*	Ag/Ab/NA	Ag/Ab/NA	Ag/Ab/NA	-/Ab/NA	-/Ab/NA

[a] Positive-stranded-RNA

[b] Replication cycle includes reverse transcription; the viral genome within the nucleocapsid is a DNA molecule

[c] circular negative-stranded-RNA

*Ag, Antigen; Ab, Antibody, NA, Nucleic acid; α-Ifn, Alpha-Interferon; ddI, Didesoxyinosin

HCV is a major cause for chronic liver disease. About 180 million people worldwide are infected with this insidious agent. Although HCV infections are frequently asymptomatic or associated only with mild symptoms, 80% of infected patients are unable to eliminate the virus. These people are at high risk of developing chronic liver disease, which in 10 to 20 % of cases leads to liver cirrhosis or hepatocellular carcinoma. HCV infection is primarily transmitted by parenteral routes i.e. blood and blood products. Sexual transmission and intrauterine infections play only a minor role. As early as 2 days after intravenous infection of chimpanzees, HCV RNA becomes detectable in the liver, the primary site of HCV replication. However, it is not clear which cells are in addition infected in vivo. Low replication of the virus prevents direct detection of viral proteins or RNA in infected cells. Based on kinetic studies and mathematical modeling it was estimated that about 10^{12} virus particles are produced per day in an infected individual (104, 120). A human adult liver consists of ca. 2 x 10^{11} hepatocytes. Assuming that 10% of hepatocytes are infected (161) on average 50 particles are produced per cell per day.

Currently the only available therapy for chronically infected patients is a combination treatment of interferon-α (IFN-α) and the nucleoside analogue ribavirin. However, only ~50% of all patients develop a sustained response. In addition, the therapy has a lot of side effects,

demonstrating the need for more effective antiviral therapeutics. Currently, no effective vaccine has been developed. The high variability of the virus, the lack of small animal models and the lack of a suitable *in vitro* cell culture system that allows propagation of the virus have hampered developments in this area.

However, in recent years significant advances have been achieved by the development of the replicon system, that allows autonomous multiplication of subgenomic viral RNAs (91). Using this system some light was shed on the process of intracellular RNA replication and it turned out to be a valuable tool for the development of new therapeutics.

1.2 Genomic organization of HCV

HCV is a positive-stranded RNA virus that has been classified to the genus *Hepacivirus* (153). Together with the genera *pestivirus*, *flavivirus* and the unassigned species GB virus A, -B, and -C it belongs to the family of *Flaviviridae*. Six closely related viral genotypes, classified by nucleotide sequence homology (about 25-35% at the nucleotide level), comprise the HCV genus (153). The most abundant worldwide is genotype 1 that also represents the least responsive to current therapy. Furthermore, within an HCV genotype, several subtypes can be defined, again based on nucleotide sequence homology (131).
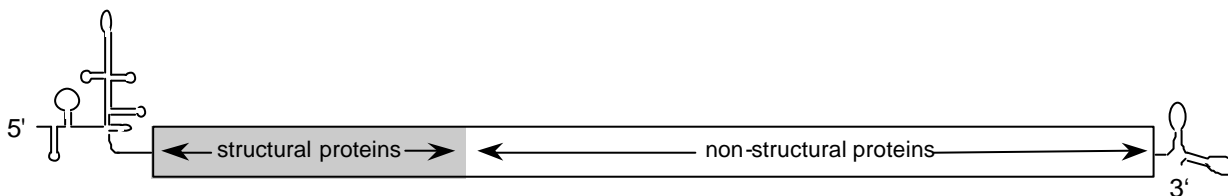
FIG. 1: HCV genome organization. Schematic presentation of the HCV genome, divided into structural and non-structural protein regions. The ORF is flanked at the 5` and 3` end by short, highly structured non-translated regions.

The HCV genome is a linear molecule of positive polarity that has a length of approximately 9600 nucleotides (nt) and that carries a single long open reading frame (Fig.1). The ORF is flanked at the 5` and 3` ends by short highly structured non-translated regions (NTR) that are important for viral replication and translation. The 341 nt long 5´NTR contains an internal ribosome entry site (IRES), which directs cap-independent translation of one large polyprotein. The IRES permits direct binding of the 40S ribosomal subunit in the absence of additional translation factors in a way that the initiator AUG start codon is placed in the P-site of the ribosome (38, 149, 155). Moreover, genetic analysis of the 5`NTR revealed that the first 125 nts are indispensable for RNA replication, showing that signals required for RNA replication overlap with those necessary for translation (35). The 3`NTR is about 350 nts long and has a

tripartite structure composed of an ~40 nts variable region downstream of the HCV coding sequence, a polypyrimidine tract of heterogeneous length, and a highly conserved 98-nt sequence designated X tail (77, 140, 141, 164). The latter two regions were shown to be essential for recovery of infectious HCV in chimpanzees (165). Apart from sequences located in the 5´ and 3´NTR, further RNA structures within the coding region may contribute to RNA replication. One such cis-acting RNA element (CRE) has been identified within the NS5B coding region (36, 152).
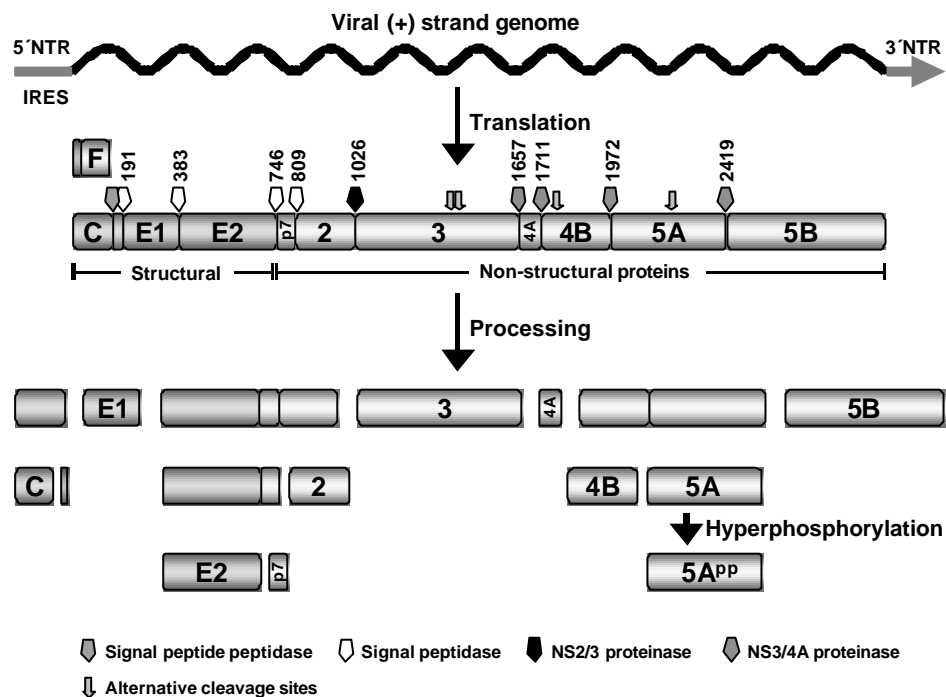
FIG. 2: Genomic organization of HCV and processing pathways of the polyprotein (adapted from Bartenschlager et al., Adv. Virus Res., in press, with permission). A schematic representation of the HCV genome with the 5' and 3' NTRs is shown at the top, the translation products are given below. Proteinases involved in processing of the polyprotein are indicated by arrows that are specified at the bottom of the figure. The major cleavage pathways leading to distinct processing intermediates, most notably E2-p7-NS2 and NS4B-5A are indicated. The hyperphosphorylation of NS5A probably occurs after complete proteolytic cleavage.

The HCV polyprotein is co- and posttranslationally processed into at least 10 different cleavage products that are ordered within the polyprotein from the NH_2 to the COOH terminus as follows: structural proteins core, envelope protein (E)1, E2, p7, non-structural (NS) protein 2, NS3, NS4A, NS4B, NS5A and NS5B (Fig.2). In addition, a novel HCV protein has recently been reported. Translation of this protein also initiates at the core gene AUG start codon, but due to ribosomal frame shifting into an alternative reading frame this additional protein, designated F (frameshift) protein is produced (154, 163).

The structural protein region up to NS2 is processed by cellular signal peptidases and a signal peptide peptidase (Fig.2). Cleavage between NS2 and NS3 is mediated by the NS2-3 proteinase. NS3 together with its co-factor NS4A represent the main viral proteinase complex that is responsible for processing at the NS3/4A, NS5A/5B, NS4A/4B, and NS4B/5A junctions with the indicated order. The core, E1 and E2 proteins are supposed to be the major constituents of the virus particle. The core protein that resides at the very N-terminus of the polyprotein, is a basic 21 kDa protein known to bind nucleic acids (34, 126, 129). Furthermore, the core protein was shown to homo-oligomerize presumbly required for assembly of nucleocapsid-like structures and it interacts with the E1 glycoprotein (88). The E1 and E2 proteins are highly glycosylated type I transmembrane proteins (23). It has been demonstrated that they are capable of forming non-covalently linked heterodimers probably representing the native pre-budding form of the glycoproteins (22, 24, 118). However due to the absence of a fully permissive cell culture system for HCV, detailed studies of the functions of E1 and E2 are limited. Nevertheless, there is evidence that E2 is responsible for the attachment to a receptor on host cells, whereas E1 may function as a fusion protein (44, 124). The small hydrophobic peptide p7 is an integral membrane protein carrying two transmembrane domains leading to a double membrane-spanning topology. Recently, two groups independently showed that p7 forms an ion channel in artificial lipid bilayer systems by hexamerisation suggesting that it may function as a viroporin (52, 110). These proteins are known to enhance membrane permeability and are believed to be required for late steps in virus assembly.

For most of the non-structural proteins defined functions have been described. NS3 is an enzymatic bifunctional protein whose amino-terminal domain acts as a proteinase together with NS2 or NS4A, whereas its carboxy-terminal domain possesses helicase and NTPase activity (49, 53, 56, 58, 74, 136, 137). NS4A is a cofactor of NS3 that activates the NS3 proteinase function (33, 83, 142). NS5B is the RNA dependent RNA polymerase (7). It forms the catalytic center of the HCV replication machinery and is responsible for the synthesis of negative-strand intermediates that in turn serve as a template for the generation of new genomic plus strands. The genomic variability of HCV is due to the high error rate of the viral RNA dependent RNA polymerase, which has been calculated to be in the range of about 10^{-4} substitutions per nucleotide per round of RNA replication. This property of NS5B is also one cause for the creation of a viral quasispecies meaning a pool of microvariants within an infected patient (96, 133). The very hydrophobic protein NS4B seems to have no obvious enzymatic activities, but induces the formation of intracellular membranous vesicles, so called membranous web structures (27). These membrane alterations have shown to be the site of intracellular RNA

replication (48, 101). There is evidence that in analogy to other positive-stranded RNA viruses, a membrane associated multiprotein complex is built up. This complex is supposed to consist of the non-structural proteins NS3 to NS5B, as well as cellular components and the viral RNA.

1.3 The HCV non-strucutral protein 5A

A still enigmatic protein is the HCV phosphoprotein NS5A that is also supposed to be part of the replication complex (RC). NS5A has been the subject of intensive research over the last decade. The protein is predicted to be predominantly hydrophobic and contains an amphipathic α-helix at the N-terminus that was shown to be necessary and sufficient to mediate membrane association (11). However, the three-dimensional structure of the protein has not yet been solved. Two phosphorylated forms of NS5A, termed p56 and p58, according to their electrophoretic mobility can be distinguished by one-dimensional SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) (68, 144). Whether these two forms share the same phosphorylation sites, is still unknown. NS5A is mainly phosphorylated on serine and to a much lesser extent, on threonine residues (for review, see reference 111). The enzyme responsible for this phosphorylation is not known, although there is evidence that members of the CMGC group of serine-threonine kinases including casein kinase II (CKII), cyclin-dependent kinases (CDKs) and mitogen-activated protein kinase (MAPKs) are involved in this process (61, 75, 121, 123). Moreover, the p70S6K kinase or enzymes with the same specificity seem to be involved in NS5A phosphorylation (17). The p56 form of NS5A is a basal phosphorylated protein, modified by phosphorylation most likely in the center of the molecule (amino acids 2200-2250) and near its C-terminus (amino acids 2351-2419). P58 is supposed to be the hyperphosphorylated form of p56 (68, 68, 115, 144). Centrally located serine residues 2197, 2201 and 2204 are important for this modification, although it is not clear whether these sites themselves are phosphorylated or just affect phosphorylation of other serine residues (144). The only phospho acceptor sites that have been mapped with biochemical approaches are serine residues 2321 and 2194 (70, 122). However, only the latter is highly conserved among HCV strains.

Both the degree and the requirements for hyperphosphorylation vary between different HCV isolates. For the HCV-H isolate (genotype 1a) at least two phosphorylated forms of NS5A were observed irrespective of its expression alone or within the context of the polyprotein (123). In contrast, for the genotype 1b HCV-BK, HCV-J and HCV-Con-1 isolates, more stringent requirements were found. For the HCV BK strain generation of functional NS2 from the NS2-3 precursor is necessary for the hyperphosphorylation of NS5A (86). In case of the HCV-J isolate

the presence of fully processed NS4A is required for the hyperphosphorylation of NS5A, although, it could also be mediated in trans (3, 68). For the Con-1- and BK-strains NS5A hyperphosphorylation is only found when NS5A is expressed in the context of a fully processed NS3 to NS5A polyprotein fragment. Small deletions in NS4B and even single amino acid substitutions in NS3 or NS4A that do not affect polyprotein cleavage abolish formation of p58 arguing that rather subtle changes have a tremendous effect in cis on the accessibility of NS5A to phosphorylation (76, 103). For two NS5A proteins of different HCV 2a genotypes basal phosphorylation, but not hyperphosphorylation has been reported (57, 123). In contrast, a recently described genotype 2a consensus genome designated JFH-1 expresses a basal and a hyperphosphorylated NS5A protein (69). Since phosphorylation of NS5A is a conserved feature among different isolates and genotypes and also among other members of the *Flaviviridae*, it is supposed to play an important role in the viral life cycle (121).

Multiple cellular proteins interacting with NS5A and resulting in very different effects on cell functions have been described (reviewed in146). For instance, NS5A appears to play a role in resistance against IFN-α by interfering with the activity of the protein kinase PKR that is responsible for the translational arrest and the induction of apoptosis in IFN-α treated cells (39, 40). Further evidence supporting this notion came from patient studies performed in Japan. A stretch of 40 amino acids in the center of NS5A conserved between HCV isolates from patients resistant to IFN-α therapy was identified. HCV variants carrying mutations in this region appeared to be more sensitive to IFN therapy. Therefore, this region was termed the IFN-sensitivity determining region (ISDR) (30). Since infection with HCV often leads to chronic persistence, the virus is supposed to modulate host cell signaling pathways in order to regulate cell growth and activation. Therefore, intensive investigation of the interactions between NS5A and cellular signaling pathways were conducted. Using different approaches such as yeast two-hybrid screens, NS5A has been shown to bind to a range of cellular signaling molecules like growth factor receptor binding protein 2 (Grb2) (139), Phosphoinositide-3-kinase p85 subunit (55), p53 (95), cyclin-dependent kinase 1 (Cdk1) (2), tumor necrosis factor receptor associated factor 2 (TRAF-2) (108) and Bax. A cellular transcription factor (45), as well as a putative nucleoplasmic transporter, karyopherin β3 (16), were also found as NS5A interaction partners. Due to these interactions NS5A is supposed to perturb kinase signaling cascades or cause alterations in cell growth and cell signaling.

Finally, several lines of evidence underscore the importance of NS5A for RNA replication. NS5A is part of the membrane-associated RC (48). This complex is supposed to consist of the non-structural proteins NS3 to NS5B, as well as cellular components and is responsible for the

replication of the viral genome. Furthermore, isolated replicon cell membrane fractions contained both p56 and p58 forms of NS5A, and were competent for synthesis of HCV RNA in vitro (54). However, the exact mode of action remains to be discovered.

1.4 HCV Replication cycle: a hypothetical model

Our current understanding of HCV replication is mainly hypothetical, since there is no fully permissive cell culture system that allows efficient propagation of the virus. However, some details begin to emerge due to the availability of efficient *in vitro* systems. Of major importance are replicons that recapitulate the intracellular steps of RNA replication, and HCV pseudoparticles that are instrumental for analyzing the early events in the infectious process. The overall outline of the HCV replication cycle is depicted in Fig. 3.
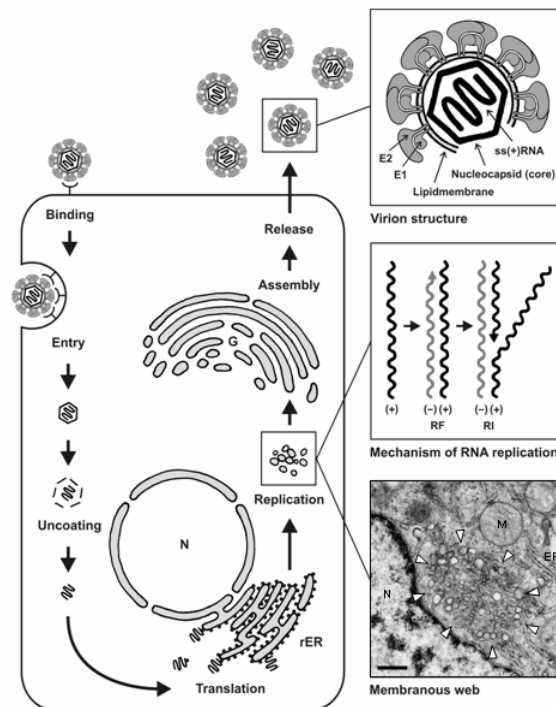
FIG. 3: Hypothetical HCV replication cycle (kindly provided by M. Frese). After infection of the host cell, particles are probably internalized by receptor-mediated endocytosis, the viral genome is liberated from the nucleocapsid (uncoating) and translated at the rough ER. NS4B induces the formation of membranous vesicles (referred to as a membranous web; electron micrograph lower right), which are supposed to serve as scaffold for the viral replication complex. After genome amplification and HCV protein expression, progeny virions are assembled and newly produced virus particles may leave the host cell by the constitutive secretory pathway. The upper right panel of the figure shows a schematic representation of an HCV particle. Note that the envelope proteins E1 and E2 are drawn according to the proposed structure and orientation of the TBEV envelope proteins M and E, respectively (Yagnik *et al.,* 2000). The middle panel shows a model for the synthesis of negative stranded (-) and positive stranded (+) progeny RNA *via* a double stranded replicative form (RF) and a replicative intermediate (RI). The bottom panel shows an electron micrograph of a membranous web (arrow heads) in an Huh-7 cell containing subgenomic HCV replicons. Bar, 500 nm. N, nucleus; ER, endoplasmic reticulum; M, mitochondria. The electron micrograph is adapted from Gosert *et al.* 2003, J Virol 2003;77:5487-5492, with permission from the American Society for Microbiology.

First insights into the biophysical properties of HCV particles emerged from chimpanzee experiments and electron microscopy analysis, demonstrating that HCV is a spherical enveloped particle with a size of approximately 40 to 70 nm in diameter. In analogy to closely related viruses like Dengue and Tick-borne encephalitis virus and according to structure predictions it can be assumed that the HCV envelope contains the viral glycoproteins E1 and E2 that form a homodimeric pair of E1- and E2-heterodimers present at the surface of the virus particle (Fig. 3). Enclosed by the viral envelope is a spherical nucleocapsid that is composed of multiple copies of the core protein and a single copy of the RNA genome. HCV particles bind to the host cell *via* a specific interaction between the HCV envelope glycoproteins and an as yet unknown cellular receptor. Receptor candidates identified by screening of a human cDNA expression library with recombinant E2 protein or binding assays with recombinant E2 are CD81 and human scavenger receptor class B type I (SR-BI) (116, 127). After binding, particles are probably internalized by receptor-mediated endocytosis (60). During the process of uncoating, viral genomic RNA is liberated from the nucleocapsid into the cytoplasm and translated *via* the IRES at the rough ER. During and after translation of the polyprotein a series of proteolytic cleavages takes place and the viral multiprotein RC is supposed to build up. The viral protein NS4B, perhaps in conjunction with other viral or cellular factors, induces the formation of membranous vesicles (27). The resulting membranous web is thought to serve as scaffold for the viral RC (48). By bromodesoxyuridine labeling of newly synthesized RNA, the web was shown to be the site of RNA replication. All viral non-structural proteins (NS3 to NS5B) colocalize with these structures, too. RNA synthesis of new viral genomic RNA occurs through the synthesis of full-length negative-sense RNA by the RNA-dependent RNA-polymerase. One molecule of negative-sense RNA serves as a template for the generation of large numbers of new positive-stranded RNAs. Thereby a ~10 fold excess of positive- versus negative-strand RNA is produced within the cell (91). After genome amplification and HCV protein expression, progeny virions are assembled. The site of virus particle formation has not yet been identified. It may take place at intracellular membranes derived from the ER or the Golgi compartment. Newly produced virus particles may leave the host cell by the constitutive secretory pathway.

1.5 Experimental systems

A major hurdle in studying HCV replication has been, and still is, the lack of efficient and simple virus cultivation systems. Numerous attempts have been made to propagate HCV in animals or

in cell culture. Until recently, these efforts either failed or replication levels were too low to allow reproducible studies (for reviews see 114). Thus far, the only animal that can reliably be infected is the chimpanzee. However, ethical concerns as well as high costs limit the number of experiments to an absolute minimum. Further attempts to develop a convenient animal model with primate species failed. Tupaias (Tupaia belangeri chinesis; tree shrew) became of interest for HCV infection experiments, since they can be infected with HBV (162). However, these animals have not achieved widespread use, as HCV infection is sporadic even in immunocompromised animals. Since the most common and amenable model in virus research is the laboratory mouse (Mus musculus domesticus), attempts were also made to establish an HCV mouse model. However, infection of the mouse is not possible due to the narrow host range of HCV. To overcome this problem, xenotransplantation systems have been developed that are based on the engraftment of human liver tissue into immunocompromised mice. In the so-called trimera system, mice are irradiated and subsequently reconstituted with bone marrow cells from a severe combined immunodeficiency (SCID)-mouse. Finally, ex-vivo infected human liver fragments are transplanted under the kidney capsule. In this system, HCV RNA can be detected for 10 to 50 days after transplantation by RT-PCR in up to 50% of the animals (64). A more robust mouse system is the Albumin-uroplasminogen activator (Alb-uPA) mouse (97). By selective backcrossing of an Alb-uPA-mouse, expressing a hepatotoxic protein with a SCID-mouse, homozygous immunodeficient SCID-Alb-uPA-mice were generated. Newborns of this mouse line suffer from liver damage due to liver-specific expression of the uPA transgene. However, mice can be rescued by efficient repopulation with human cells after transplantation of primary human hepatocytes. Efficient infection of human liver cells in these chimeric mice was observed in ~75% of the cases.

Apart from the animal models, several cell culture propagation systems for HCV have been developed within the last few years. These systems are often based on the infection of primary cell cultures or cell lines or the cultivation of primary cells from chronically infected patients. Unfortunately, all these systems suffer from poor reproducibility and low level of HCV replication that can only be detected by very sensitive, but error prone techniques. Compared to the infection of cell lines with HCV containing patient material, the introduction of cloned genomes into cells has several advantages. The RNA can be generated in nearly unlimited quantities by in vitro transcription, it is well defined and can be manipulated at will, permitting a detailed genetic analysis of viral functions. Furthermore, for many positive-strand RNA viruses such as poliovirus (PV), introduction of cloned genomes into cells was shown to result in the production of infectious virus progeny. However, in the case of HCV this approach turned out to

be rather difficult. Up to now only two reports have been published that describe the successful replication of transfected HCV genomes (20, 168). However, in both studies truncated HCV genomes lacking the authentic 3`NTR were used, raising justified concerns, whether true replication was achieved, because the poly(U)-tract and the X-tail are essential for RNA replication. Based on the observations that for several positive-strand RNA viruses such as alpha-, flavi- and pestiviruses expression of the structural proteins is not required for RNA replication and high expression levels of these proteins might have cytotoxic effects, in 1999 a corresponding cell culture system for HCV was developed that is based on the self-replication of selectable subgenomic HCV RNAs (replicons) in the human hepatoma cell line Huh-7 (91). These RNAs were derived from a cloned consensus genome of genotype 1b in which the region encoding the core to NS2-region was replaced by the selectable marker neomycin-phosphotransferase *(neo)* (Fig. 4). At the 5´and 3´end the ORF is flanked by the authentic HCV NTRs to ensure efficient replication. Furthermore, the first 48 nucleotides of the core coding sequence were maintained at the 3`end of the HCV IRES in order to guarantee full IRES activity. A heterologous IRES element derived from the Encephalomyocarditis virus (EMCV) was inserted upstream of the NS3 to NS5B coding region (Fig. 4), resulting in a bicistronic construct. Translation of the first cistron (*neo*) is mediated by the HCV IRES whereas translation of the HCV replication factors NS3 to NS5B is governed by the EMCV IRES.
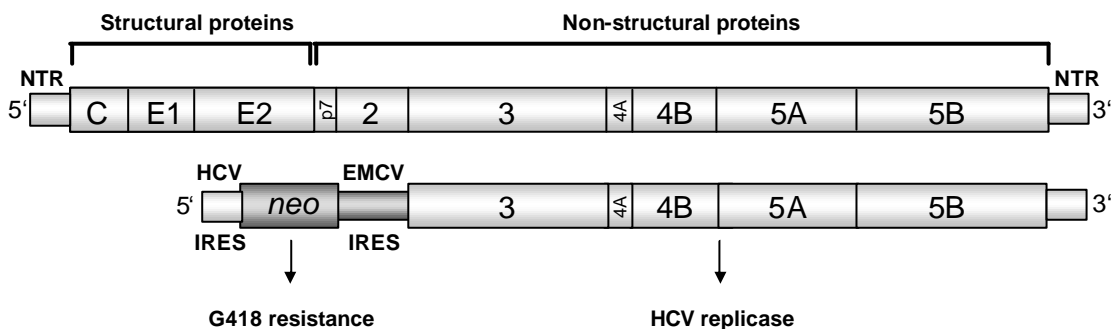
FIG. 4: Schematic representation of the HCV genome and a selectable subgenomic HCV replicon. The RNAs are composed of the HCV 5' NTR, the *neo* gene encoding the neomycin phosphotransferase, the EMCV IRES, the HCV coding sequence from NS3 to NS5B and the HCV 3' NTR.

Upon transfection of Huh-7 cells by electroporation and subsequent selection with G418, only those cells supporting replication of the replicon RNAs developed a resistance to G418, conferred by the amplified neo-gene. Non-transfected cells or cells unable to support replication were eliminated during selection (Fig. 5).
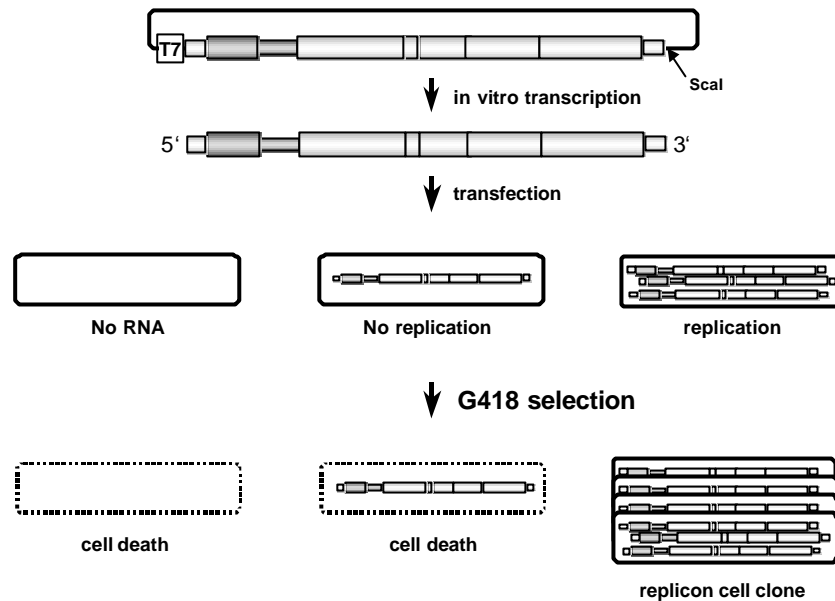
FIG. 5: Experimental approach for the generation of Huh-7 cell clones carrying persistently self-amplifying HCV replicons. A replicon plasmid shown in the upper panel was used for synthesis of in vitro transcribed replicon RNAs that were transfected into naive Huh-7 cells. If the RNA replicates within transfected cells, G418 resistance is conferred to the cells by neo and cells will survive G418 selection. Later on, G418 resistant cell colonies can be isolated and persistent replicon cell clones established. Non-transfected cells or cells that do not support RNA replication cannot survive G418 selection. T7, T7 RNA polymerase promoter; ScaI, the ScaI restriction site was used to linearize the plasmid DNA for the generation of an authentic HCV 3´end.

Using this approach a low number of cell clones could be established carrying high amounts of viral subgenomic RNA. Based on quantification by Northern hybridization, an average copy number of 1000 to 5000 positive strand RNA molecules per cell was determined. Replicon cell clones continuously passaged under selective pressure maintain the viral RNA for many years. Further analysis of replicon sequences from selected cell clones revealed that the viral RNA had aquired mutations in the HCV coding region that lead to a higher number of G418 resistant colonies upon retransfection of these replicons (8, 90). The adaptive mutations are a key determinant for efficient RNA replication in cell culture and give the answer to the puzzling discrepancy between the low number of G418 resistant colonies obtained after transfection, but high amounts of amplifying viral RNA within G418-resistant cells. The underlying mechanism of cell culture adaptation remains to be determined.

1.6 Subject of the Thesis

The overall aim of this PhD thesis was to study the role of NS5A for HCV RNA replication. In a first step, a reliable assay had to be established that would allow monitoring of transient RNA replication and thereby avoid time-consuming and error-prone selection of G418-resistant cells after transfection with selectable neo-replicons. To achieve this goal, highly adaptive mutations had to be identified and used to develop a reporter-gene based replication assay that facilitates mutation analysis of NS5A. In addition, these newly identified adaptive mutations should be used to develop alternative subgenomic as well as genomic replicons, in order to broaden the scope of the replicon system.

By using these tools a functional and structural analysis of NS5A should be performed. Phosphorylation of NS5A, as a potential regulatory mechanism to control RNA replication should be studied, as well as potential complementation of replication-functions in trans. Furthermore, membrane association of NS5A and its role for the formation of the HCV replicase complex and RNA replication should be investigated. Finally, cellular interaction partners of NS5A should be identified to gain insights into interactions with cellular kinase signaling networks and to evaluate potential functions of NS5A besides its potential role in RNA replication.

2. Materials and Methods

Centrifugation steps described in the materials and methods section were performed in different centrifuges. If no information about the rotor is given, an Eppendorf 5417 centrifuge was used. All rotors from high-speed centrifuges are made by Kendro.

2.1 Materials

2.1.1 Viruses

<u>MVA:</u> recombinant attenuated *Vaccinia-Virus* (strain Ankara) that constitutively expresses T7-RNA-polymerase; not pathogenic for humans; attenuated by multiple passage on chicken cells

2.1.2 Cells

<u>Bacteria</u>

E. coli Sure (Stratagene, Heidelberg): e14⁻(McrA⁻) λ(mrcCB-hsdSMR-mrr)171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5(Kan^r) uvrC [F´proAB lacI^{q}ZλM15 Tn10 (Tet^r)]

<u>Mammalian –cell lines</u>

Huh-7: human hepatoma cell line

Huh-7/T7 cells: Huh-7 cells were transduced with engineered murine-leukemia-virus particles that allow nuclear expression of T7 RNA polymerase after integration of the reotroviral genome into the cellular DNA.

2.1.3 Media

<u>Bacteria</u>

LB-Medium: 10 g trypton, 5 g yeast-extract, 5 g NaCl per 1 l medium; ampicillin was added at a concentration of 50-100 µg/ml. For hardening 1,5% agar-agar was added to the liquid medium.

<u>Cell cultures</u>

Cell monolayers of the human hepatoma cell line Huh-7 were routinely grown in Dulbecco's modified minimal essential medium (DMEM; Life Technologies, Karlsruhe, Germany) supplemented with 2 mM L-glutamine, nonessential amino acids, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10% fetal calf serum. G418 (Geneticin; Life Technologies) was added at a final concentration of 250 to 1,000 µg/ml to cell lines carrying HCV neo-replicons. Huh-7-hyg/ET and Huh-7-hyg/5.1 cells which contain the subgenomic hyg-replicons hyg-ET and hyg-5.1, were selected in medium supplied with 50 µg/ml hygromycin B. Transient replication assays were performed with a highly permissive Huh-7 cell clone

that was generated by the curing of a stable replicon cell clone (luc-ubi-neo-replicon) with a polymerase-inhibitor.

2.1.4 Antisera

<u>Primary antibodies</u>

Antibody	Specificity	Species	Subtype	Comments
NS3B	NS3 Helicase, 1230-1562	Rabbit	Polyclonal	1:100 IF 1:2500 Western
NS4B	4B/C-His from baculo, denat. Ag	Rabbit	Polyclonal	1:250 IF 1:2000 Western
NS5A	NS5A, 2102-2235, pool 5	Rabbit	Polyclonal	1:100 IF 1:2500 Western
NS5B-12B7.54.1	NS5B conf. epitope	Mouse	IgG monoclonal	1:2 IF
NS5B.1.5.3	NS5B linear epitope	Mouse	IgG monoclonal	1:1500 Western

<u>Secondary antibodies</u>

α-rabbit IgG, HRP conjugate (Sigma): 1:20000 for Western Blot

α-mouse IgG, HRP conjugate (Sigma): 1:20000 for Western Blot

α-mouse IgG , Alexa 488 conjugate (Molecular Probes): 1:1000 for IF

2.1.5 Vectors

pFK1-389neoEI3420-9605/wt: subgenomic HCV-replicon

pFK1-389 neoEI3420-9605/9-13F: first identified subgenomic replicon

pFK1-389neoEI3420-9605/ΔGDD: subgenomic HCV replicon carrying a 10-amino acid deletion at aa position 2731-2741 within the active center of the RNA dependent RNA polymerase (RdRp); serves a negative control for transfection experiments

pFK1-389lucEI3420-9605/wt: subgenomic HCV replicon carrying a luciferase reporter gene instead of the neomycin-phosphotransferase

pFK1-341spacerPILucEI3420-9605/ET: most adapted subgenomic reporter replicon carrying two adaptive mutations in NS3 (E1280I, T1280I) and one in NS4B (K1846T); second generation reporter replicon containing the PV-IRES (PI) for translation of the reporter gene, higher replication efficiency compared to pFK1-389lucEI3420-9605/ET

pFK1-389neoEI342-9605/wt: genomic HCV replicon, contains structural- and non-strucutral proteins

pTM/NS3-3`: expression vector that contains a T7 promoter for transcription of the HCV non-structural proteins NS3 to NS5B. Translation of the generated RNAs is mediated by the EMCV-IRES that is located downstream of the T7-promoter and upstream of the HCV proteins.

2.1.6 Primer sequences

Name	Sequence	Comments
A9386	5`TTA GCT CCC CGT TCA TCG GTT GG 3`	Long-PCR
A9413	5`CAG GAT GGC CTA TTG GCC TGG AG 3`	RT
AhygroPmeIStop	5`ACA AGA GTT TAA ACT CAG TTA GCC TCC CCC ATC TC3`	Cloning primer
AhygroScaI	5`CAC TAT CGG CGA GAA CTT CTA CAC AG 3`	Mutagenesis primer used for removal of the ScaI restriction site in the hygromycin-gene
AzeoPmeIstop	5`ACA AGA GTT TAA ACT CAG TCC TGC TCC TCG GCC AC3`	Cloning primer
S59	5`TGT CTT CAC GCA GAA AGC GTC TAG 3´	Long-PCR
ShygroAscI	5`TTG GCG CGC CAT GAA AAA GCC TGA ACT CAC CGC G3`	Cloning primer
ShygroScaI	5`CTG TGT AGA AGT TCT CGC CGA TAG TG3`	Mutagenesis primer used for removal of the ScaI restriction site in the hygromycin-gene
SzeoAscI	5`TTG GCG CGC CAT GGC CAA GTT GAC CAG TGC CG3`	Cloning primer

2.1.7 Buffers and solutions

30% Acrylamide stock solution: acrylamide and bisacrylamide were mixed in a concentration 29:1; solution was filtered and degassed before use

Coomassie staining-solution: 0,6 g/l Coomassie brilliant blue R250 (Serva) dissolved in 50% methanol/10% acetic acid

Cytomix: 120 mM KCl; 0,15 mM $CaCl_2$; 10 mM potassiumphosphate-buffer (pH 7,6); 25 mM Hepes (pH 7,6); 2 mM EGTA; 5 mM $MgCl_2$; pH of the solution was shifted to pH 7,6 with KOH; before use 2 mM ATP (pH 7,6) and 5 mM glutathione were freshly added.

DNA-loading buffer: 1 mg/ml bromphenolblue; 2 mg/ml xylene-cyanol; 1mM EDTA; 50% sucrose

Glyoxal-loading buffer: 0,25 mg/ml bromphenolblue; 0,25 mg/ml xylene-cyanol; 10 mM $NaPO_4$ (pH 7,0); 50% (v/v) glycerol

Guanidine-solution: 4 M guanidine-thiocyanate; 25 mM sodium citrate; 0,5% sarcosyl; 0,1 M 2-ß-mercaptoethanol (70µl per 10 ml solution) added before use, solution is stable for one month

Hybridization-solution: 5xSSC; 5xDenhardt-Solution; 50% (w/v) formamide; 1% (w/v) SDS; after addition of 100µg/ml salmon sperm DNA solution was used for prehybridization

Luciferase lysis-buffer: 1% triton-x-100; 25 mM glycyl-glycine; 15 mM magnesium sulfate; 4 mM EGTA; storage at 4°C; before use addition of 1 mM DTT

Luciferase assay-buffer: 25 mM glycyl-glycine; 15 mM potassium phosphate-buffer (pH7,8); 15 mM magnesium sulfate; 4 mM EGTA; before use addition of 1 mM DTT and 2mM ATP

Luciferase substrate-solution: 1:5 dilution of 1mM luciferine-solution with 25mM glycyl-glycine-solution

Methylene blue: 0,03% methylene blue; 0,3 M NaAc; pH 5,2

Mops-buffer (10x): 0,4 M mops (pH 7), 0,1 M NaAc, 0,01M EDTA; pH adjusted with NaOH

NP-buffer: 50 mM tris/HCl (pH 7,5), 150 mM NaCl, 1% nonidet P-40, 1% sodium desoxycholate, 0,1% SDS; for cell lysis add proteinase inhibitors 1 mM PMSF; 0,001 U/ml aprotinin and 4µg/ml leupeptin

PCR-buffer (10x): 100 mM tris/HCl (pH 8,3); 500 mM KCl; 15 mM $MgCl_2$; 0,01% gelatine

PBS (10x): 80 mM di-sodiumhydrogenphosphate; 20 mM sodium-di-hydrogenphosphate; 1,4 M NaCl

Protein sample-buffer: 200 mM tris/HCl (pH8,8); 5 mM EDTA; 0,1% bromphenol blue; 10% Sucrose; before use add 3% SDS and 2% ß-ME

RNA loading-buffer for formaldehyde gels: 50% (v/v) glycerol; 0,25mg/ml bromphenol blue; 0,25mg/ml xylencyanol; 1 mM EDTA (pH 8,0)

SSC (20x): 3M NaCl; 0,3 m sodium citrate

TAE (40x): 193,82 g tris-OH; 65,62 g NaAc; 29,78 g EDTA dissolved in water; adjust pH with ~50 ml acetic acid to 8,3 and fill up with water to the final volume of 1 liter.

TBE (10x): 250 mM tris-OH; 55 g boracid; 8,3 g EDTA dissolve in a total volume of 1 liter

TE: 10 mM Tris/HCl (ph 8,0); 1 mM EDTA

TGS (10x): 250 mM tris-OH; 1,92 M glycine; 1% SDS

Transcription-buffer RRL (5x): 400 mM Hepes (pH 7,5); 60 mM magnesium chloride; 10 mM spermidine; 200 mM DTT

2.2 Nucleic acid standard methods

2.2.1 Isolation of DNA

All protocols for the preparation of plasmid-DNA from bacteria are based on the principle of alkaline lysis first demonstrated by Birnboim and Doly (1979). After alkaline cell lysis plasmid DNA is released and can be separated from the chromosomal DNA that is attached to the cell wall by centrifugation.

Small scale preparation of plasmid DNA

Small scale preparations of plasmid DNA were performed with „Spin-Columns" from Sigma according to the manufacturer's protocol.

Bacteria from 5ml LB-culture were pelleted twice by centrifugation into 2ml tubes. After careful resuspension of the bacteria with 250µl resuspension buffer, 250µl of lysis buffer (NaOH/SDS) were added. Lysis was stopped after 5 min. incubation at RT by addition of 350µl neutralization buffer. Then, the suspension was centrifuged for 10 min. at maximum speed and the plasmid containing supernatant was transferred onto a spin-column that was pretreated with a column preparation solution. After 1 min. centrifugation at 8000g, the flow-through was thrown away and the column-bound DNA was washed by centrifugation steps one time with 500µl of an optional washing-buffer and one time with 750µl of an ethanol-containing buffer. After drying of the silica membrane by further 1 min. centrifugation step, plasmid-DNA was eluted in 100µl 10mM tris pH 7,5 solution. To increase the yield of plasmid DNA, elution can be perfomed by 2-time centrifugation with 50µl buffer per step. Capacity of the columns according to the manufacturer was around 40µg of DNA. Usually 5 to 10 µg of low copy plasmid DNA were isolated by using this protocol.

Large scale preparation of plasmid DNA

Large scale preparation of plasmid DNA was performed by using „Nucleobond AX 500-Cartridges" from Macherey&Nagel (Düren) according to the manufacturer's protocol.

Bacteria from 500 ml of overnight culture were pelleted by 10 min. centrifugation at 8000 g (GS-3, 4°C) and resuspended in 12 ml buffer S1. Then, cells were lysed by addition of 12 ml buffer S2 and incubated for 5 min. at RT. After addition of 12 ml buffer S3 and further 5 min. incubation on ice, solution was clarified by centrifugation for 45 min. at 4°C (> 10000g). In the meantime a „NucleoBond-cartridge" was

equilibrated with 5ml of buffer N2. To circumvent loading of SDS-precipitates onto the cartridge, the supernatant was filtered through a gauze. The cartridge material was ion exchange silica. After loading of the clear lysate on the cartridge, the bound DNA was washed twice with 12 ml of buffer N3 and eluted with 12 ml of the low salt and high pH elution-buffer N5. For DNA precipitation 0,7 vol. isopropanol were added, followed by centrifugation at >15000 g, 4°C for 30 min.. The resulting DNA pellet was washed once with 70% ethanol and finally dissolved in 500µl 10mM tris-buffer pH 7.5.

2.2.2 Agarose gel electrophoresis

DNA molecules can be separated for their length by agarosegel electrophoresis. Therefore DNA was mixed with 1/10 Vol. 10x bromphenol blue loading dye and loaded onto an agarose gel. A molecular weight marker from MBI Fermentas (Lambda-DNA/Eco130I/MluI and pUC19 DNA/MspI) was used for comparison to determine the size of the DNA fragments. In general 1 to 2% agarose gels were prepared for separation of DNA fragments. 1xTAE was used as running buffer, running-conditions were 5-10V/cm. For a better separation of very small fragments agarose concentration was increased up to 2.5%. In the case of very large fragments it was decreased to 0.8% agarose. For visualization of the DNA, ethidium bromide was added to the gel in a final concentration of 1µg/ml. Gels were documented by a video supported system after radiation with UV-light (312 nm).

2.2.3 DNA extraction from agarose gels

Extraction and purification of DNA from agarose gels was performed by using the „QIAEX II Gelextraction Kit" from Qiagen (Hilden) according to the manufacturer's protocol. An agarose gel slice containing the identified DNA fragment was solubilized by addition of at least 3 volumes of buffer QX1 and incubation for 10 min. at 50°C. During this time 5µg of DNA can be adsorped to 10µl QIAEX II suspension added to the mixture. After centrifugation (all centrifugation-steps were done for 30sec, 10000RpM) QIAEX II particles carrying the adsorbed DNA were washed once with 500µl of buffer QX1 and twice with 500µl of the ethanol-containing buffer PE. After the second wash with buffer PE the pellet was air-dried and DNA finally eluted by addition of 20µl 10mM tris-buffer. The supernatant containing pure DNA could be removed after centrifugation and the DNA was ready for further applications.

2.2.4 Ligation of DNA-fragments

For standard ligation 0,1pmol DNA of the digested vector and 0,3 pmol of insert-DNA-fragment were used. Furthermore, 1µl 10x ligase-buffer (MBI-Fermentas), 1U T4-DNA-ligase (MBI-Fermentas) and water to a final volume of 10µl were added to the DNA-fragments. After incubation at 22°C for 2h the

ligation-mix was added directly to competent bacteria for transformation. To avoid a high number of background colonies caused by religation of the vector, the linearized vector-DNA was pretreated with alkaline phosphatase. In more detail, 0,5-1 pmol DNA were incubated for 30 min. at 37°C in a 1x reaction buffer containing 1U calf intestinal alkaline phosphatase (cip; NEB). After preparative gel-electrophoresis DNA was extracted, purified and used for ligation.

2.2.5 Transformation of E. coli

Competent bacteria DH5α were generated by the CaCl$_2$-method. For transformation 50µl of these bacteria were incubated for at least 25 min. on ice with plasmid DNA or ligation reactions. After heat shocking of the bacteria at 42°C for 2 min., cells were mixed with 800µl of LB-media and incubated for a further 30 min. at 37°C. After centrifugation for 2 min. at 6000 rpm, supernatants were removed and cells resuspended in 50µl of LB. After plating of the cells on an antibiotic-containing LB-agar plate, colonies were grown over night at 37°C.

2.2.6 Restriction analysis of DNA

Restriction digests of DNA were performed with enzymes from the manufacturers NEB (Schwalbach) and Böhringer (Mannheim). Depending on the amount of DNA, restriction was performed in a total volume of between 15 and 200 µl ($\leq$ 0,1µg/µl, standard: 15µl analytic digestions, preparative digestions 100µl). Reactions were performed in a 1x reaction buffer for one or more hours at the recommended temperature. The amount of enzyme added to the reactions was dependent on the DNA concentration as well as on the unit definition given by the manufacturers. One unit of endonuclease activity is defined as the amount of enzyme that is needed to digest 1 µg substrate DNA (often DNA of the λ-bacteriophage) completely within 1 hour. 1µg λ-DNA corresponds to 1/32 pmol. Therefore, to digest 1pmol λ-DNA completely within 1 hour with an enzyme that cuts the DNA once, 32 units of the enzyme are necessary. Hence, 32U of an enzyme are recommended to digest 1pmol of plasmid DNA if it has one restriction site. If the plasmid DNA contain two restriction sites for the enzyme, 64U of the enzyme are necessary for a complete digestion.

2.2.7 Purification and precipitation of DNA

Purification of DNA for example after restriction digest was performed by phenol/chloroform extraction. Therefore the DNA was mixed with 1/10 vol. of 3 M NaAc pH 6.0 and extracted twice with 1 vol. TE saturated phenol and once with 1 vol. chloroform. The DNA containing upper phase was always

transferred into a new tube. Finally the DNA was precipitated by addition of 2.5 vol. ethanol or 0.7 vol. isopropanol. In the case of the ethanol precipitation the sample was incubated for 30 min. at –20°C and centrifuged for at least 15 min. at 13000 rpm. For precipitation with isopropanol, the sample was directly centrifuged for at least 30 min. at 13000 rpm, 4°C. Afterwards, the pelleted DNA was washed once with 70% ethanol and dissolved in an apropriate volume of water or buffer. As an alternative method we used the „QIAEX II Gelextraction Kit" of Qiagen (Hilden) to purify and concentrate DNA in a small volume of buffer.

2.2.8 Polymerase Chain Reaction (PCR)

PCR is a method to multiply a defined DNA sequence from a complex template DNA selectively. It is necessary that the flanking sequences of the target DNA are known, to generate two oligonucleotide primers (18-30 bp). One of these single stranded primers is complementary to the sequence of interest (sense primer) and the other to the complementary strand of the target sequence (antisense primer). Therefore the opposed primers surround the target sequence. The primers are used by the polymerase as the starting point (free 3´OH end) of amplification. Chain elongation is performed by addition of dNTPs in 5´ to 3´ direction. The steps of PCR amplification are as follows:

- heat denaturation of the double-stranded template DNA at 95°C
- primer annealing to the complementary sequences of the single stranded target sequence (45-59°C)
- primer extension by the action of DNA polymerase at 68° or 72°C (synthesis efficiency around 1kb/min.)

After primer extension the mixture is heated again to separate the strands. Then it is cooled to allow the primers to hybridize with the complementary regions of newly synthesized DNA, and the whole process is repeated. Each cycle literally doubles the content of the original target DNA. In general, 20-30 cycles are run, yielding a 10^6- to 10^9-fold increase in the target sequence.

The reaction mixture was prepared on ice. Each reaction contained 250µM dNTPs (each dNTP), 1x buffer, 1pmol/µl primer, H₂O, 100ng-1µg template DNA (for cloning-PCR) and lastly added 1µl of polymerase. The total volume of the reaction was in the range of between 25 and 100µl. Annealing temperature as well as elongation time were dependent on the primer composition and the length of the amplified sequence, respectively. All nucleotide sequences of PCR products were confirmed by sequencing.

Standard-program for cloning-PCR :

95°C	60 sec.	
48°C	30 sec.	5x
72°C	60 sec.	
95°C	20 sec.	
50°C	30 sec.	10x
72°C	60 sec.	
72°C	120 sec.	
10°C	∞	

2.2.9 PCR-based mutagenesis

PCR based mutagenesis (59) was performed to generate plasmids carrying point mutations, deletions or insertions at the desired site. For each mutagenesis PCR two complementary mutagenesis primers were required carrying the necessary nucleotide alterations. Furthermore two primers flanking the target sequence at the 5´and 3´end were ordered. In the first round two PCRs were performed (as described above). One reaction contained the sense primer for the flanking 5´sequence and the antisense mutagenesis primer, whereas the second contained the mutagenesis sense primer and the flanking 3´antisense primer. Both PCR products overlapped in the region where the alteration was introduced (mutagenesis primer sequences). The amplified fragments were separated by agarose gel electrophoresis and purified by using the „QIAEX II Gelextraction Kit" from Qiagen. In a third modified PCR 1µl of each of the purified DNA fragments was mixed. These fragments then hybridized to each other by their overlapping regions and the DNA polymerase finalized the complete sequence. After 5 cycles of amplification the flanking 5´sense and 3´antisense primers were added to the reaction and further 15 cycles were performed. The generated PCR fragment now carries the desired alteration and can be used for cloning after purification.

2.2.10 DNA sequencing analysis

Nucleotide sequences of the final constructs were confirmed by automated nucleotide sequencing using an ABI 310 sequencer (Applied Biosystems). Big dye version 1.1 (Applied Biosystems) was used for cycle sequencing according to the instruction of the manufacturer with slight modifications. 200-500ng of plasmid DNA were mixed with 2µl big dye mix (containing buffer, deoxy- and di-deoxy-nucleotides and

polymerase), 1µl sequencing primer (5pmol/µl), 1µl 5x buffer and water to a final volume of 10µl. The following program was used for cycle sequencing:

95°C	20 sec.	
50°C	20 sec.	30x
60°C	240 sec.	
10°C	∞	

To remove unincorporated dye-labeled di-deoxy nucleotides the reaction was scaled up to 100µl, mixed with 10µl 3M NaAc, 1µl 20% SDS and boiled for 5 min. at 98°C. Afterwards DNA fragments were precipitated with 2.5 vol. ethanol by centrifugation for 20 min. at maximum speed, RT. Finally the DNA pellet was washed once with fresh 70% ethanol, air-dried and dissolved in 20µl formamide. Analysis of the resulting sequences was done by using vector NTI (invitrogen).

2.2.11 Preparation of total RNA from cells

Total RNA from mammalian cells was prepared by a single-step isolation method first described by Chomczynski und Sacchi (14). 1×10^7 cells were lysed with 750µl of GITC solution. Pipetting the solution up and down degraded the genomic DNA. Then 1/10 vol. 2 M NaAc (pH4) and 1 vol. acidic phenol were added to the mixture. After addition of 1/5 vol. chloroform the mixture was vortexed and incubated on ice for 15 min. The phases of the milky solution were separated again by 10 min. centrifugation at 13000RpM, 4°C and the RNA containing upper phase was transferred into a new tube. During this step, the interphase should not be swirled up, since it contains the degraded DNA. Now the RNA was precipitated by addition of 1 vol. isopropanol. After 30 min. incubation at –20°C, the RNA was pelleted by centrifugation for 15 min. at maximum speed, 4°C. Finally the pellet was washed once with 70% ethanol and dissolved in an appropriate volume of water. The RNA was stored at –70°C, whereas 2µl of it were used for quantification.

2.2.12 Quantification of DNA and RNA with absorption spectroscopy

The concentration of nucleic acid was determined by their absorption at different wavelengths. A_{260} measurements are quantitative for nucleic acid preparations in microgram quantities, but absorbancy readings at 260nm cannot discriminate between DNA and RNA. The ratio of absorbance at 260 and 280nm can be used as an indicator of nucleic acid purity. Proteins for example, have a peak absorption at 280nm that will reduce the A_{260}/A_{280} ratio. Absorption at 320nm is an indicator for particulates in the solution or dirty cuvettes. Contaminations by aromatic moieties such as phenol absorb at 230nm. Before

measurement, DNA samples were diluted with buffer 1:50. The concentration of the sample was calculated by using the Lambert-Beersche law $c_{DNA}[\mu g/ml]=OD_{260}$ x ε x dilution factor. (ε_{DNA}: 40; ε_{RNA}: 50)

2.2.13 Reverse transcription of RNA and amplification of c-DNA (RT-PCR)

Amplification of replicon RNAs was done by long-distance RT-PCR using a mixture of polymerases. One microgram of totalRNA and 50 pmol of primer A9413 (CAG GAT GGC CTA TTG GCC TGG AG) were mixed in a total volume of 10.5 µl and denatured for 10 min at 65°C. Afterwards the samples were put directly on ice to prevent renaturation. To each sample, 4µl RT-buffer, 2µl 100mM DTT, 2µl 10 mM Na-dNTP and 0,5µl RNasin (1 U/µl) were added. Reverse transcription was performed with Expand-RT (Roche Biochemicals, Mannheim, Germany) in a total volume of 20 µl and after 1 h at 42°C, different amounts of the reaction mixture (0.5 to1µl of c-DNA) were used for PCR with the Expand Long Template PCR system (Roche Biochemicals). The total volume of the reaction was 25µl. Two separate mastermixes were made, one containing 1,25µl 10mM dNTP (each dNTP 10mM); 6,25µl water, and primers S59 (TGT CTT CAC GCA GAA AGC GTC TAG) and A9386 (TTA GCT CCC CGT TCA TCG GTT GG), the other containing 2,5µl 10 x Expand^{TM}Long Template PCR buffer 3, 0,75µl 25 mM MgCl$_2$, 8,75µl water and 0,5µl Expand^{TM}Long Template polymerase-mix. 9,5µl of mastermix 1 and 12,5µl of mastermix 2 were added to the template-DNA. Cycle conditions were two min of initial denaturation at 94°C and 30 cycles of 10 s at 94°C, 90s at 54°C, and 540 s at 68°C. After 10 cycles, the extension time was increased 10 s for each additional cycle. After a final 10-min incubation at 68°C, PCR products were purified by preparative agarose gel electrophoresis, restricted with *Sfi*I, AscI or SpeI and inserted into the parental construct pFK-1-389neoEI3420-9605/wt.

2.2.14 In vitro transcription

Linearized plasmid DNA was used for the generation of "run off" transcripts. Therefore, plasmid DNA was restricted with *Ase*I and *Sca*I (New England Biolabs, Bad Schwalbach/Taunus, Germany) and after extraction with TE saturated phenol and chloroform and precipitation with ethanol dissolved in 40µl RNase-free water. The in vitro transcription reaction had to be mixed at RT, because otherwise spermidine present in the buffer would precipitate the DNA. Each reaction had a volume of 100µl, containing 20µl of 5x RRL (400mM HEPES (pH 7.5), 60 mM MgCl$_2$, 10 mM spermidine, 200 mM dithiothreitol), 6,25µl 25mM each nucleoside triphosphate, 50 U of RNasin (Promega, Mannheim, Germany), 6 µg of restricted plasmid DNA, and 4µl of T7 RNA polymerase (15U/µl, Promega). After 2 h at 37°C, an additional 2µl (30U) of T7 RNA polymerase per µl was added and the reaction mixture was incubated for another 2h. Transcription was terminated by the addition of 6µl of RNase-free DNase (Promega) (1.2U per µg of plasmid DNA) and 30 min of incubation at 37°C. After addition of 60µl 2M

NaAc pH 4,5 the total volume was scaled up with water to 600µl. Then, 2/3 vol. acidic phenol was added to the reaction mixture, mixed and incubated on ice for 15 min. After 10 min. centrifugation at maximum speed, 4°C, supernatant was extracted with 1 vol. chloroform, DNA was precipitated with 0.7 vol. isopropanol at RT and dissolved in 50µl RNase-free water. The RNA concentration was determined by measurement of the optical density at 260 nm, and RNA integrity was checked by denaturing agarose gel electrophoresis. By this protocol around 1-2µg RNA per µl are produced.

To generate ^{32}P-radio-labeled riboprobes for Northern-hybridization, 0,5µg linearized, phenol/chloroform extracted DNA was added to 4µl 5x transcription-buffer (Promega), 2µl 100mM DTT, 40U Rnasin (Promega), 2µl 5 mM non radioactive NTPs, 2µl 100 µM CTP, 5µl 50 µCi α^{32}P-CTP and 1µl RNA-polymerase (T3- or T7-RNA-Polymerase, Promega) in a total volume of 20µl. The mixture was incubated for 1h at 37°C. Afterwards, DNA was degraded by addition of 1µl RQ1 DNase (Promega) and the probe was clarified from free radioactive nucleotides by using a Sephadex G25 column (Amersham). The probe was eluted from the column with 1ml water (protocol according to the manufacturer).

2.2.15 RNA-formaldehyde-gel electrophoresis

To obtain 100ml of a 1% gel, 1g agarose and 72g water were weighed, the agarose dissolved by boiling and the gel-solution cooled down to 60°C. After addition of 10 ml 10x mops-buffer and 18 ml 12,3 M formaldehyde the gel was filled into the chamber. For preparation of the samples, 4µl of in vitro transcript were mixed with 2,5µl 10x mops-buffer; 4,5µl 12,3 M formaldehyde and 12,5µl formamide and incubated for 15 min. at 55°C. Then the mixture was incubated for 5-10 min. at RT and 1µl ethidium bromide-solution (10mg/ml) was added. After further 5 min. incubation time, 5µl RNA-loading dye was added and the sample was loaded onto the gel. Electrophoresis was performed in 1x mops-buffer at 5V/cm.

2.2.16 RNA-transfection and selection of G418-resistant cell lines

Transfection of in vitro transcripts into Huh-7 cells was performed by electroporation. Therefore, $2x10^6$ cells were seeded on 15 cm dishes 4 days prior electroporation. At the time of electroporation cells were grown up to 80-90% density. They were washed once with PBS, trypsonized with 0,05% trypsin/0,02% EDTA, resuspended in complete media and centrifuged for 5 min. at 500g. An aliquot was used to determine the number of cells. After two further washing steps by resuspension and centrifugation of the cells in PBS, the number of cells was adjusted to $1x10^7$cells/ml with a special electroporation buffer (cytomix). 10 to 1000 ng of in vitro transcripts adjusted with total RNA from naive Huh-7 cells to a final amount of 10 µg was mixed with 400 µl of the cell suspension. Electroporation conditions were 960 µF and 270 V using a Gene pulser system (Bio-Rad, Munich, Germany) and a cuvette with a gap width of

0.4 cm (Bio-Rad). Cells were immediately transferred to 8 ml of complete DMEM and seeded in a 10-cm-diameter cell culture dish. After 24 h, medium was replaced by complete DMEM supplemented with 250 -500 µg of G418 per ml. Medium was changed weekly, and 3 to 4 weeks after electroporation, colonies were stained with Coomassie brillant blue (0.6 g/liter in 50% methanol-10% acetic acid). To determine the number of CFU of a given RNA, serial dilutions from 300 to 0.25 ng of RNA were transfected into Huh-7 cells and processed further as described above. For the transcomplementation assay selection conditions were slightly modified. In pilot experiments, we found that the number of G418-resistant colonies obtained after transfection of naive Huh-7 and Huh-7-hyg cells depends on the amount of RNA transfected into the cells, the G418 concentration and the duration of selection. Optimal results with respect to lowest colony formation of the mutants after transfection into naive cells and highest colony numbers when using Huh-7-hyg cells carrying the helper replicon were obtained with 250 µg/ml G418 and 1 µg mutant replicon RNA. Under these conditions it took ~6 months to establish a cell clone from one individual colony.

2.2.17 Transient replication assay

Huh-7 cells were transfected by electroporation using 5 µg of a luciferase reporter replicon. After addition of 12 ml complete DMEM, 1 ml aliquots of the cell suspension were seeded into a 10-cm^2 culture dish and harvested at different time points after transfection (given in the result section). For determination of luciferase activity, cells were washed 3 times with phosphate-buffered saline (PBS) and scraped off the plate into 350 µl ice-cold lysis buffer (1% Triton X-100, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA and 1 mM DTT). One hundred microliters of lysate was mixed with 360 µl of assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, 2 mM ATP and 15 mM K_2PO_4 [pH 7.8]) and, after addition of 200 µl of a 200 µM luciferin stock solution, measured in a luminometer (Lumat LB9507 from Berthold, Freiburg, Germany) for 20 s. Values obtained with cells harvested 4h after electroporation were used for normalization since they reflect transfection efficiency.

In the case of the transient trans-complementation assay, Huh-7 cells were co-transfected by electroporation as described above using 5 µg of a neo-replicon as helper RNA (or 10 µg of a helper RNA encoding the NS3 to NS5A sequence) and 1 µg of a replication-deficient luciferase reporter replicon.

2.2.18 Glyoxal agarose gel electrophoresis and Northern-hybridization

To determine plus stranded-HCV-RNA within total RNA, Northern hybridization was performed using α^{32}P-CTP labeled minus strand riboprobes. Total RNA was prepared by a single-step isolation method

(see above). For sample preparation, 5 to 20µg of total RNA were mixed with 4,1µl 100 mM Na-phosphatebuffer pH7; 20,5µl DMSO; 6µl 6M deionized glyoxal and water up to a volume of 40.6µl. Samples were denatured for 1h at 50°C and afterwards 10,9 µl of loading dye were added. The samples were loaded onto a denaturing 1% glyoxal-agarose gel. The gel contained 1% agarose and 10mM Na-phosphate buffer, pH 7. For quantification 10^9, 10^8 and 10^7 molecules of in vitro transcribed RNA were also loaded onto the gel. Electrophoresis was performed in a 10 mM Na-phosphate buffer, pH7. To maintain the pH at 7, buffers were mixed during the run by magnets. After electrophoresis, RNA was transferred to a positively charged nylon membrane (Hybond) *via* vacuum blotting using 50mM NaOH (0,3 bar pressure). Then, the RNA was immobilized on the membrane by incubation at 80°C for 2 hours. Prior to hybridization, the membrane was stained with methylene blue and cut ~0.5 cm below the 28S rRNA band. Both membranes were pre-hybridized for at least 20 min. with hybridization-solution containing 10µg/ml salmon-sperm DNA. For hybridization, the pre-hybridization solution was replaced by hybridization solution and the radio labelled probes were added to the membranes. The upper strip containing the HCV replicon RNA was hybridized with a ^{32}P-labeled negative-sense riboprobe complementary to the *neo*-gene, the *hyg*-gene or HCV specific sequences. The lower strip that was hybridized with a β-actin-specific antisense riboprobe was used to correct for total RNA amounts loaded onto each lane of the gel. Hybridization was performed over night at 68°C. Next day, the membranes were washed twice for 15 min. with 2 x SSC, 0,1%SDS solution and once with a more stringent washing solution containing 0,2 x SSC, 0,1%SDS. Signals were detected by autoradiography.

2.2.19 Transfection of Huh-7/T7 cells with Lipofectamine 2000

$1x10^5$ Huh-7/T7 cells were seeded on 10^2cm-dishes and grown until the next day to a density of around 90-95%. Medium was exchanged against 1ml DMEM complete without Penicillin and Streptomycin. For transfection two mixes were prepared in luminometer tubes according to the manufacturer's protocol. One mix contained 1.6µg DNA and the other 4µl Lipofectamine 2000. Both mixes were filled up with Optimem medium to a total volume of 100µls. After 5 min. incubation, both solutions were mixed together and incubated for maximal 30 min. at room temperature. During this time the solution becomes cloudy. Then the 200µl transfection-mix were added to the cells and after a further 3 to 4 hours' incubation at 37°C transfection-medium was replaced by complete DMEM. For radioactive labelings medium was again replaced by label-medium. For immunofluorescence analysis, the cells were fixed with 3% paraformaldehyde 8 hours after transfection.

2.3 Expression and Analysis of Proteins

2.3.1 SDS-polyacrylamide -gel electrophoresis (SDS-Page)

Proteins can be separated by their molecular weight under denaturing conditions in a sodium dodecylsulfate-polyacrylamide gel. Therefore gels with a size of 18 x 40cm were made using an acrylamide concentration of between 7 and 12% for the separating gel. The acrylamide/bisacrylamide solution was a 30% stock solution, containing a 29:1 composition of acrylamide:bisacrylamide. The polymerisation reaction was started after addition of 1/1000 vol. TEMED and 1/1000 vol. saturated ammoniumpersulphate to the separating gel and it was poured immediately into gel casts. 5 ml of isopropanol was applied to remove air bubbles, and the gel was left to set for around 30 minutes. The stacking gel contained 5% acrylamide stock solution. Protein samples were denatured for 5 min. at 95°C before loading onto the gel. Electrophoresis was performed in 1x TGS buffer at a current strength of 45mA. To determine the molecular weight of the sample -proteins, a protein standard designated "Prestained Protein Marker" (NEB, Schwalbach) was used for comparison. This standard was composed of a mixture of proteins with defined molecular weight. After electrophoresis the gels were stained with Coomassie blue solution for 20 min. at 60°C. Thereby proteins were fixed within the gel. For destaining, gels were incubated for 20 min. at 60°C in a 5% methanol/5% acetic acid solution. For analysis of immunoprecipitated or radiolabelled proteins, the resolution gel was dried and signals detected by autoradiography.

2.3.2 Western blot

Cells were lysed by a 1-min sonification in denaturing protein sample buffer (200 mM Tris-HCl [pH 8.8], 5 mM EDTA, 0.1% bromophenol blue, 10% sucrose, 3.3% sodium dodecyl sulfate [SDS], and 2% 2-mercaptoethanol). Aliquots of cell lysates corresponding to 10^6 cells were loaded onto SDS–8% or 10% polyacrylamide gels and after electrophoresis transferred to a polyvinylidene difluoride membrane (PolyScreen; NEN Life Science Products, Zaventem, Belgium) using a semidry blotter (Bio-Rad, Munich, Germany). Blotting conditions were 1mA/cm^2 for at least 1h. Membranes were incubated overnight in blocking buffer (PBS containing 0.5% Tween 20 and 5% milk powder [wt/vol]) to saturate unspecific binding-sites, and a primary antibody was added thereafter at a given dilution for 1 h (for dilutions of antibodies see Table). After being washed three times with 0.5% Tween 20 in PBS, the membrane was incubated with a second antibody conjugated with peroxidase (Sigma, Deisenhofen, Germany) in blocking buffer for 1 h and washed three times as described above. Bound antibodies were detected by chemiluminescence using luminol and a specific enhancer (SuperSignal West Dura Extended

Duration Substrate; KMF Laborchemie, St. Augustin, Germany). Chemiluminescence was detected by autoradiography.

2.3.3 Vaccinia virus (VV) T7-expression system

For transient expression of HCV proteins in cell cultures the vaccinia virus-T7 hybrid system was used. Therefore 2.5×10^5 Huh-7cells were seeded in $10cm^2$ diameter dishes and infected with 25µl MVA the next day. After 1 h incubation at RT the inoculum was removed and cells were incubated in complete DMEM for 1h at 37°C. Then cells were transfected with 0.4 µg DNA and 10µl Effectene (Qiagen, Hildesheim, Germany) as instructed by the manufacturer. After incubation overnight at 37°C, cells were washed with prewarmed DMEM lacking cysteine/methionine and incubated for 6 h in DMEM supplemented with 2 mM glutamine, 10 mM Hepes and 100 µCi of $[^{35}S]$ protein labeling mixture (Express; NEN Life Science, Köln, Germany) per ml. For radiolabeling of proteins with $[^{32}P]$orthophosphate (NEN Life Science, Köln, Germany), cells were transfected as described above, washed three times with phosphate-free medium (Sigma, Deisenhofen, Germany), and labeled for 6 h in the same medium supplemented with 100 µCi of $[^{32}P]$orthophosphate per ml. After labeling, cells were washed once with PBS and lysed with 1 ml NPB. HCV-specific proteins were isolated by immunoprecipitation from cell lysates under denaturing conditions. Immunoprecipitation was performed overnight at 4°C and proteins were separated by SDS-PAGE. For optimal separation of the two NS5A phosphoprotein variants, 8 to 10% SDS-PAGE was performed. Dephosphorylation of NS5A was performed with lambda protein phosphatase (New England Biolabs GmbH, Schwalbach/Taunus, Germany), using immunoprecipitated proteins as instructed by the manufacturers. Immunocomplexes were washed three times with lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) and once with the corresponding phosphatase assay buffer. Samples were treated with 400 U of phage l phosphatase or 10 U of CIP. After 1 h at 30°C, samples were centrifuged, supernatants were removed, and dephosphorylated immunocomplexes were analyzed by SDS-PAGE and autoradiography.

2.3.4 Immunoprecipitation

Preadsorption was performed by mixing 46µl antibody with 25µl protein-A sepharose (1:1 slurry, Biorad) per reaction. After addition of an appropriate volume of lysis buffer (50 mM Tris (pH7,5); 150 mM NaCl; 1% NP40; 1% NaDesoxycholate; 0,1% SDS), the mix was incubated for at least 1.5h at 4°C in a shaker. Then the beads were washed once with 500µl lysis buffer by centrifugation for 2 min. at 6800g and resuspended in 50µl lysisbuffer per reaction. 900µl cell lysate was added and the

immunoprecipitation performed over night at 4°C in a shaker. Next day, immunoprecipitated proteins were washed at least 3-5 times with lysis buffer by centrifugation and resuspension as described above. Finally, immunoprecipitated proteins coupled to the beads were resuspended in an appropriate volume (70-100µl) of protein sample-buffer and denatured for 5 min. at 95°C. During this step, proteins disassociate from the beads and can be analyzed after SDS-PAGE by western blot or autoradiography.

2.3.5 Immunofluorescence analysis (IF)

Huh-7/T7 cells were seeded on glass cover slips and transfected with lipofectamine 2000 according to the manufacturer's instructions. 6-8h after transfection, cells were washed three times with prewarmed PBS and fixed with 500µl of a prewarmed 3% paraformaldehyde solution for about 10 min at RT. Thereafter cells were again washed three times with PBS and then stored for 2-3 days at 4°C or directly used for further preparation. For permeabilization cells were incubated for 5 min. with 500µl of a 0.5% triton-x-100/PBS solution and washed 3 times with PBS prior to incubation with the first antibody. Different antibodies directed against the HCV non-structural proteins (NS3, NS4B, NS5A and NS5B) or cellular genes (bin 1) were used as primary antibodies. The primary antibody was diluted to the desired concentration (often 1:100) with a 1x PBS buffer containing 5% goat serum, to prevent unspecific binding of the antibody. After 45 min. incubation at RT the cells were washed 3 times 10 min. with 1x PBS and incubated with the second antibody. This antibody was conjugated with fluorescent dyes (Alexa 488, Alexa 546, Cy3 and fluorescein isothiocyanate) and again diluted with a 1x PBS buffer containing 5% goat serum in the range of 1:1000. After 45 min. incubation in the dark, the cells were washed once with 1x PBS and counterstaining of the nucleus was performed using DAPI. Therefore the cells were incubated for 1 min. with a 1:3000 diluted DAPI solution and immediately washed 3 times for 10 min. with 1x PBS. Finally, the cells were washed once with water and mounted on glass slides with Fluoromount.

3. Results

3.1 Identification of highly adaptive mutations in NS5A and improvement of the HCV replicon system

Studies of the HCV replication cycle have been made possible with the development of subgenomic selectable RNAs (replicons) that replicate autonomously in cultured cells. In these replicons the region encoding the HCV structural proteins up to NS3 was replaced by the neomycin phosphotransferase gene, allowing the selection of transfected cells that support high-level replication of these RNAs. Analyses of the sequences of HCV RNAs isolated from selected cell lines (e.g. 9-13 and 5-15) revealed that the replicons had gained cell culture-adaptive mutations (8, 90). For instance, a single amino acid substitution in the NS5B RdRp increased the efficiency of colony formation (ECF) ~500-fold compared with that of the unaltered replicon RNA (90). However, the mechanism by which this cell culture adaptation was achieved remained unknown. To further improve the subgenomic replicon system, it was of great interest to identify adaptive mutations that enhance RNA replication further and therefore would allow the establishment of a transient replication assay. This kind of assay would be a valuable tool, since it does no longer depend on cumbersome and time consuming selection of cells and therefore permits rapid analysis of mutants. Furthermore, it may help to shed some light into the mechanism of adaptation. Such mutations were also critical for the generation of replicons carrying alternative resistance genes besides the neo-gene. These replicons would allow screening of a broader panel of G418 resistant cell lines for their ability to support HCV replication. This aspect was of interest, since the human hepatoma cell line Huh-7 was the only available cell line at that time, efficiently replicating HCV RNA. Finally, the generation of selectable adapted genomic replicons was not possible with wildtype or poorly adapted HCV sequences. Consequently, the main aim of the first part of this thesis was the development of highly efficient HCV replicons, as well as adapted authentic and selectable genomes.

3.1.1 Identification of cell culture-adapted HCV replicon RNAs

Although we had recently identified a single amino acid substitution in NS5B that increased the ECF ~500-fold (90), we attempted to develop an even more efficient replicon. Based on the assumption that adaptive mutations might accumulate during serial passage of replicon RNAs, total RNA was isolated from a cell line that harbored an NS3-5B replicon (cell line 5-15) (91)

and transfected into naive Huh-7 cells. After stringent selection with 1 mg of G418 per ml, a fast-growing cell clone was isolated and again total RNA was prepared and transfected into parental Huh-7 cells (Fig. 6A). After three successive passages, cell line 5-15-9-2-3 was obtained.
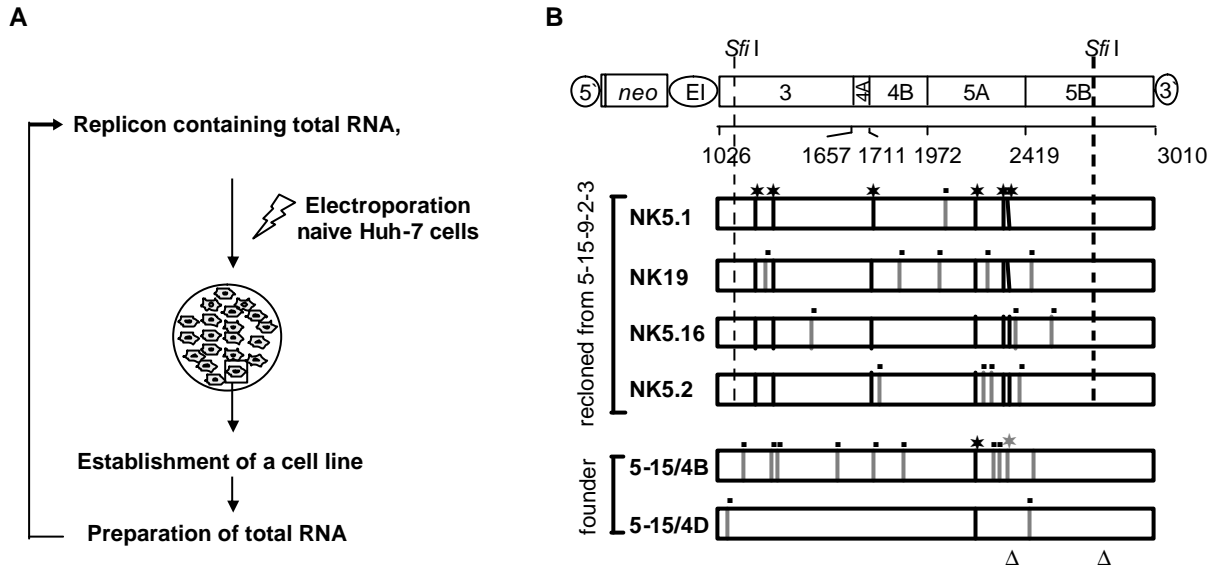
FIG. 6: (A) Experimental approach for the generation of highly adaptive replicons. Total RNA including adapted HCV replicons was isolated from replicon cell clone 5-15 and transfected into naive Huh-7. Upon G418 selection, single G418 resistant colonies grew up that were used for the establishment of new replicon cell clones. Again total RNA from theses cells was used for transfection of naive Huh-7 cells. (B) Sequence analysis of HCV replicons isolated from replicon cell lines. The structure of the selectable replicon construct is shown at the top with numbers below the NS3-5B polyprotein referring to the P1 positions of the cleavage sites. *neo*, neomycin phosphotransferase gene; EI, EMCV-IRES. Since for optimal HCV IRES activity ~48 nucleotides of the core open reading frame are required, 16 amino acid residues of the core protein are fused to the amino terminus of the neomycin phosphotransferase. Coding regions of the NS3-5B polyprotein cloned from cell line 5-15-9-2-3 and the founder line 5-15 are drawn below. Differences in the amino acid sequences are indicated by vertical lines. A black line labeled with a star refers to an amino acid substitution that was conserved within the 4 sequences isolated from cell lines 5-15-9-2-3 and grey lines labeled with a dot indicate non-conserved mutations. Note that both replicons cloned from the founder cell line 5-15 carried the proline substitution in NS5A at pos. 2197 whereas the glutamate substitution in NS5A at pos. 2350 (labeled with a grey star) was only found in one of these replicons (clone 5-15/4B). The positions of single nucleotide deletions in the polyprotein coding sequences of clone 5-15/4D are indicated with triangles.

To identify cell culture-adaptive mutations, nearly full-length replicon RNAs were amplified by long-distance RT-PCR using primers S59 and A9386 and, after restriction of the amplified DNA fragments with *Sfi*I, almost the complete HCV ORF was inserted into the parental replicon construct (Fig. 6B). In vitro transcripts from 82 different clones were prepared and transfected into naive Huh-7 cells to determine their ECFs. As summarized in Table 2, 69 clones were replication defective, because no G418-resistant colonies were obtained. Nine of the tested clones were comparable to the wildtype (20 to 50 colonies), whereas four clones were more efficient.

TABLE 2. ECF of HCV RNAs derived from replicon sequences recloned from cell line 5-15-9-2-3

No. of tested clones	cfu/µg RNA
69	0
9	1 – 100
1	100 – 1,000
1	1,000 - 10,000
2	> 10,000

Sequence analysis of the *Sfi*I-fragments of these clones revealed several amino acid substitutions (Fig. 6B). Six of them were found in all four clones derived from cell line 5-15-9-2-3, whereas the number of non-conserved amino acid substitutions was variable. A careful titration of in vitro transcripts derived from the two most efficient clones, which were designated 5.1 and 19, revealed that replicon 5.1 had the highest ECF (~500,000 CFU per µg of RNA) and that replicon 19 was about sevenfold less efficient (~70,000 CFU/µg).

3.1.2 Determination of adaptive mutations in the HCV coding sequence

To identify the adaptive among all obtained mutations, we focused our further analyses on the conserved amino acid substitutions. Based on the assumption that adaptive mutations should be generated early during selection to allow survival of transfected cells under G418 selection and become fixed in all RNA progeny, we analyzed the following conserved mutations in more detail. Three of the six substitutions were located in NS5A, and two were located in NS3. Only one conserved mutation was found in NS4B, and none were found in NS4A and NS5B. Interestingly, the conserved mutation in the center of NS5A at position 2197 of the HCV polyprotein was also present in two clones that were isolated from the founder cell line 5-15 (Fig. 6B). In contrast, a second conserved substitution in NS5A at position 2350 was found in only one of these two clones (5-15/4B). To identify which of the six conserved mutations were responsible for cell culture adaptation, they were introduced individually into the parental replicon construct and serial dilutions of in vitro transcripts were transfected into naive Huh-7 cells. After selection with G418, the ECF was determined.
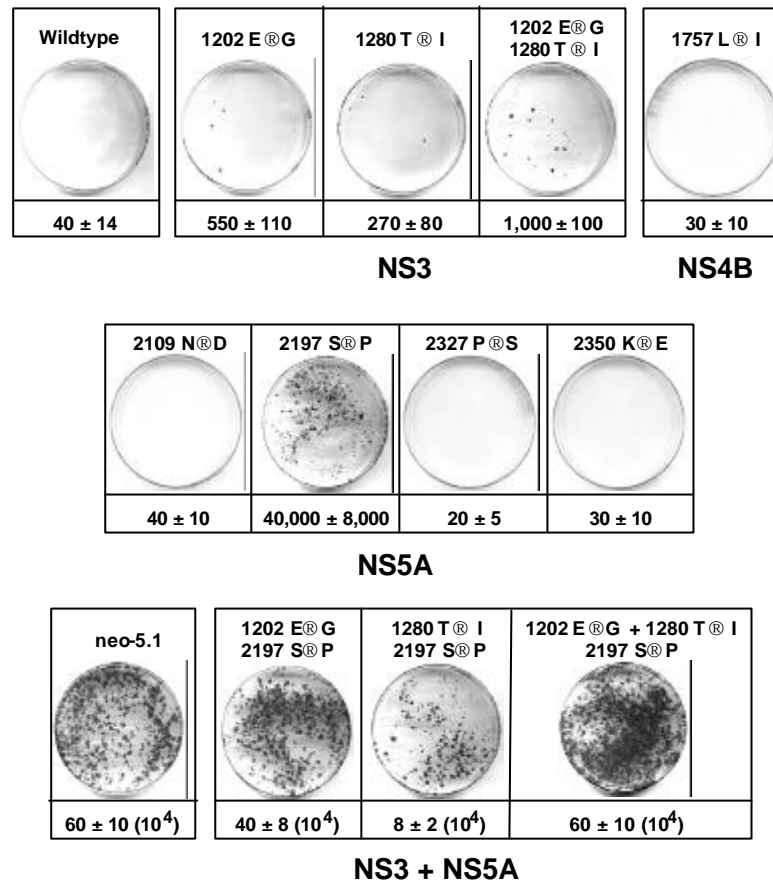
FIG. 7: Identification of cell culture adaptive mutations. Huh-7 cells were transfected with the parental replicon 5.1 or replicon RNAs carrying the mutations given above each plate. Numbers below the plates refer to the cfu/µg of *in vitro* transcribed replicon RNA. For comparison representative plates are shown that were obtained after transfection of each 10 ng in vitro transcribed replicon RNA. Note that for the determination of the CFU, serial titrations down to 0.5 ng of each RNA were transfected.

The results shown in Fig. 7 demonstrate that the most adaptive mutation was the one located in the center of NS5A at amino acid position 2197 of the polyprotein. In addition, the glycine substitution for glutamic acid and the isoleucine substitution for threonine in NS3 at positions 1202 and 1280, respectively, increased the ECF too, albeit to a much lesser extent. All other mutations did not affect the number of G418-resistant cell colonies. Interestingly, the highly adaptive NS5A substitution, but not the NS3 mutations, was already found in the replicons isolated from the founder cell line 5-15. Thus, the initial adaptive mutation was the one found in NS5A, and this mutation remained conserved during cell culture passage of the replicon RNAs. The additional adaptive mutations in NS3 must have been acquired at a later stage or were present only in a minor fraction of replicons in the founder cell line and accumulated during successive cell culture passage. Owing to the high selective pressure we used, the presence of these three mutations in the replicon RNA appeared to confer a selection advantage. In agreement with this assumption, we found that these substitutions were synergistic. When the two NS3 mutations were combined, the ECF of the replicon was ~4-fold higher than that of the RNA

harboring only the adaptive substitution at position 1280. However, when both NS3 mutations were combined with the adaptive NS5A substitution at position 2197, the ECF of the triple mutant was dramatically increased compared with the replicon that harbored only this NS5A substitution (Fig. 7). In fact, the G418 transduction efficiency of the triple mutant was as high as the one obtained with replicon 5.1. Since the analysis of sequences of the HCV ORF flanking the *Sfi*I fragment did not reveal additional amino acid substitutions in NS3 and NS5B that were conserved between the four replicons recloned from cell line 5-15-9-2-3, we had identified the major adaptive mutations (result of the sequence analysis of the adapted replicons see Table 7, appendix).

3.1.3 Lack of correlation between the ECF and replicon RNA copy number in G418-selected cell lines

To analyze whether an increase in the ECF would directly correlate with the copy number of the HCV RNA in a selected cell line, we took advantage of several HCV replicons that differed in their levels of cell culture adaptation. Replicons 5.1, 19 were the ones described above. Replicon 9-13F and replicon 5B2884Gly are two replicons isolated from replicon cell clone 9-13. While 9-13F carries one essential adaptive mutation in NS5A at position 2163 of the polyprotein that enhances replication efficiency ~100 fold compared to wildtype, replicon 5B2884Gly harbored a single glycine substitution in NS5B at position 2884 that led to an ~500-fold increase in the ECF compared to that of the wildtype (90). In vitro transcripts of these constructs were transfected into naive Huh-7 cells, and for each replicon at least three G418 resistant colonies were isolated and expanded to replicon cell clones. To determine the copy numbers of these RNAs in selected cells, we had to consider that the replication levels of the replicons depend on host cell metabolism, in a way that the amounts of HCV RNA are higher in exponentially growing cells than in resting cells. Therefore, copy numbers were determined from serial measurements using cells that had been harvested 3, 4, 5, and 6 days after seeding. A summary for all cell lines is given in Table 3. In agreement with the ECFs, the highest copy number was found in cell lines harboring replicon 5.1. However, in spite of the dramatic differences in the numbers of colonies obtained with a given replicon RNA, the replication levels within a selected cell line differed much less. Even between the replicons with the highest and the lowest levels of adaptation (5.1 and 9-13F, respectively), which varied in their ECFs by a factor of ~100, the differences in the copy numbers were only ~2-fold. With the other replicons, the replication levels were comparable. This result suggests that the ECF is primarily determined by the initial level of RNA

replication and that, during selection, either particular host cells that support high-level replication of the HCV RNA are enriched or further adaptive mutations are acquired.

TABLE 3. CFU of adapted replicons and copy number of HCV RNAs in cell lines obtained after transfection of given replicons and G418 selection (mean values and standard deviations)

replicon	cfu/µg RNA	copy number[1]
19	$72,000 \pm 5,000$	$5 \pm 1.6 \times 10^7$
5.1	$510,000 \pm 75,000$	$6.9 \pm 1.4 \times 10^7$
9-13F	$4,250 \pm 650^2$	$2.6 \pm 0.5 \times 10^7$
5B2884Gly	$23,000 \pm 3,000^2$	$5 \pm 2.5 \times 10^7$

[1] Number of HCV replicon RNA molecules per µg total RNA as determined by Northern-blot (see Fig. 3).
[2] Data from Lohmann et al., 2001.

3.1.4 Transient replication of HCV replicons carrying a luciferase reporter gene

To gain first insights into the mechanism of cell culture adaptation and to simplify the subsequent analyses, we established a transient replication assay that allowed monitoring of the RNA replication by measuring of a reporter gene. Therefore, we replaced the *neo* genes in the various cell culture-adapted replicons by the gene encoding the luciferase of the firefly *Photinus pyralis* (Fig. 8A). Likewise the neo-replicons, the luciferase replicons also contain 16 amino acid residues of the core protein fused to the amino terminus of the luciferase gene for optimal HCV IRES activity. Since the copy number of the reporter gene should be determined by the replication levels of the corresponding replicon RNAs, the luciferase activity in a lysate of transfected cells could be used to directly monitor the replication of an RNA. To confirm this hypothesis, we performed an experiment where we transfected different luciferase-replicons, varying in their ECF. The most adapted RNAs were 5.1 and 19. As a moderate adapted replicon we used replicon 9-13F which had an ~100fold lower ECF compared to RNA 5.1. Furthermore, we transfected a wildtype luciferase-replicon, carrying no adaptive mutation and a replication deficient RNA designated GND in parallel. The latter, is a derivative of the wildtype replicon that carried a single amino acid substitution changing the GDD motif of the RdRp active site to GND and therefore served as a negative control.
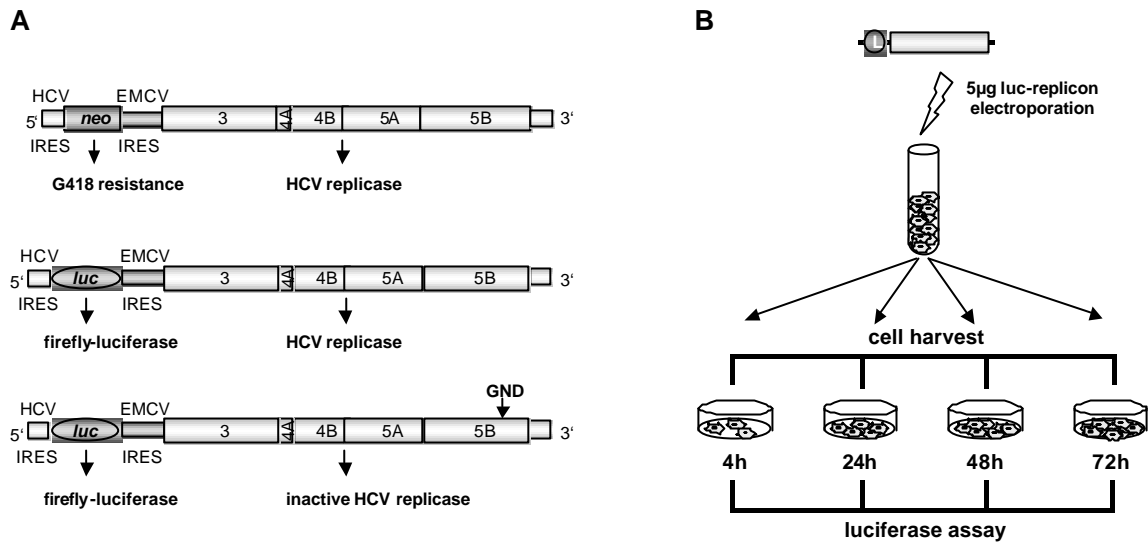
FIG. 8: (A) Structure of a replicon construct harboring a luciferase gene. Shown in the upper panel is a subgenomic neo-replicon. For generation of a reporter replicon, the luciferase gene of the firefly (*Photinus pyralis*) was introduced instead of the neomycin-phosphotransferase. As a negative control, a replication deficient luciferase replicon was generated carrying a point mutation in the active site (GDD motif) of the polymerase. This mutation leads to an amino acid exchange of aspartic acid for asparagine inactivating the RdRp. *luc*, firefly luciferase gene; GND, inactivating mutation of the polymerase GDD-motif. (B) Experimental approach of the transient replication assay. After electroporation of Huh-7 cells with 5µg luciferase-replicon, equal numbers of cells were seeded into 10cm^2 dishes and harvested 4, 24, 48 and 72h after transfection for measurement of luciferase activity. As a negative control the replication deficient luc-replicon GND was used.

Huh-7 cells were transfected by electroporation, and 1/10 of the cells was seeded into each cell culture dish. Cells were harvested at various time points, and luciferase activities were determined. Luciferase activities measured 4 h after transfection were used to quantify transfection efficiencies (Fig. 8B).

To compare transient RNA replication of the luc-replicons with the neo-replicons, we also performed an electroporation with the analogous neo-RNAs. In this case, Huh-7 cells were transfected and total RNA prepared 3.5, 15, 24, 48, 72 and 96 h later was analyzed by Northern-hybridization in order to determine the copy number of the replicons. In case of the neo-replicons, a replicon that carried a 10 amino acid residues deletion spanning the active site of the NS5B RdRp served as a negative control (rep5.1/ΔGDD; Fig. 9B). A representative result of the above-described experiment is shown in Fig. 9.
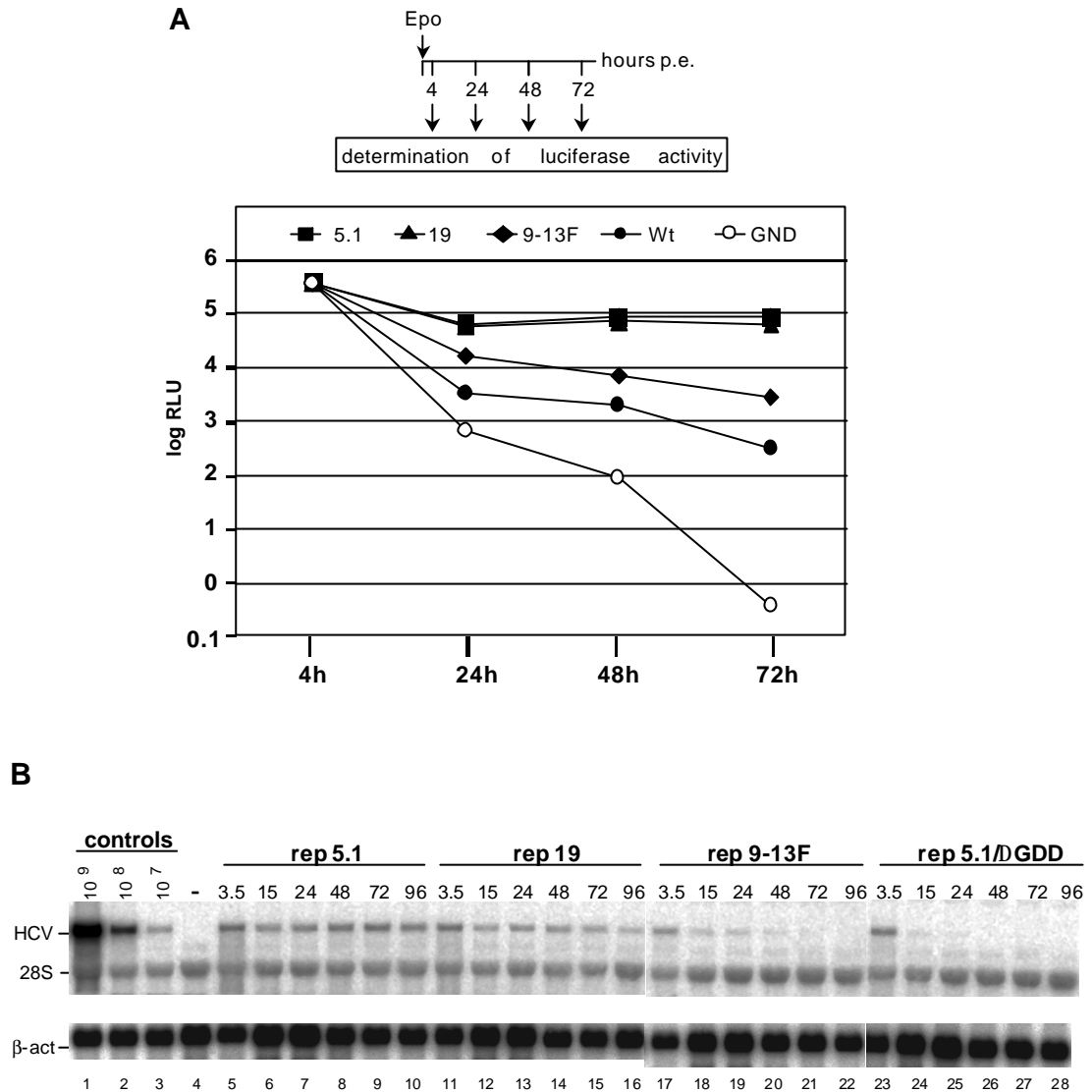
FIG. 9: Transient replication of cell culture adapted replicons. (A) Flow chart of the experimental approach. Huh-7 cells were transfected by electroporation (epo) with luciferase-replicons and 1/10 was seeded in each culture dish. After 4, 24, 48 and 72 h cells were lysed and luciferase activities were determined. The result obtained with the replicons specified in the top of the graph is shown below. Values are corrected for transfection efficiency as determined by measuring the luciferase activity 4 h after transfection. (B) Huh-7 cells were transfected with selectable neo-replicon RNAs and harvested at time points indicated above each lane. HCV RNAs were analyzed by Northern-blot. Serial dilutions of *in vitro* transcripts served as controls for the Northern hybridization (lanes 1 - 3), and β-actin RNA as a control to correct for the amount of total RNA loaded in each lane of the gel (~2 µg). The result obtained with total RNA from naive Huh-7 cells is shown in lane 4 (-). The positions of replicon RNAs, 28S ribosomal RNA and β-actine m-RNA are given at the left. The replicon carrying a deletion that spans the active site of the NS5B RNA-dependent RNA polymerase served as a negative control (rep5.1/ΔGDD).

Overall, the replication kinetics observed with these *luciferase* replicons were very similar to the ones obtained with the *neo* replicons that were analyzed by Northern hybridization up to 96 h after transfection (Fig. 9B). At every determined time point (except 4 h post transfection), luciferase activities obtained with the most highly adapted RNA, 5.1, were consistently the highest whereas those found in 9-13F-transfected cells were much lower. Owing to the high

sensitivity of the luciferase reporter, we were also able to determine the replication kinetic of the parental, non-adapted replicon RNA. As shown in Fig. 9A, at 24, 48, and 72 h post transfection a difference was found between this replicon and the replication-defective negative control (GND). However, compared to the least-adapted RNA, 9-13F, a significant lower replication was found. Therefore, we can conclude that cell culture-adaptive mutations strongly increase initial RNA replication and they are generated during a low-level replication of the non-adapted parental replicon. It should be noted that in more than 10 independent experiments, the absolute values of luciferase activities varied significantly, but in all cases, the relative differences between the individual replicons remained the same. In summary, luciferase-replicons can be used for transient replication assays since luciferase activity directly correlates with replication efficiency. The greatest difference in luciferase activities was observed 48h after transfection, where relative light units of the GND negative control already had decreased to nearly background levels, whereas luciferase activity of the most adapted replicon 5.1 was 1000-fold higher.

In a further experiment we also analyzed the transient replication of the different adapted HCV RNAs in cells after passage. We recently found that replication efficiency of the replicons in stable cell clones strongly depends on the cell growth. If cells are grown to confluence and left for prolonged times in this confluent or senescent state, HCV RNA levels drop. Therefore, in order to avoid a reduction of replicon RNA levels when the cells reached confluence in the transient assay, we passaged transfected cells 72h and a second time 168h post transfection (Fig. 10A). As shown in Fig. 10B, luciferase activity declined from 72h to 96h after electroporation. At the same time, 72h after transfection cells are grown to confluence. Interestingly, in the cells passaged at 72 h post transfection, an increase in luciferase activity was clearly visible with replicons 5.1, 19, and 9-13F (compare the values obtained 127 and 144 h post transfection). When these cells were passaged a second time 96 h after the first passage, only in cells transfected with the highly adapted replicons 5.1 and 19 a second increase of luciferase activity could be observed (compare the values at 216 and 244 h post transfection). These results show that in unselected cells, the replicons were gradually lost, with the level of decline being determined by the level of cell culture adaptation.
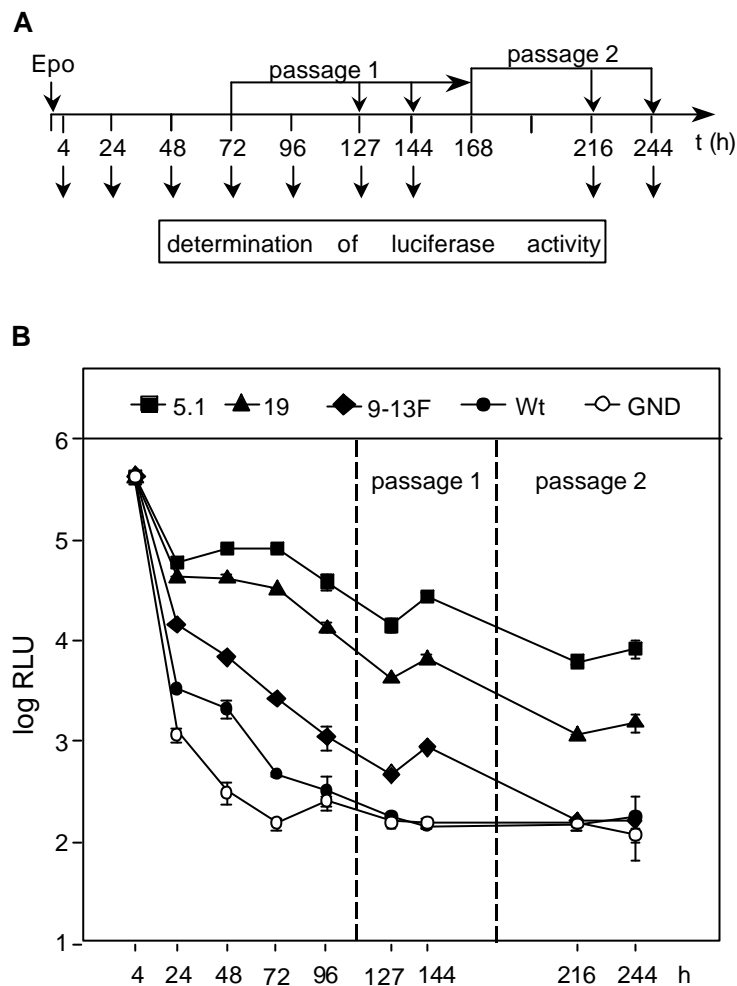
FIG. 10: Transient replication of HCV luciferase replicons after cell passage. (A) Flow chart of the experimental approach. Huh-7 cells were transfected with 5µg of the different luciferase replicons in parallel and each 1/10 was seeded in a culture dish. After 4, 24, 48, 72 and 96 h cells were lysed and luciferase activities were determined. Cells that were cultured for 72 h were passaged at a dilution of 1:3. One third was each harvested at time points corresponding to 127 and 144 h after transfection whereas the remainder was again passaged at a time point corresponding to 168 h post transfection. Cells of this second passage were lysed at time points corresponding to 216 and 244 h after transfection. (B) Results obtained with the replicons specified in the top. Values are corrected for transfection efficiency as determined by measuring the luciferase activity 4 h after transfection. The dilution factor of 3 for the first passage and 6 for the second passage were taken into account.

3.1.5 Construction of subgenomic replicons with alternative selection markers

So far, all described selectable subgenomic replicons carried the neo-gene conferring resistance to G418. Since many cell lines already contain a copy of this resistance gene, e.g. PH5CH by stable expression of an oncogene, alternative resistance genes like hygromycin-phosphotransferase and zeocin were introduced into the subgenomic replicon and analyzed for their ability to replicate. A schematic drawing of the structure of the constructed selectable replicons is shown in Fig. 11.
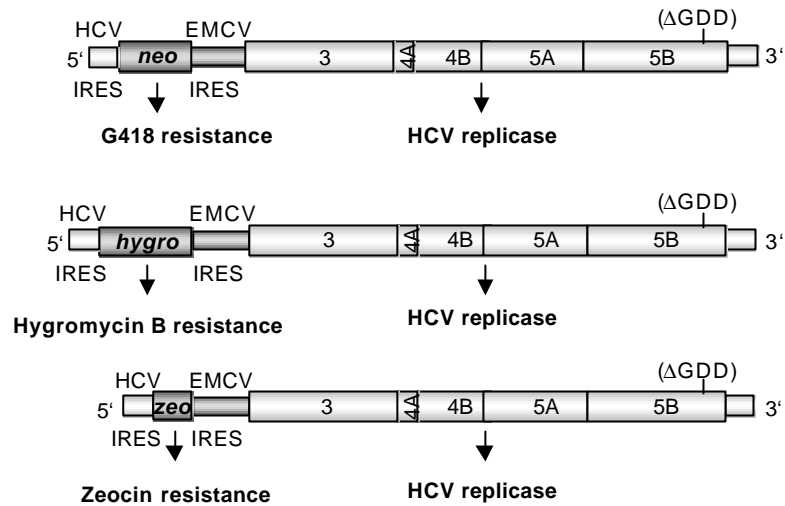
FIG. 11: **3.1.6 Construction and characterization of genomic replicons**

To allow analysis of the complete viral life cycle in vitro, we wished to establish cell lines that harbor autonomously replicating full-length HCV genomes. Prior to the identification of adaptive mutations, a selectable HCV genome was generated that derived from the original Con-1 HCV isolate and was constructed similar to the subgenomic replicons. However, experiments to establish viable cell clones after transfection of this wildtype RNA and selection with G418 failed (data not shown). Assuming that this was due to insufficient RNA replication, a selectable full-length genome (sfl) was constructed that carried the highly adaptive mutations of replicon 5.1 (Fig. 13). As a result, this HCV genome differed from the original one by 6 amino acid substitutions. While three of them did not affect replication, the combination of the three remaining synergistically increased RNA replication (E1202G and T1280I in NS3 and S2197P in NS5A; see Fig. 7).
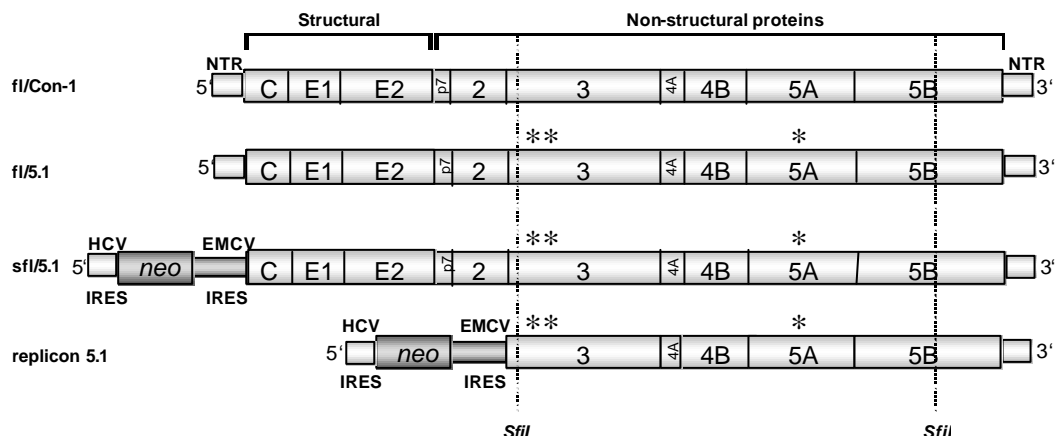
FIG. 13: Schematic drawing of adapted full length genomes. The original Con-1 wildtype genome is shown at the top. A full length genome (fl) carrying the 5.1 adaptive mutations as well as an adapted selectable full length genome (sfl/5.1) are shown below. The selectable subgenomic replicon 5.1 carrying two weakly adaptive mutations

in NS3 (E1202G+T1280I) and a highly adaptive mutation in NS5A (S2197P) is depicted at the bottom. Positions of the *SfiI* restriction sites are indicated by vertical lines.

Upon transfection of Huh-7 cells with this selectable full-length (sfl) genome, we were able to generate a low number of G418-resistant colonies. In fact, the number of resistant colonies obtained per microgram of transfected RNA was about 3 to 4 orders of magnitude lower as compared to the ECF of the respective subgenomic replicon rep 5.1. The reason for this reduction is unclear. Nevertheless, several independent cell clones could be established, that all carried HCV RNA of correct length, demonstrating that the sfl-genomes were capable to replicate efficiently in Huh-7 cells (Fig. 14).
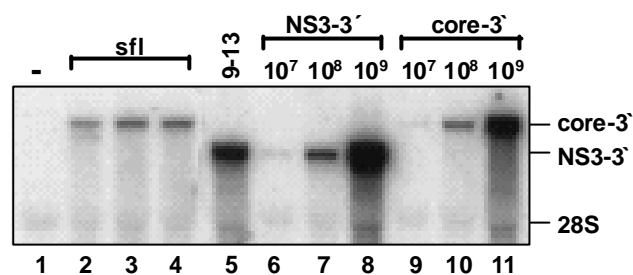
FIG. 14: Northern blot analysis of cell lines carrying an sfl genome. Total RNA from three different selectable full-length cell lines (sfl) was prepared, and 2µg was analyzed by Northern blotting using a ^{32}P-labeled HCV specific riboprobe (lane 2-4). For a reference, 2 µg of total RNA derived from naive Huh-7 cells (-) or from subgenomic replicon cell line 9-13 (lane 5) as well as serial dilutions of the original in vitro transcripts (subgenomic replicon and sfl genome) were analyzed in parallel (lane 6-8, and 9-11, respectively).

Unfortunately, all attempts to detect particle production of genomic replicon cell clones failed. To exclude the possibility that the heterologous sequences abrogated particle formation, an adapted full-length genome was generated (Fig. 13, fl-5.1) and tested in transient assays. Although replication of this adapted genome was detectable, no evidence for particle formation was observed.

3.2 Role of NS5A phosphorylation for RNA replication

As alluded to in the introduction, NS5A is a highly phosphorylated protein, supposed to be implicated in RNA replication (for review see 94). In chapter 3.1 we describe the identification of a highly adaptive mutation in NS5A that leads to the substitution of a potential phospho acceptor site. To address the question of direct correlation between the phosphorylation status of NS5A and HCV RNA replication, a series of NS5A mutations affecting serine residues was analyzed for replication and phosphorylation.

3.2.1 Alanine substitutions for highly conserved serine residues in cluster 1 of NS5A and their effects on hyperphosphorylation and RNA replication

In order to study the role of NS5A phosphorylation for RNA replication we first searched for highly conserved serine residues that could serve as phospho acceptor sites. Based on amino acid sequence alignments of about 80 different HCV isolates, we identified 24 highly conserved serine residues in NS5A that were grouped in 3 clusters as shown in Fig. 15.
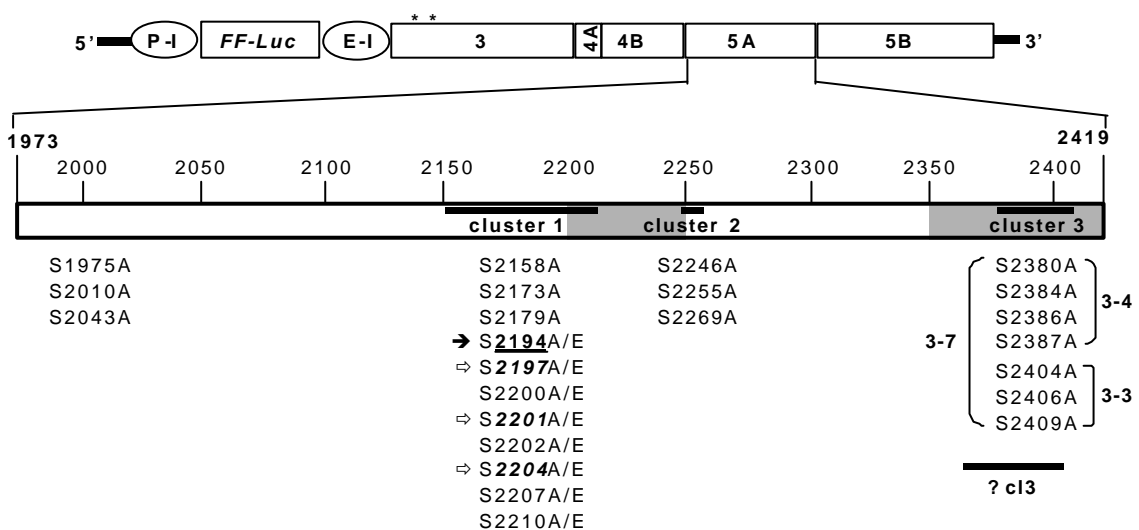
FIG. 15: Amino acid substitutions affecting highly conserved serine residues in NS5A. Structure of the subgenomic HCV replicon used for transient RNA replication analyses in this study. The HCV 5'and 3'NTRs are indicated by thick lines, the PV IRES (P-I) directing the translation of the reporter gene firefly luciferase (luc) is depicted as an oval. The EMCV IRES (E-I) directs the translation of the HCV NS3 to 5B coding sequence. The positions of cell culture adaptive mutations are indicated with asterisks. The lower panel shows a schematic representation of NS5A indicating the major phosphoacceptor site (S2194, closed arrow) and sites required for hyperphosphorylation (S2197, S2201, and S2204, open arrows). Regions important for basal phosphorylation are shaded in grey. Serine residues highly conserved between multiple HCV isolates and genotypes were grouped into 3 clusters with cluster 1 including the sites involved in hyperphosphorylation as well as the major phospho acceptor site. Amino acid substitutions generated in this study are indicated below the scheme. In case of cluster 1, in addition to alanine substitutions, selected replacements by glutamic acid were analyzed. In case of cluster 3, substitutions affecting 3, 4 or 7 amino acid residues were combined (mutants 3-3, 3-4, and 3-7, respectively). The deletion removing cluster 3 completely is indicated below (Δcl3). Numbers refer to the positions within the polyprotein of the HCV Con-1 isolate (EMBL data base accession number AJ238799).

Cluster 1 includes the major phospho acceptor S2194 and the sites required for hyperphosphorylation (S2197, S2201 and S2204) whereas cluster 3 resides at the C-terminus of NS5A and overlaps with the region required for basal phosphorylation. Given their high degree of conservation, 3 distantly spaced serine residues in the N-terminus of NS5A flanking the amphipathic α-helix that anchors NS5A to intracellular membranes (11) were included in the analysis.
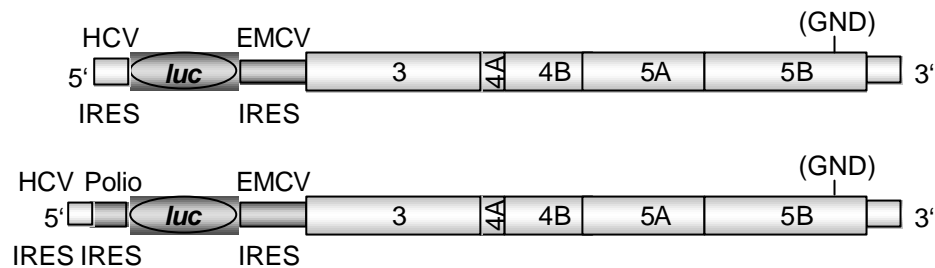
FIG. 16: Comparison of a second-generation luciferase replicon (lower panel) with the original luciferase replicon. They differ by the additional IRES element from the PV in the first cistron that directs translation of the luciferase gene. It is separated from the HCV 5´NTR (nt 1-341) by a random 63-nt-long spacer sequence.

The importance of these residues for RNA replication was studied by introducing alanine substitutions into a second-generation subgenomic reporter replicon and subsequent transient transfection assays (Fig. 16). This replicon is composed of the complete HCV 5' NTR, the PV-IRES that directs the translation of the firefly luciferase reporter gene, the EMCV-IRES mediating translation of the NS3 to NS5B coding region and the authentic 3'NTR. Since the HCV 5´NTR was only needed for RNA replication, but no longer for translation of the reporter gene, the replication level of the second-generation construct was higher compared to the corresponding original reporter replicon that has been described in the first chapter of the result section. The parental construct used for all further analyses was designated luc-T and carried two low adaptive mutations in NS3 (E1202G, T1280I) (89), which together with adaptive NS5A mutations enhance RNA replication synergistically. Using this construct allowed identification of even weakly adaptive NS5A mutations that otherwise could not be detected. Replication efficiencies of the RNAs carrying various single amino acid substitutions were determined in a transient replication assay. Therefore in vitro transcribed replicon RNA was transfected into Huh-7 cells by electroporation, aliquots of the transfected cell suspension were seeded and incubated for 4, 24, 48, and 72 h. Luciferase activities were determined in cell lysates and the results were normalized for transfection efficiency by using the 4 h value.
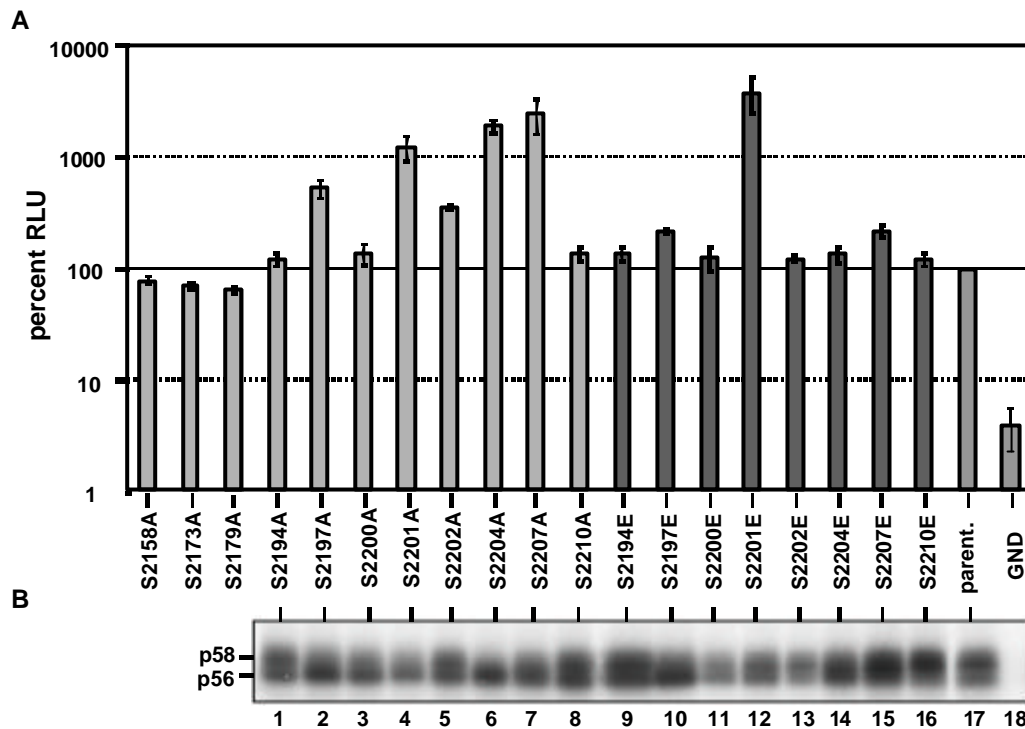
FIG. 17: Results of transient replication assay and phosphorylation analysis of mutants from cluster 1. (A) Mutants specified below the graph were transfected into Huh-7 cells. Replication was measured by determining the relative light units (RLU) at 48 h post electroporation and normalization for transfection efficiency by using the luciferase activity measured 4 h after transfection. The value determined with the parental replicon (parent.) was set 100% and used as a reference to normalize the replication of all other replicons. The replicon carrying an inactivating mutation in the NS5B RNA polymerase (GND) served as a negative control. Values are means and standard deviations from at least 3 independent experiments each measured at least in duplicate. (B) Phosphorylation analysis of NS5A by using the vaccinia virus T7 hybrid system. NS3 to NS5B polyprotein fragments carrying a given mutation in NS5A were transfected into Huh-7 cells and proteins were radiolabelled by using [^{32}P] orthophosphate. NS5A proteins were isolated by immunoprecipitation and separated by SDS 10% -PAGE. Mock transfected cells served as a negative control (lane 18). The two phosphoprotein variants p56 and p58 are marked.

Alanine substitutions for the highly conserved serine residues at the N-terminus of NS5A (S1975, S2010, S2043) did not affect RNA replication significantly (data not shown). The same was true for the analogous replacements of S2158, S2173 and S2179 at the N-terminus of cluster 1 (Fig. 17A). In agreement with an earlier report that demonstrated no absolute requirement of the major phospho acceptor site for RNA replication, we found that replacement of S2194 by alanine had no effect on RNA replication (9) (Fig. 18A). A different picture was found for most of the remaining serine residues in cluster 1. In case of the known adaptive sites S2197 and S2204 alanine substitutions enhanced RNA replication although this substituting residue has so far not been found in replicon cell clones. Instead, proline and cysteine residues at position 2197, or isoleucine, arginine and valine residues at position 2204 have been described showing a high flexibility for mutations at these sites that confer cell culture adaptation. A peculiar phenotype was observed with the alanine substitutions affecting the 3 consecutive serine residues at position 2200-2202. While the mutations affecting S2201 and S2202 increased RNA

replication, albeit to different extents, the replacement affecting S2200 had no effect. Deletion of one serine residue of this triple was also found to be the major adaptive mutation in a replicon cell clone, established after transfection of wildtype RNA. Since S2201 and S2202 are determined by the same nucleotide codon (TCA), one can not distinguish which amino acid exactly is missing. Surprisingly, the alanine substitution for S2207 that so far has not been identified in replicons isolated from Huh-7 clones, enhanced RNA replication as efficiently as the most adaptive alanine substitution in NS5A (S2204A) that is the also the most prevalent one in Huh-7 replicon cell clones (8, 90). To study the effect of these mutations on hyperphosphorylation of NS5A, NS3 to 5B polyprotein fragments carrying defined NS5A mutation(s) were expressed with the vaccinia virus/T7 hybrid system. Using this system allowed us to study phosphorylation of NS5A independent of RNA replication. Metabolic labeling with [^{32}P] orthophosphate and [^{35}S] cysteine/methionine was performed and NS5A proteins were analyzed after immunoprecipitation by SDS 10% PAGE and autoradiography. As shown in Fig. 17B, the alanine substitutions affecting residues S2197, S2201, S2204 and S2207, which enhance RNA replication most efficiently, led to a significant decrease or loss of NS5A hyperphosphorylation, which was not the case for the poorly adaptive mutation S2202A. This result indicates that mutations affecting those serine residues in cluster 1 that strongly increase RNA replication (about 10-fold or more as compared to the parental replicon) correlate with a significant reduction or loss of NS5A hyperphosphorylation. Moreover, the data show that the serine residue at position 2207 is also involved in hyperphosphorylation as well as cell culture adaptation.

3.2.2 Combination and deletion of alanine substitutions for highly conserved serine residues of cluster 1

In an attempt to further increase RNA replication and to analyze the correlation with NS5A hyperphosphorylation, combinations of highly adaptive mutations were generated. In addition, we also deleted small parts of the central region of NS5A and analyzed these mutants for RNA replication and NS5A phosphorylation pattern. Shown in Fig. 18 is the result of a transient replication assay of various combination mutants.
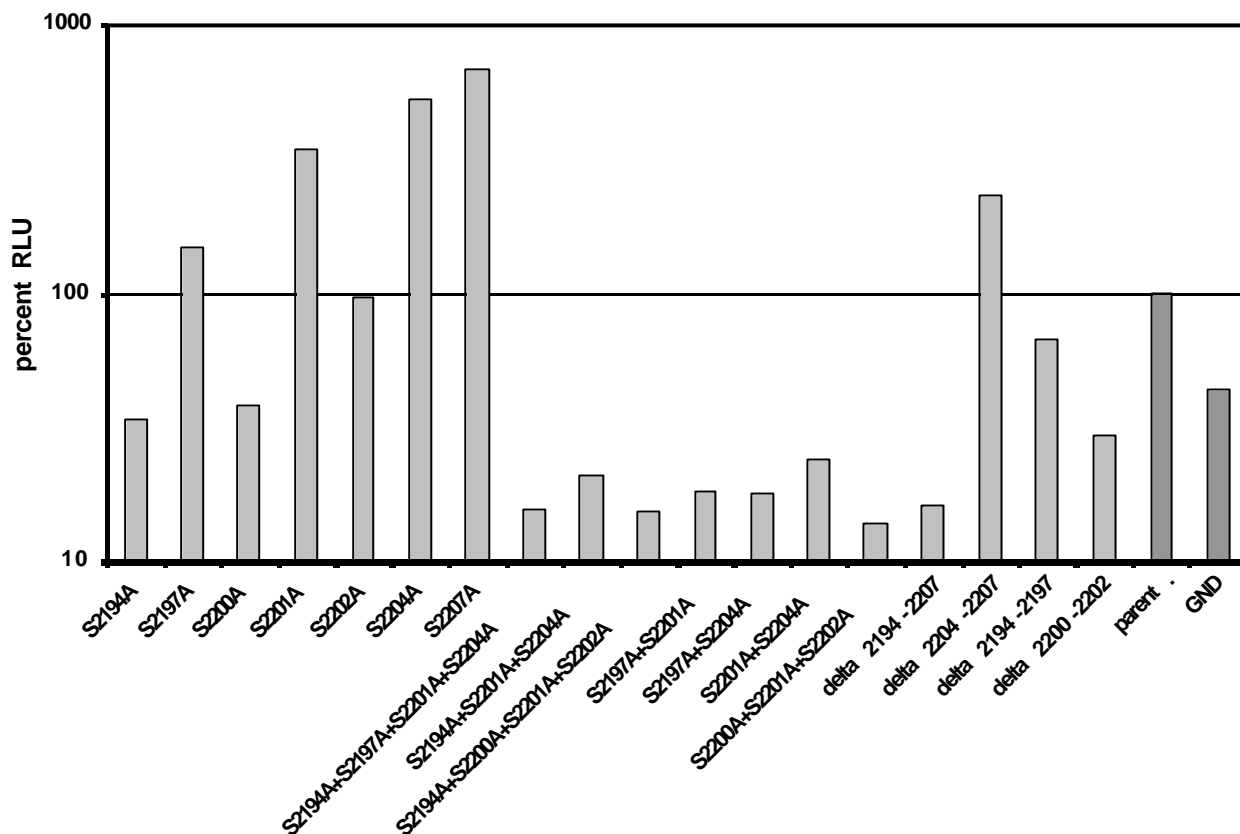
FIG. 18: Representative result of a transient replication assay of combination mutants of cluster 1. Mutants specified below the graph were transfected into Huh-7 cells. Luciferase activity was determined 4 and 48h after transfection and values were normalized for transfection efficiency that is reflected by the 4h value. The value determined with the parental replicon (parent.) was set 100% and used as a reference to normalize replication of all other replicons. The replicon carrying an inactivating mutation in the NS5B RNA polymerase (GND) served as a negative control.

Combinations of alanine substitutions affecting two potential hyperphosphorylation sites such as S2197, S2201 and S2204 consistently abrogated RNA replication. These results were confirmed by the more sensitive G418 selection assay, using neo-replicons that carried the desired mutations. In addition, deletion of the central region including the major phospho acceptor site S2194, as well as all hyperphosphorylation sites (Δ2194-2207) blocked RNA replication, too. Only a low level RNA replication was observed for the smaller deletions Δ2194-2197 and Δ2200-2202. Surprisingly, deletion of amino acids 2204 to 2207 (Δ2204-2207) was weakly adaptive as compared to the parental replicon, although two sites important for hyperphosphorylation were deleted. Analysis of the phosphorylation pattern of some of these mutants revealed a strong reduction of NS5A hyperphosphorylation in all cases. No clear double band was visible in any case as shown in Fig.19.
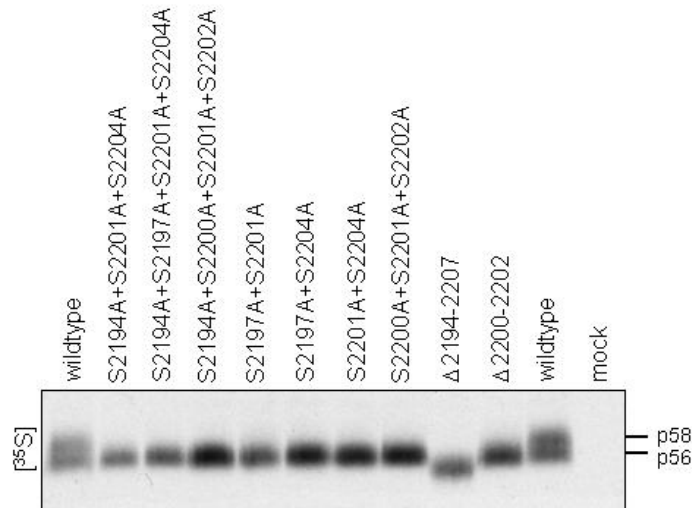
FIG. 19: Phosphorylation analysis of NS5A carrying multiple mutations or deletions by using the vaccinia virus T7 hybrid system. NS3 to NS5B polyprotein fragments carrying a given mutation in NS5A were transfected into Huh-7 cells and proteins were radiolabelled by using [^{35}S] cysteine/methionine. NS5A proteins were isolated by immunoprecipitation and separated by SDS 10%-PAGE. Mock transfected cells served as a negative control. The two phosphoprotein variants p56 and p58 are marked.

This result leads to the conclusion that a minimal hyperphosphorylation of NS5A may be required for efficient RNA replication. Combination of highly adaptive mutations usually blocks RNA replication, probably because they follow the same mechanism of adaptation.

3.2.3 Glutamic acid substitutions for highly conserved serine residues in cluster 1 of NS5A and their effects on hyperphosphorylation and RNA replication

The results described thus far do not discriminate whether the loss of a negative charge or the loss of a potential phosphate group is required for the increase of RNA replication. To address this question in more detail, highly conserved serine residues in cluster 1 were replaced by glutamic acid residues and analyzed for RNA replication and NS5A phosphorylation as described above. By introduction of a glutamic acid instead of a serine residue, phosphorylation at a defined position is mimicked by the negative charge of the glutamic acid. As shown in the right panel of Fig. 17A, save for one the glutamic acid substitutions did not lead to increases of RNA replication. The exception was the S2201E mutant that replicated as efficiently as the replicons carrying the most adaptive alanine substitutions (S2204A and S2207A).

Analysis of the phosphorylation pattern of the substitutions after expression in the context of an NS3 to 5B polyprotein fragment revealed an almost invariable double band of p56 and p58. However, we can not exclude, that the protein migration was affected by the introduction of a glutamic acid. It is possible that the presence of the negatively charged glutamic acid residue at position 2201 resulted in the production of a p58 form that may represent a fraction of basal

phosphorylated NS5A. To address this possibility a metabolic labeling of NS3 to 5B and a subsequent immunoprecipitation of NS5A and phosphatase treatment was performed. As shown in Fig. 20 only one protein species with an apparent molecular weight with of ~54kD was found after dephosphorylation of the S2201E NS5A variant.
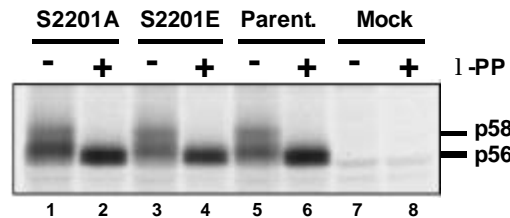
FIG. 20: Effect of adaptive mutations at amino acid position 2201 on hyperphosphorylation of NS5A. T7-based plasmids encoding NS3 to NS5B and carrying the mutations specified above the lanes were transfected into Huh-7 cells that stably express T7 RNA polymerase. Proteins were radiolabeled metabolically with [^{35}S] cysteine and methionine and NS5A proteins were isolated by immunoprecipitation. One half of the immunocomplexes was treated with λ-phosphatase (+), the other half was mock treated (-) and the samples were separated by SDS 10% PAGE. Mock transfected cells served as a negative control. The positions of p56 and p58 are indicated at the right.

Therefore a change of the electrophoretic mobility due to this particular amino acid substitution is unlikely.

During the course of this work, Blight and coworkers reported (9) that alanine or aspartic acid substitutions affecting the major phospho acceptor site S2194 in combination with the highly adaptive NS5A mutation S2204I nearly abrogated RNA replication. Moreover, we found that combination of S2194A and S2201A reduced RNA replication if compared to the single substitution S2201A. Therefore, we wanted to know, whether combination of the adaptive mutation S2201E and the conservative substitution of the major phospho acceptor site S2194E also had an effect on RNA replication. A transient replication assay was performed with the mutants S2194E+S2201E and S2197E+S2204E.

As shown in Fig. 21 combination of glutamate substitutions had no effect on the adaptive phenotype of S2201E. Conservative exchange of two potential hyperphosphorylation sites (S2197E and S2204E) had no influence on the replication level, too.
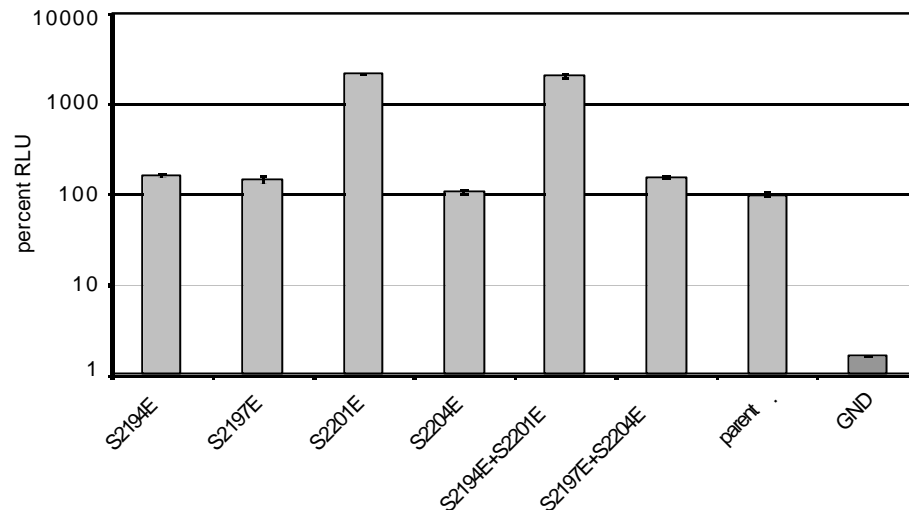
FIG. 21: Transient replication of mutants harboring combinations of glutamate substitutions. Mutants specified below the graph were used for transfection of Huh-7 cells. Luciferase activity was determined 48h post transfection and normalized to the 4h values that reflect transfection efficiency. The value determined with the parental replicon (parent.) was set 100% and used as a reference to normalize the replication of all other replicons. The replicon carrying an inactivating mutation in the NS5B RNA polymerase (GND) served as a negative control.

In summary, most glutamic acid substitutions for serine residues in cluster 1 of NS5A do not confer cell culture adaptation and have no impact on hyperphosphorylation. The only exception was S2201E that resulted in an enhanced RNA replication without obvious effects on phosphorylation of NS5A. In contrast to recent reports that performed similar experiments, combination of the highly adaptive exchange S2201E with S2194E did not affect replication efficiency underlining the special role of this adaptive mutation.

3.2.4 Influence of adaptive mutations in NS3, NS4B and NS5B on NS5A hyperphosphorylation

For most HCV isolates analyzed thus far, at least one additional viral protein is required for NS5A hyperphosphorylation. In the case of our Con-1 genome expression of NS5A in the context of the NS3 to NS5A polyprotein is required to obtain p58 (76). Mutations in NS3, NS4A and NS4B have been shown to dramatically decrease hyperphosphorylation of NS5A. This observation prompted us to test whether adaptive mutations in NS3, NS4B, and NS5B would affect p58 production and whether the mutations in NS3, NS4A and NS4B that reduce formation of p58 lead to an adaptive phenotype. To this end, we first focused our analysis on two substitutions in NS3, 4 mutations in NS4B and one in NS5B that enhance RNA replication to various extents (89). The two mutations in NS3 have almost no effect on their own, but cause a profound enhancement when combined with highly adaptive mutations. The substitutions in

NS4B increase RNA replication most efficiently whereas the NS5B mutation has an intermediate phenotype.

To study the impact of these mutations on NS5A hyperphosphorylation, NS3 to NS5B polyprotein fragments carrying these substitutions individually were expressed in Huh-7 cells and analyzed after radiolabeling with [^{35}S] cysteine/methionine and immunoprecipitation by SDS PAGE.
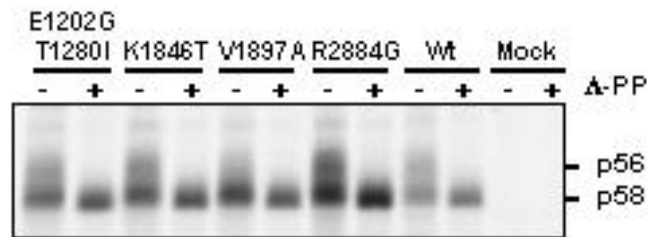

FIG. 22: Effect of adaptive mutations in NS3, NS4B and NS5B on hyperphosphorylation of NS5A. T7-based plasmids encoding NS3 to NS5B and carrying the mutations specified above the lanes were transfected into Huh-7 cells that stably express T7 RNA polymerase. Proteins were radiolabeled metabolically with [^{35}S] cysteine and methionine and NS5A proteins were isolated by immunoprecipitation. One half of the immunocomplex was treated with λ-phosphatase (+), the other half was mock treated (-) and the samples were separated by SDS 8% PAGE. Mock transfected cells served as a negative control. The positions of p56 and p58 are indicated at the right.

As shown in Fig. 22 substitutions in NS3 and NS5B led to a phosphorylation pattern comparable to wildtype NS5A, whereas a reduction of NS5A hyperphosphorylation was found with all adaptive mutations in NS4B albeit to different extents. While the exchange of K1846 caused only a minor reduction of p58, Ala-, Met- or Leu-substitutions for V1897 strongly decreased hyperphosphorylation of NS5A. This result is consistent with the observation that alanine substitutions in NS5A that have the highest impact on RNA replication significantly reduce p58 formation (S2201A, S2204A and S2207A). On the other hand the data obtained with the K1846T and S2201E mutants show that production of p58 does not exclude the possibility of enhanced RNA replication like in the case of K1846T and S2201E.

In addition we analyzed a panel of amino acid exchanges in NS3, NS4A and NS4B that caused a nearly complete loss of NS5A hyperphosphorylation for their ability to enhance RNA replication. The proline to histidine mutation affecting the second amino acid of NS3 had no effect on the enzymatic proteinase activity. The mutations in NS4A were selected to preserve the functional domains of NS4A. The leucine substitution for a glycine residue was located in the central proteinase activation domain (G1678L) and does not affect polyprotein processing (7). Furthermore, multiple alanine substitutions were introduced into the C-terminus of the molecule (1690AAA, 1697AAA, 1703AAA and 1707AAAA). For NS4B, two in-frame deletions (Δ1782-1821 and Δ1903-1933) were analyzed that based on secondary structure predictions and the hydrophobicity profile of NS4B, should not affect the overall protein structure. None of the

described mutations in NS3, NS4A or NS4B had an effect on polyprotein processing, but all of them reduced or completely blocked hyperphosphorylation of NS5A.
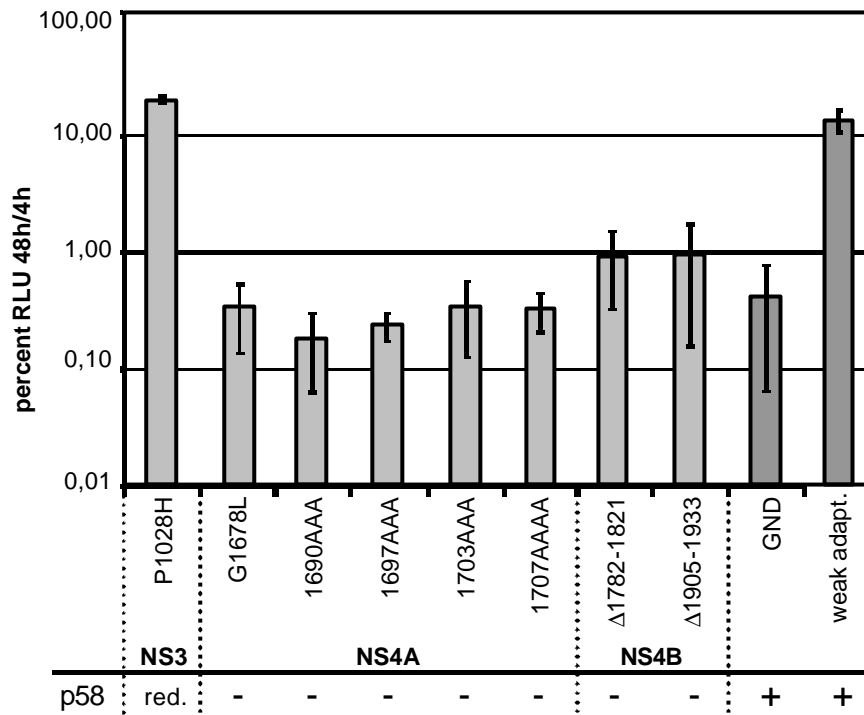
FIG. 23: Transient replication of mutants dramatically reducing formation of p58. Huh-7 cells were transfected with the mutants indicated below the graph. Luciferase activity determined 48h post transfection was determined and normalized to the 4h values that reflect transfection efficiency. Replicon GND was used as a negative control, a replicon carrying two weakly adaptive mutations in NS3 (E1202G+1280I) served as a positive control.

For the transient replication assay the mutations were each inserted into a subgenomic reporter replicon and analyzed for their influence on RNA replication. As shown in Fig. 23, except for the NS3 mutation, none of the mutants was able to replicate. This result confirmed that a complete loss of hyperphosphorylation might not be compatible with efficient RNA replication. Furthermore, reduction of p58 formation in general does not allow drawing conclusions about the replication ability of a replicon.

3.2.5 Replication and phosphorylation analysis of cluster 3 located at the C-terminus of NS5A and involved in basal phosphorylation

Basal phosphorylation of NS5A requires two regions in NS5A that reside in the center (amino acid 2200-2250) and at the C-terminus of the molecule (amino acid 2350-2419; Fig. 15). To analyze the role of phosphorylation in these regions for RNA replication, we replaced single or multiple serine residues by alanine residues and analyzed the corresponding replicons and polyprotein fragments as described above.
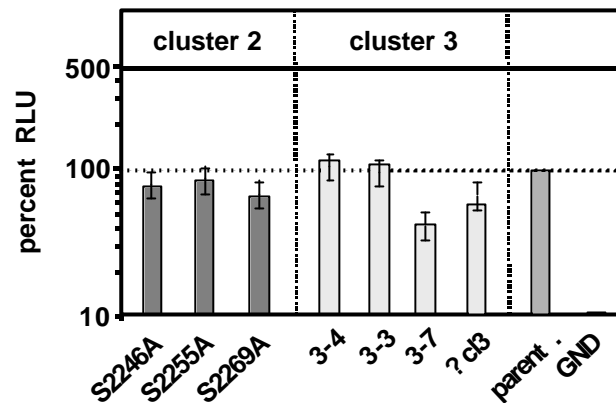
FIG. 24: Mutations at the C-terminus of NS5A reduce phosphorylation, but have no impact on RNA replication. (A) Substitutions affecting single serine residues in cluster 2 or multiple serine residues in cluster 3, as well as a deletion of cluster 3 (Δcl3), were analyzed in a transient replication assay. The parental replicon (parent., carrying two weak adaptive mutations in NS3) and the inactive mutant (GND) served as positive and negative control, respectively. All values were measured 48 h after electroporation and, after correction for their transfection efficiency, normalized with respect to the parental replicon, which was set 100%.

Mutations in cluster 2 that resides at the 3'border of the central region did not affect RNA replication (Fig. 24). The same result was found with replicons, in which 3, or 4, or even 7 serine residues in cluster 3 were simultaneously replaced by alanine residues (mutant 3-7; Fig. 24). During the course of these studies, we identified a Huh-7 cell clone that carried a stably replicating genomic replicon with an in-frame deletion in NS5A from amino acid 2370 to 2412. This mutation led to a complete removal of cluster 3 suggesting that this region is dispensable for RNA replication. In order to support this conclusion we introduced the deletion into a subgenomic reporter replicon and found that the replication efficiency of this replicon (?cl3) was comparable to the parental one in our transient assay (Fig. 24). These results clearly show that serine cluster 3 in NS5A is dispensable for RNA replication.
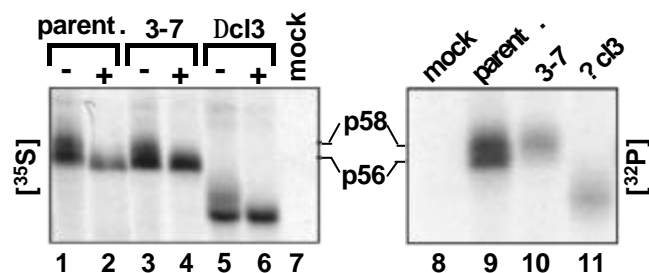
FIG. 25: Phosphorylation analysis of cluster 3 mutants. NS3 to NS5B constructs carrying the mutations specified above the lanes were transiently expressed in Huh-7 cells by using the vaccinia virus T7 hybrid system. Proteins were radiolabeled with [^{35}S] cysteine and methionine (left panel) or [^{32}P] orthophosphate (right panel) for 6h and NS5A proteins were isolated from cell lysates by immunoprecipitation. For the [^{35}S]-labeled proteins one half of the samples was treated with phosphatase from bacteriophage ? (+) whereas the other half was mock treated (-) prior to SDS-10% PAGE.

The effect of these substitutions on NS5A phosphorylation pattern was studied upon expression of the NS3 to NS5B polyprotein fragment in Huh-7 cells expressing T7 RNA polymerase after

MVA infection. Analysis of [^{35}S] radiolabelled proteins revealed expression of NS5A proteins to about the same level (Fig. 25, left panel). Only in case of the unaltered NS5A the p56/p58 double band was detected (lane 1) that was sensitive to treatment with phosphatase as deduced from the disappearance of p58 and the increase in the electrophoretic mobility of phosphatase-treated p56 (lane 2). No such shift of p56 was found with the two mutants 3-7 and Δcl3 after phosphatase treatment, respectively, arguing that the mutations affected basal phosphorylation (lane 4, 6). This conclusion was confirmed by the analysis of the [^{32}P] labeled proteins (Fig. 25, right panel). While a clear double band was found with the unaltered NS5A (lane 9), in case of mutant 3-7 radiolabel was incorporated almost exclusively into the NS5A species that corresponds to p58 (compare lane 9 with lane 10). Given the higher electrophoretic mobility of the NS5A protein carrying the C-terminal deletion (Δcl3), no firm conclusions could be drawn from the labeling pattern. However, the production of a phosphatase-sensitive higher molecular weight species that corresponds to p58 of full length NS5A suggests that the truncated protein is also hyperphosphorylated (compare lane 5 with lane 6). In summary, these data show that the C-terminal serine cluster of NS5A is involved in basal phosphorylation but non-essential for RNA replication.

In order to address the question whether NS5A phosphorylation is required at all for RNA replication, we generated a panel of replicons in which the deletion (Δcl3) or substitutions in cluster 3 (mutant 3-7) were combined with single or multiple substitutions that reduce or block hyperphosphorylation (S2197A, S2201A, S2204A and S2207A). These mutants were again analyzed for their replication efficiency and NS5A phosphorylation pattern. The result of the transient replication assay is shown in Fig. 26.
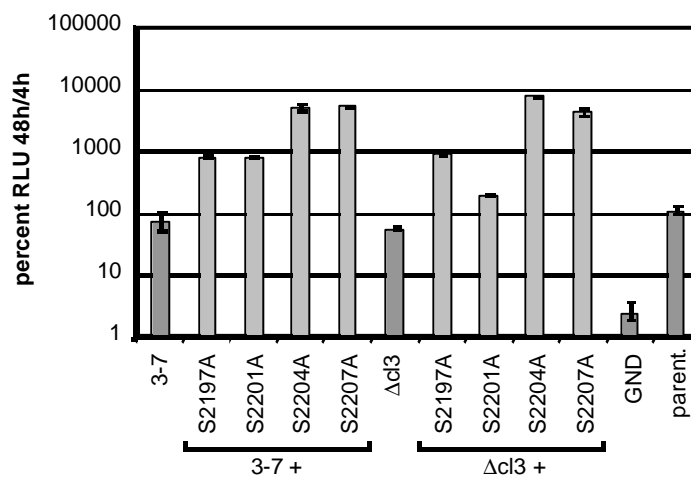
FIG. 26: Transient RNA replication of mutants carrying double mutations in NS5A affecting its basal- and hyperphosphorylation. Huh-7 cells were transfected with 5μg of the corresponding RNA indicated below the lanes and luciferase activity was determined 4 and 48h after electroporation. All values were normalized for transfection efficiency and the value determined with the parental replicon (parent.) was set 100%. Replicon GND served as a negative control. Replicons 3-7 and Δcl3 were used as positive controls for the RNAs carrying double mutations.

Insertion of the adaptive mutations S2197A, S2201A, S2204A or S2207A into mutant 3-7 or combination with the C-terminal deletion Δcl3 resulted in efficiently replicating replicons. The adaptive phenotype caused by single substitutions in the center of NS5A was preserved with the mutants harboring additional mutations at the C-terminus (3-7 or Δcl3). Like for the single substitutions, combinations of these mutations with S2204A and S2207A caused the strongest enhancement of RNA replication (Fig. 26).

Thus, mutations at the C-terminus of NS5A are neutral and do not affect RNA replication cooperatively or negatively when combined with highly adaptive mutations in the center of NS5A.

Since the adaptive mutations in the center of NS5A led to a decreased hyperphosphorylation and the alterations at the C-terminus reduced basal phosphorylation, we were interested whether combination of both would prevent NS5A phosphorylation. Therefore, $[^{35}S]$ cysteine/methionine and $[^{32}P]$ orthophosphate labeling experiments were performed by using the vaccinia virus T7 system for expression of the different NS5A proteins, in the context of NS3 to 5B polyproteins. The results of the $[^{32}P]$ orthophosphate and $[^{35}S]$ labeling experiments are shown in Fig. 27.
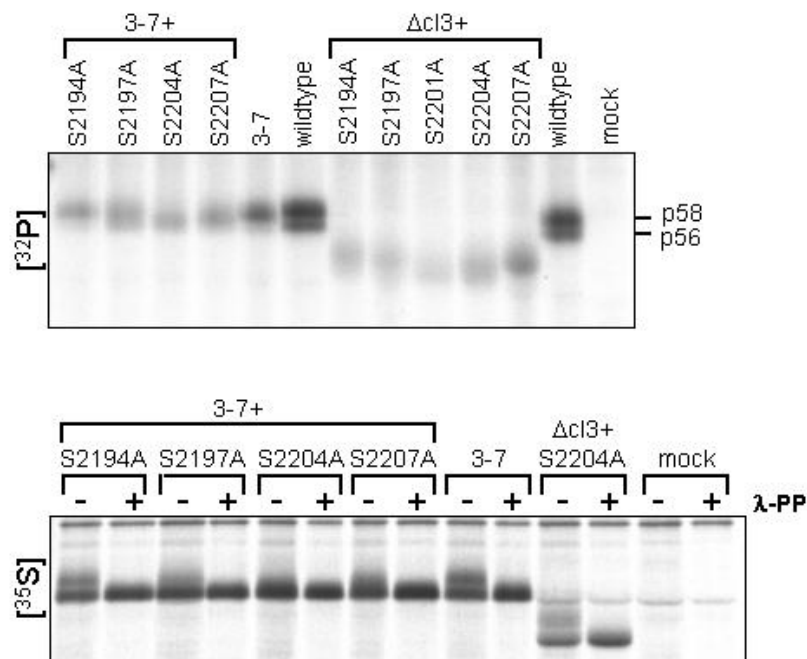

FIG. 27: Phosphorylation analysis of cluster 3 combination mutants. NS3 to NS5B constructs carrying the mutations specified above the lanes were transiently expressed in Huh-7 cells by using the vaccinia virus T7 hybrid system. Proteins were radiolabeled with $[^{32}P]$ orthophosphate (upper panel) or $[^{35}S]$ cysteine and methionine (lower panel) for 6h and NS5A proteins were isolated from cell lysates by immunoprecipitation. For the $[^{35}S]$-labeled proteins one half of the samples was treated with phosphatase from bacteriophage ? (+) whereas the other half was mock treated (-) prior to SDS-10% PAGE.

In all cases NS5A phosphorylation was still detectable but with some mutants the efficiency was very low (e.g. combination of Δcl3 with S2204A) arguing that a very low phosphorylation of NS5A is sufficient for high level RNA replication. Interestingly, for mutant S2204A+3-7 we observed a phosphorylated p56 form after [32P] orthophosphate labeling, although substitution 3-7 alone nearly blocked phosphorylation of p56.

Further attempts to create a replicon capable of replication, but carrying a non-phosphorylated NS5A protein were not successful. In all cases we still detected phosphorylated NS5A. Thus, the question, whether phosphorylation of NS5A is required at all for replication could not be answered with this approach.

3.2.6 Analysis of the genetic flexibility of the C-terminus of NS5A by introduction of heterologous sequences

The finding that multiple substitutions introduced simultaneously into the C-terminus of NS5A as well as a complete deletion of cluster 3 do not affect RNA replication indicated that this region of the molecule is genetically highly flexible and may tolerate even more substantial genetic manipulations. In fact, during the course of this work, Moradpour and colleagues (99) reported that the insertion of the enhanced green fluorescent protein (eGFP) into NS5A did not abrogate replication of a subgenomic replicon. We therefore, wanted to know whether an insertion of heterologous sequences into the C-terminal deletion identified in our study is tolerated. In the first set of experiments we introduced a convenient multiple cloning site carrying recognition sequences for *Asc*I, *Xba*I and *Pme*I with a total length of 37 nucleotides (Fig. 28). For reasons of easy readout of RNA replication, we inserted the luciferase gene from *Renilla reniformis*, the enhanced green fluorescence protein gene (eGFP) from *Aequorea victoria* or the gene encoding for *Discosoma species* red fluorescent protein (dsRed). Insertions were engineered in frame into the multiple cloning site of NS5A.
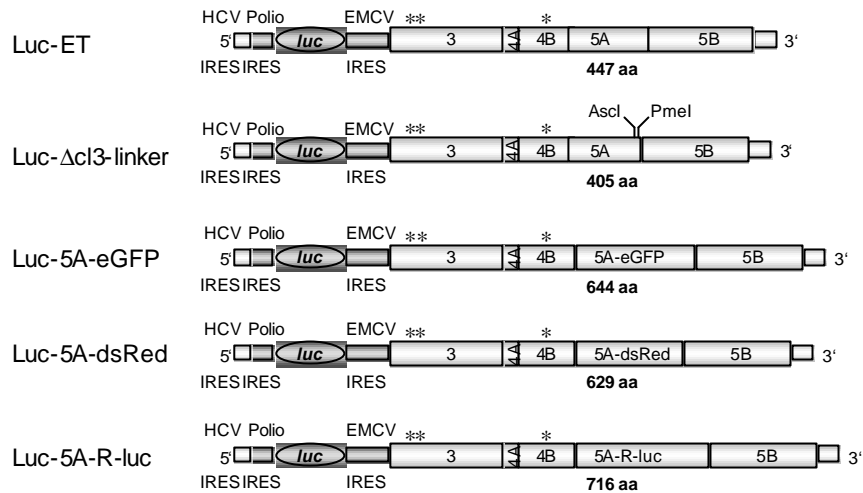
FIG. 28: Structure of luciferase-replicons carrying insertions of heterologous sequences within the C-terminus of NS5A. The upper construct represents an adapted luc-replicon, termed Luc-ET. Adaptive mutations in NS3 (E1202G and T1280I and NS4B (K1846T) are indicated by stars. This replicon was used as a positive control for the transient replication assay. The replicon in the second upper lane is the parental construct used for the generation of the NS5A-reporter gene fusion constructs. It contains a linker sequence introduced by PCR into the C-terminal deletion Δcl3. By using the *AscI* and *PmeI* restriction sites the reporter genes eGFP, dsRed and Renilla-luciferase were inserted into replicon Luc-Δcl3-linker, leading to Luc-NS5A-eGFP, Luc-NS5A-dsRed and Luc-NS5A-R-luc. Numbers below the NS5A proteins refer to the length of the NS5A protein.

These insertions led to an extension of the total length of NS5A by 239 amino acids in case of eGFP, 224 amino acids for dsRed and 311 amino acids in the case of the renilla luciferase (Fig. 28). To test the replicons for their ability to replicate, we performed a transient replication assay.
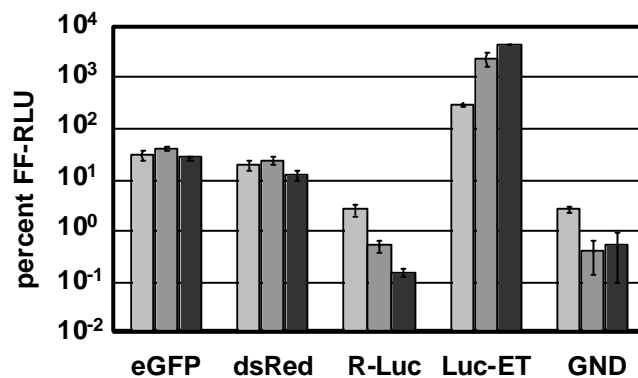
FIG. 29: Time course of a transient replication assay. Transfected Huh-7 cells were harvested 4, 24, 48 and 72 h after electroporation and luciferase activities in the cell lysates were determined. Values normalized for transfection efficiency by using the 4h value are shown. The adaptive replicon carrying authentic NS5A (Luc-ET), as well as the inactive replicon (GND), served as positive and negative controls, respectively.

As shown in Fig. 29, the insertion of Renilla luciferase completely blocked RNA replication but did not affect activity of this reporter because high levels of renilla luciferase activity were detected at 4 h post transfection (not shown). In contrast, an about 100-fold reduced but clearly

detectable replication was found with the two RNAs that carried the insertions of eGFP or dsRed confirming the high genetic and presumably also structural flexibility of the NS5A C-terminus. To study, whether the insertions had an influence on polyprotein processing, [^{35}S] labeling was performed and NS4B, NS5A and NS5B proteins were analyzed by immunoprecipitation, SDS-PAGE and autoradiography.
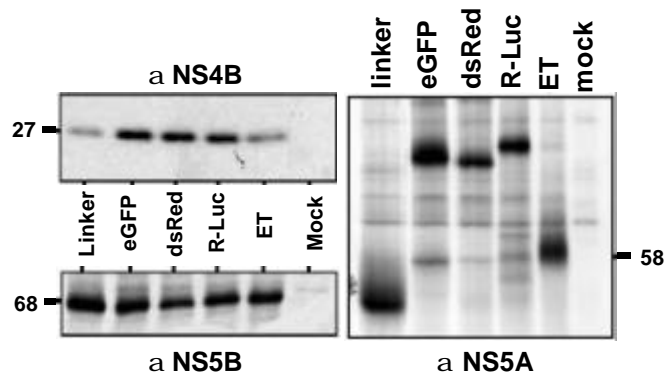
FIG. 30: Analysis of polyprotein processing by using the vaccinia virus/T7 hybrid system. NS3 to NS5B proteins carrying given insertions in NS5A were expressed in Huh-7 cells in parallel with the parental polyprotein (ET) and 4 h after radiolabeling with [^{35}S] cysteine and methionine, given HCV proteins were analyzed by immunoprecipitation and SDS 10% -PAGE.

For all replicons independent of the inserted reporter gene we were able to detect NS4B, NS5B, and the NS5A fusion proteins and observed no obvious effect on the polyprotein processing (Fig. 30). Thus, the inhibition of RNA replication by the insertion of the renilla luciferase gene is not due to a defect of polyprotein processing but rather of NS5A function.

Having established viable replicons that carry in-frame insertions of eGFP or dsRed in NS5A, we wanted to know whether these proteins retained their ability to fluoresce. To this end we performed our transient replication assay and analyzed cells 4, 24, 48 and 72h after transfection by fluorescence analysis. Unfortunately, protein levels were too low to allow detection of eGFP or dsRed. Therefore, we expressed NS3 to 5B polyproteins in Huh-7 cells that constitutively express T7 polymerase and repeated fluorescence analysis.
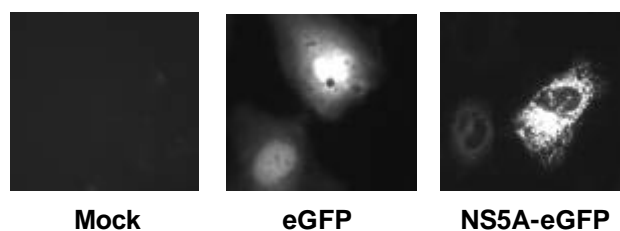
FIG. 31: Fluorescence analysis of the NS5A-eGFP fusion protein in the context of an NS3 to 5B polyprotein. Huh-7 cells constitutively expressing T7 RNA polymerase were transfected with the expression vector only (mock), the vector containing only the eGFP gene (pTM/eGFP) or the vector containing the NS3 to NS5B polyprotein with the NS5A-eGFP fusion protein. Cells were fixed 6h after transfection with 3% paraformaldehyde and analyzed by fluorescence microscopy.

The result in Fig. 31 shows that high-level green fluorescence was found with the eGFP polyprotein with a subcellular distribution pattern that is characteristic for endoplasmatic reticulum. Moreover, the spot-like appearance is typical for the membranous web that can be induced by NS4B in the absence of RNA replication (27). In contrast, no fluorescence was found with the dsRed polyprotein. This failure may be ascribed to the fact that for fluorescence, dsRed must homotetramerize in contrast to eGFP that is functional as a monomer (4). In summary these data show that the C-terminus of NS5A is genetically highly flexible and tolerates the insertion of large heterologous protein domains.

3.3 Structural and functional analysis of the NS5A membrane anchor domain

In analogy to other RNA viruses, the HCV non-structural proteins NS3 to 5B are supposed to build up a membrane associated multi-protein complex that catalyzes replication of the viral RNA genome. An important prerequisite for the formation of a functional RC is that HCV proteins and genomic RNA are recruited to intracellular membranes. In the last couple of years, several studies addressed the membrane topologies of individual HCV encoded proteins. NS3 expressed on its own localizes to the cytoplasm, however upon coexpression with NS4A, both proteins form a tight complex, in which the NS3 molecule is targeted to intracellular membranes *via* a hydrophobic transmembrane helix located in the 20 N-terminal residues of NS4A (56, 143, 161, 166). For NS4B, at least five putative trans-membrane (TM)-domains were predicted by computer-aided topology modeling. Furthermore, it induces the formation of intracellular membranous vesicles that are supposed to be the site of RNA replication, by an yet unknown mechanism.

Membrane association of NS5A is achieved by an amphipathic N-terminal α-helix. This domain is necessary and sufficient to target NS5A to the endoplasmic reticulum (ER) or an ER derived modified compartment by a post-translational mechanism, resulting in an integral membrane association. Conversely, NS5A lacking this N-terminal region is transported into the nucleus because of the loss of membrane association and the activation of a cryptic nuclear localization signal (62, 134). The NS5B RdRp carries a hydrophobic C-terminal sequence of 21 amino acids that anchors the protein into the ER membrane by a posttranslational mechanism (65, 128). It belongs to the group of tail-anchored proteins and is an integral membrane protein with a large ectodomain carrying the catalytic RdRp activity that resides on the cytoplasmic side.

The formation of a multi-protein complex requires specific interactions between individual components such as the viral proteins, as well as cellular proteins that are supposed to be involved in the formation, maintenance and functionality of the replicase complex. Besides the interaction between NS3 and NS4A, recently an interaction between NS5A and NS5B has been reported. Furthermore, both proteins were also shown to interact with the cellular SNARE-like protein h-VAPA and it was postulated that *via* this interaction the HCV proteins might be targeted to vesicle membranes and/or contribute to membrane rearrangements, although the latter process can be mediated by NS4B alone (27, 151). Nevertheless, the molecular details of the structure and functions of the replication complex (RC) remain to be discovered. Therefore, we wanted to investigate the role of NS5A membrane association for the formation of the replicase complex, RNA replication and NS5A phosphorylation.

3.3.1 Subcellular localization of NS5A membrane anchor mutants

The 3D structure information of the amphipathic α-helix (aa 5-25) was used to design a panel of NS5A mutants with aa substitutions, insertions, or deletions in the N-terminal α-helix as shown in Fig. 32.
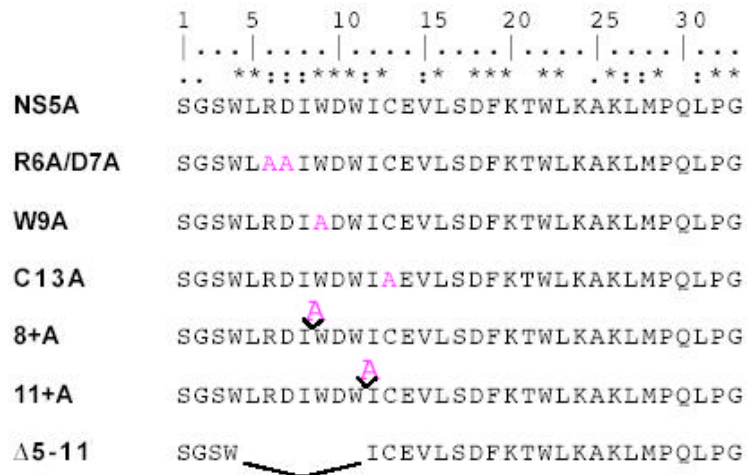
FIG. 32: NS5A membrane anchor mutants (kindly provided by V. Brass). Amino acid sequences of the different NS5A anchor mutants. The amino acid conservation among different HCV isolates (Brass *et al*, 2002) is indicated by an asterisk (*), a colon (:), and a dot (.) for fully conserved, conserved, and similar residues, respectively.

The mutations were carefully designed to preserve the overall folding of the amphipathic α-helix and taking into consideration the conservation of residues among different HCV isolates (11).
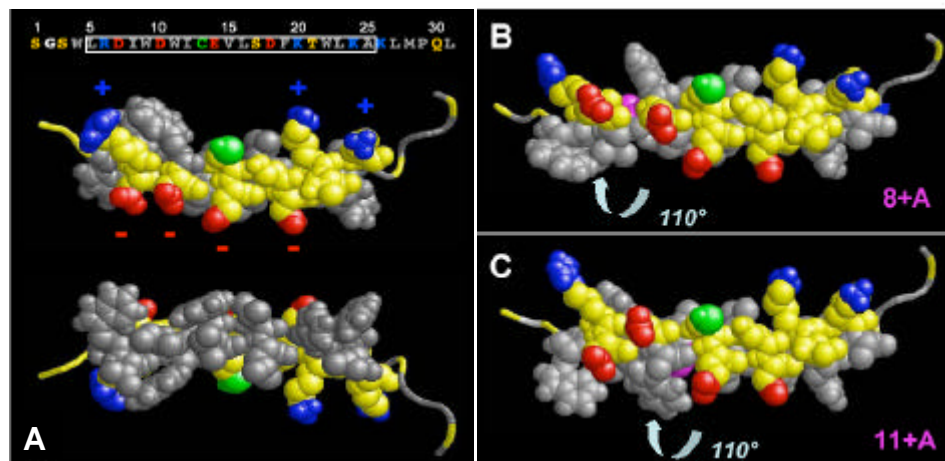
FIG. 33: Structure of the NS5A aa 5-25 amphipathic α-helix (kindly provided by F. Penin). (A) Amino acid sequence of NS5A[1-31] including helix 5-25 (white box) and space-filling representation of the polar and hydrophobic sides of this helix (model of average structure in 100 mM SDS). Residues are color coded according to their physico-chemical properties. Hydrophobic and polar residues are grey and yellow, respectively. Positive and negative charged groups of basic (K, R) and acidic (D, E) residues are blue and red, respectively. The SH group of Cys is green. **(B, C)** Theoretical models of mutants 8+A and 11+A. Ala insertion (magenta) twists the helix by 110°. The C-terminal portion of the helix is shown in the same orientation as in (A) to highlight the distortion of charged residues on the N-terminal portion. Models were constructed by using the NMR average structure of NS5A[1-31] observed in 100 mM SDS as template and SwissPDB Viewer program.

Mutation of two charged residues at position 6 and 7 to Ala resulted in construct R6A/D7A. The absolutely conserved Trp9 and Cys13 were replaced by Ala, yielding constructs W9A and C13A, respectively. To perturb the asymmetric charge distribution on the hydrophilic side of the helix Ala residues were inserted at position 8 or 11, yielding constructs 8+A and 11+A, respectively. These insertions twist the helix by 110°, as illustrated in Fig. 33B and C. Finally, construct D5-11 was obtained by the deletion of two α-helix turns within the membrane anchor domain. Subcellular localization of the NS5A mutants was analyzed by immunofluorescence microscopy following transient expression of the NS3 to NS5B polyprotein in Huh-7/T7 cells. NS5A protein was detected by a monoclonal NS5A antibody and cell nuclei were counterstained with DAPI.
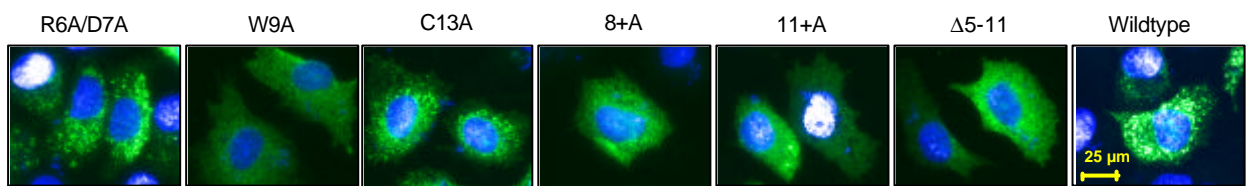
FIG. 34: Subcellular localization of NS5A membrane anchor mutants. Huh-7 cells were transiently transfected with pTM/NS3-NS5B-R6A/D7A, -W9A, -C13A, -8+A, -11+A, -Δ5-11, and –Con-1, as indicated by the captions. After fixation with 3% paraformaldehyde NS5A proteins were stained using mAb against NS5A. Bound primary antibody was revealed with an Alexa 488-conjugated secondary antibody. Slides were analyzed by fluorescence microscopy. Wildtype NS5A protein expressed from pTM/NS3-5B/Con-1 served as a control.

As shown in Fig. 34, mutants R6A/D7A and C13A showed a distribution pattern of NS5A similar to wildtype NS5A. Typical dot like structures corresponding to the membranous web were stained, indicating that these mutant NS5A proteins still localize to HCV induced vesicular structures and are probably part of the membrane associated HCV RC. For the mutants W9A, 8+A, 11+A and Δ5-11 the intracellular localization of NS5A was different compared to wildtype NS5A. Most of the protein was diffusely distributed within the cytoplasm of the cells and nearly no dot like structures were visible. In no case a nuclear staining was observed that has been described for mutants lacking the N-terminal amphipathic α-helix. Interestingly, no difference in the distribution pattern of NS5A was observed, when the mutated NS5A proteins were individually expressed in the human osteosarcoma cell line U-2 OS (data not shown). All NS5A proteins were found to localize in a reticular staining pattern, which surrounded the nucleus, extended through the cytoplasm, and included the nuclear membrane. Again, no nuclear staining was observed (data not shown). The staining pattern was indistinguishable from that of wildtype NS5A, which is associated with membranes of the ER or an ER-derived compartment (11). Thus, the designed membrane anchor mutants seemed to retain their proper subcellular localization and apparent membrane association in U-2 OS cells. However a clear difference in

the subcellular localization was observed for mutants W9A, 8+A, 11+A and Δ5-11, when expressed in the context of the polyprotein in Huh-7 cells. These mutations seem to interfere with the incorporation of NS5A into the HCV RC and were therefore most interesting candidates to study their influence on RNA replication.

3.3.2 RNA replication of NS5A membrane anchor mutants

To study the effect of mutations in the NS5A membrane anchor on RNA replication, these mutations were introduced into a reporter replicon carrying two cell culture-adaptive changes in NS3 and one in NS4B to analyze their effect on HCV RNA replication. In these replicons, translation of the firefly luciferase gene is directed by the PV-IRES, whereas the EMCV-IRES governs translation of the HCV nonstructural proteins (Fig. 35A). RNA replication was analyzed by monitoring luciferase activity at various time points after transfection into Huh-7 cells. The parental replicon and a replication-deficient RNA (GND) served as positive and negative controls, respectively. Luciferase activities 4 h after electroporation were used to determine transfection efficiencies. A representative result is shown in Fig. 35B.
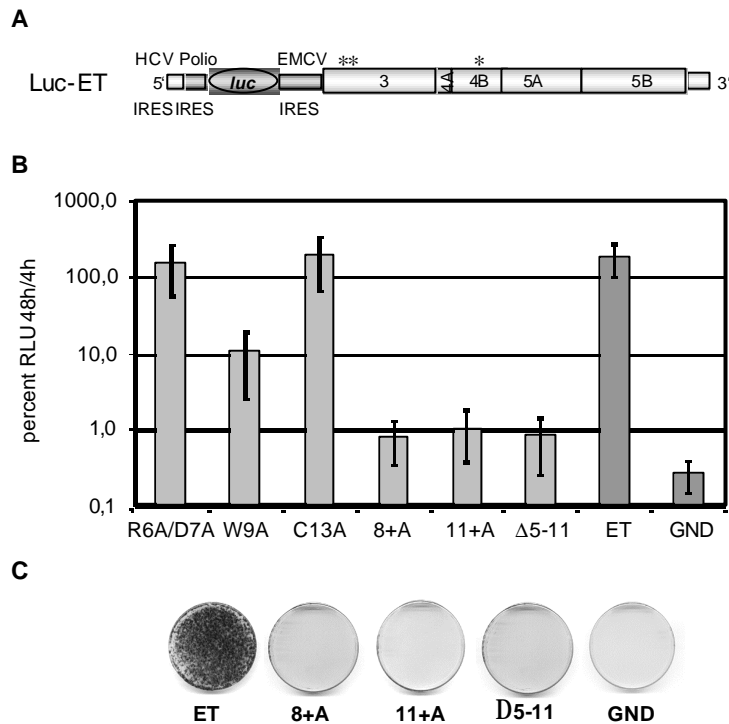
FIG. 35: RNA replication of NS5A membrane anchor mutants. **(A)** Structure of the basic replicon construct carrying the firefly luciferase gene (*luc*). Adaptive mutations in NS3 and NS4B (E1202G, T1280I, K1846T) are indicated by stars. **(B)** Representative results of a transient replication assay. Huh-7 cells were transfected with the replicon RNAs specified below each bar and luciferase activities were determined in cell lysates. Values refer to the percent ratio of the values measured 48 h and 4 h after transfection. The 4 h value was used to correct for different transfection efficiencies. ET, luc-ET replicon; GND, replication deficient RNA. **(C)** Huh-7 cells were transfected with 1 µg of G418-selectable replicons specified below each plate. After 3 weeks cells were fixed and stained with Coomassie blue.

Single and double Ala substitutions for conserved amino acids located on the cytosolic side of the helix (R6A/D7A and C13A) had no influence on replication efficiency. The Ala substitution for the fully conserved Trp9 decreased luciferase activity by about 10-fold. Insertion of Ala residues at amino acid position 8 or 11 (8+A and 11+A, respectively) abolished HCV RNA replication. The effect of these mutations was as dramatic as that observed for a deletion of two helix turns (Δ5-11). In some experiments, luciferase activities obtained with the mutants 8+A, 11+A and 5-11 were slightly higher than that of the GND negative control (Fig. 35B). Because this difference was within the range of assay variability, these mutants were reanalyzed by a more sensitive replication assay. For this purpose, they were introduced into selectable replicons in which the luciferase reporter gene was replaced by the gene encoding for neomycin-phosphotransferase. Upon transfection into Huh-7 cells and G418 selection, the number of G418-resistant colonies reflects the replication capability of a given replicon. As shown in Figure 35C no colonies were obtained under these conditions, corroborating that these mutations completely block RNA replication. In conclusion, these results indicate that insertions or deletions, leading to a twist of the helix or altering its size, have a dramatic impact on the function of NS5A for HCV RNA replication.

3.3.3 Phosphorylation analysis of NS5A helix mutants

In order to study the effect of these helix mutations on phosphorylation of NS5A, we performed metabolic [^{35}S] cysteine/methionine and [^{32}P] orthophosphate labeling experiments, upon expression of NS3 to 5B polyproteins with the vaccinia virus/T7 hybrid system in Huh-7 cells.
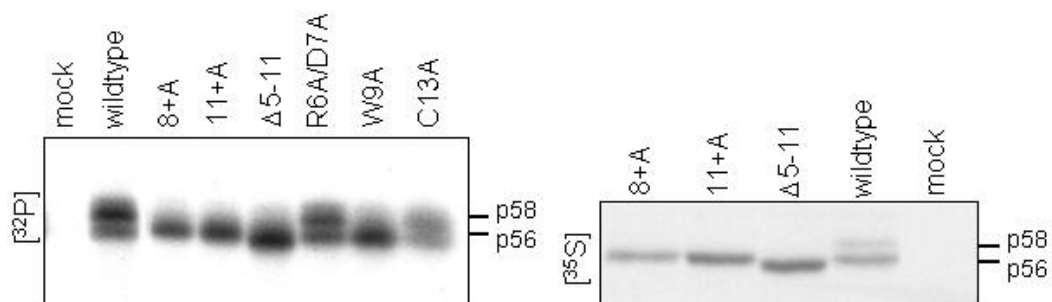
FIG. 36: Phosphorylation analysis of NS5A helix mutants. Huh-7 cells expressing T7 polymerase after infection with recombinant vaccinia virus were transfected with constructs expressing NS3 to NS5B harboring the mutations indicated above the lanes. Proteins were radiolabeled with [^{32}P] orthophosphate or [^{35}S] cysteine/methionine. NS5A proteins were isolated by immunoprecipitation and separated by 10% SDS-PAGE. Mock transfected cells were used as a negative control.

As shown in Fig. 36, mutants R6A/D7A and C13A that have no influence on RNA replication also did not affect formation of the two NS5A phosphovariants p56 and p58. Mutant W9A that

reduced RNA replication 10-fold showed a clear reduction of NS5A hyperphosphorylation, whereas for the mutants 8+A, 11+A and Δ5-11 that have the greatest impact on the structure of the helix and abrogate RNA replication, no p58 was detectable. In summary, these results demonstrate a clear correlation between reduced efficiency of RNA replication and hyperphosphorylation of NS5A.

3.3.4 Influence of N-terminal NS5A peptides on replication efficiency of subgenomic replicons

During the course of this work, Elazar and coworkers reported that a synthetic peptide representing the N-terminal amphipathic α-helix of NS5A blocked membrane association of NS5A *in vitro* in a dose-dependent and sequence-specific fashion (28). While a synthetic peptide designed to mimic the wildtype amphipathic helix inhibited membrane association of wildtype NS5A, a second peptide mimicking a mutated helix, lacking membrane association and abrogating RNA replication had no such effect on NS5A membrane association. It was therefore very likely, that disturbance of the NS5A membrane association by a synthetic peptide, may also decrease RNA replication. In order to confirm this hypothesis in a cell-based system and to study whether our helix mutations may exhibit any effects on RNA replication of a subgenomic replicon, we generated bicistronic luciferase replicons, expressing firefly-luciferase, the cellular ubiquitin sequence and N-terminal peptides of NS5A from the first cistron and NS3 to 5B from the second cistron.
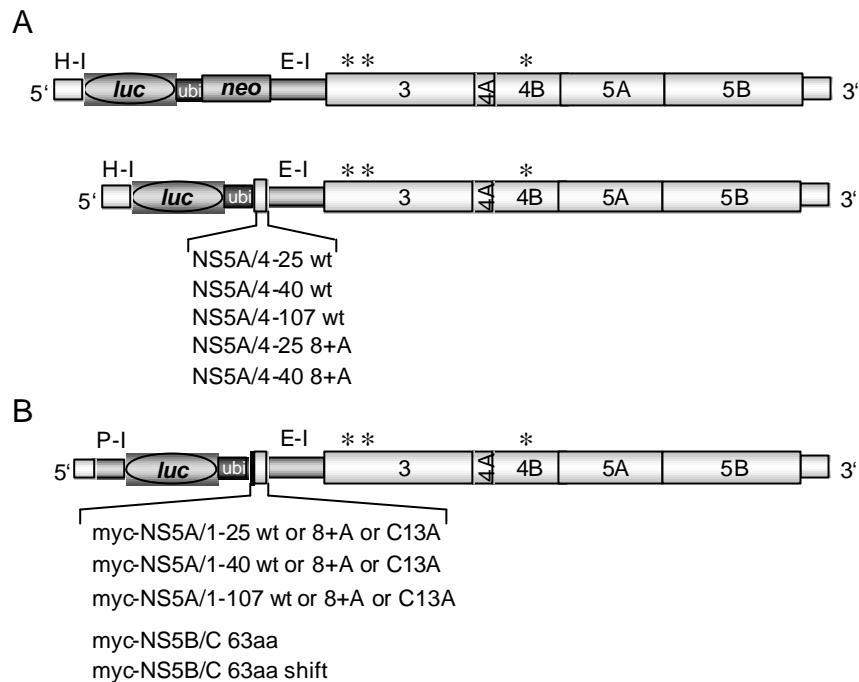
FIG. 37: Schematic representation of adapted subgenomic luc-replicons carrying a duplication of the amphipathic α-helix in the first cistron. (A) Translation of the luciferase-ubiquitin-NS5A anchor (amino acid 5-25) cassette was mediated by the HCV-IRES. NS5A fragments differed in length from amino acid 4 to 25, 4 to 40 and 4-107 and were fused in frame with the C-terminus of the ubiquitin sequence. (B) Second generation luciferase replicons, harboring the PV IRES for translation of the first cistron. N-terminal NS5A fragments were fused in frame upstream of the luciferase-ubiquitin-myc-tag sequence. NS5A fragments express wildtype NS5A sequence or carry given mutations. Numbers refer to amino acid positions within the NS5A protein (amino acid 1-447). As positive control for replicons in panel (A) a construct expressing luciferase-ubiquitin-neomycin-phosphotransferase in the first cistron was used. As controls for replicons in panel (B) constructs expressing the C-terminal 63 amino acids of NS5B (C 63aa) fused in frame to the C-terminus of the luciferase-ubiquitin-myc tag fusion protein. Replicon C 63aa shift had the same overall design, but due to a frameshift in the NS5B C-terminal fragment expressed an irrelevant luciferase-ubi-myc tag fusion protein. H-I, HCV-IRES; P-I, PV-IRES; E-I, EMCV-IRES; adaptive mutations are indicated by stars

Insertion of the ubiquitin sequence between the luciferase gene and the NS5A peptide sequence was made to ensure proper cleavage of the peptide. For better detection of the peptides we also created replicons expressing N-terminally myc tagged NS5A peptides (Fig 37B). All replicons depicted in Fig. 37A contained the authentic N-terminus of the amphipathic α-helix starting at amino acid 4 of NS5A (helix: amino acid 5-25) and varied in length up to amino acid 25, 40 or 107. In addition, these peptides corresponded to wildtype or mutated NS5A sequences. Replicons shown in Fig. 37B carried a myc-tag at the N-terminus of NS5A, followed by the NS5A sequence starting with amino acid 1. One advantage of this kind of reporter replicon is that peptide expression takes place in close proximity to HCV protein expression. Furthermore, using this kind of reporter-replicon, we were able to demonstrate that the membrane anchor of NS5B (C-teminal 21 amino acids) has a dominant negative effect on RNA replication (T. Pietschmann personal communication). Hence, in general this replicon design is usable to address the question of dominant negative inhibition.
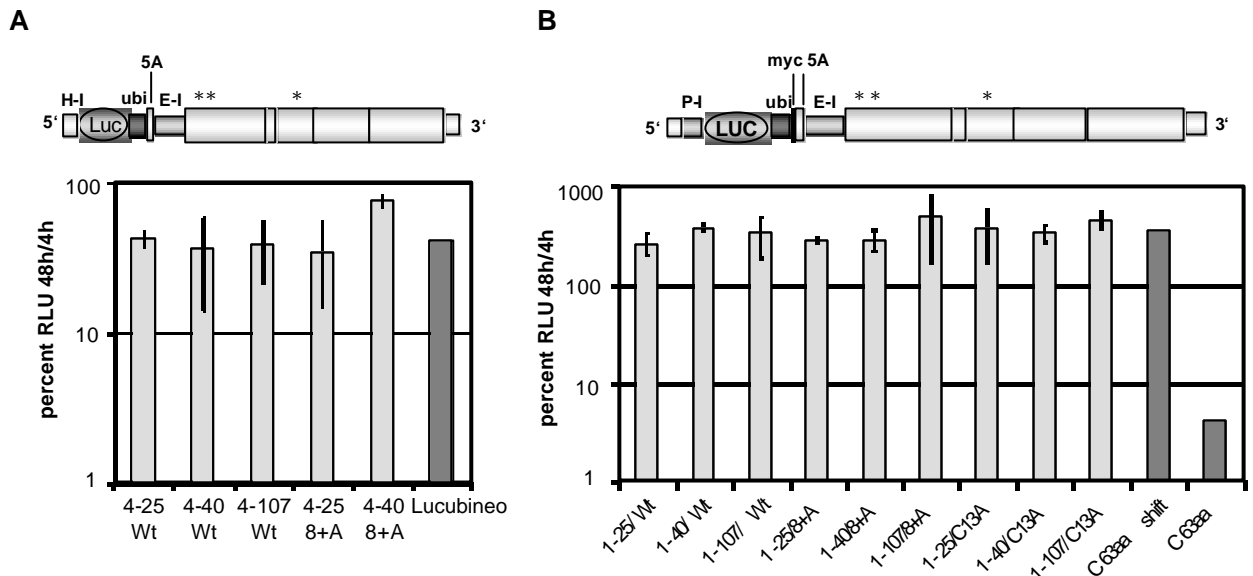
FIG. 38: Transient replication of bicistronic replicons expressing N-terminal sequences of NS5A in the first cistron. Transfected Huh-7 cells were harvested 4 and 48h after electroporation and luciferase activities in the cell lysates were determined. Values normalized for transfection efficiency by using the 4h value are shown. (A). Adaptive replicons express a luciferase-ubiquitin-NS5A amphipathic α-helix fusion protein from the first cistron and authentic NS5A within the polyprotein NS3-NS5B from the second cistron (see Fig. 37A). A construct expressing luciferase-ubiquitin-neomycin-phosphotransferase from the first cistron served as a positive control (Lucubineo). (B) Second generation reporter replicons were generated expressing a luciferase-ubiquitin-myc tag-NS5A fusionprotein under translational control of the PV-IRES from the first cistron (see Fig. 37B). NS5A fragments differed in size as indicated below each lane. Mutations within the membrane anchor region are specified in the names. A replicon expressing the last 63 amino acids of NS5B (C 63aa) in the first cistron and an arbitrary sequence due to a frameshift of the same sequence (C 63 aa shift) served as controls. Schematic presentation of the replicon constructs used for the transient replication assay described in (A) and (B). H-I, HCV-IRES; P-I, PV-IRES; EI, EMCV-IRES; adaptive mutations are indicated by stars.

As shown in Fig. 38, replication efficiencies of the various constructs were not affected by co-expressed NS5A peptides translated from the first cistron. This result was unexpected, because others had described inhibition of NS5A membrane association by analogous 5A peptides. To control for the expression levels of the peptides, we performed Western Blot analysis after transient transfection. Unfortunately, we were not able to detect the peptides, neither with a polyclonal NS5A-specific antibody, nor with a monoclonal antibody directed against the myc-epitope. However this was also true for the NS5B membrane anchor peptide that in the functional assay showed a clear dominant negative effect on RNA replication (Fig. 38B, 63aa). Since we could not exclude the possibility that expression levels or half lives of the peptides were too low to exert any effect on replication, we performed additional co-transfection experiments, in which we mixed in vitro transcribed luc-replicon RNAs with a CMV promoter-driven plasmid encoding the first 44 amino acids of NS5A fused C-terminal with GFP and repeated the transient replication assay. However, even under these conditions, we observed no significant effect on RNA replication (data not shown). Although, expression of the NS5A-GFP fusion protein could be detected by fluorescence microscopy, albeit at rather low levels, we

did not observe a dominant negative effect of the NS5A membrane anchor on RNA replication. However, we can not exclude the possibility that expression levels of the peptides were too low to exert an effect on RNA replication. Further studies will therefore be required to see, whether NS5A peptides interfering with membrane association inhibit RNA replication.

3.4 Rescue of HCV RNA replication by trans-complementation of NS5A

As described above, virtually all positive-strand RNA viruses replicate their genomes on virus-induced vesicular membrane compartments that present rather enclosed vesicular structures with a limited possibility for exchange of viral RNA and proteins. With the aim of gaining insights into the functional organization of the HCV replication complex (RC) and the relationship between its individual components, we wanted to establish a trans-complementation assay. In general, complementation describes the ability of gene products from two different viruses to interact functionally in the same cell, permitting virus replication of otherwise defective mutants. Complementation is a wide spread method originally used in virology for functional analysis of mutants that were organized into complementation groups defining separate viral functions. Naturally occurring complementation has been described for several plant- and RNA viruses. For instance, in the case of *Poliovirus* (PV) complementation of mutations in proteins 2C and 3D (RdRp) was found (148), although complementation efficiency was rather low. In case of the flavivirus *Kunjin virus* (KV), efficient trans-complementation was reported for mutations affecting NS1, the NS3 helicase or the NS5 RdRp (71 - 73, 87). For a close relative of HCV, the pestivirus *Bovine viral diarrhea virus* (BVDV) Grassmann and coworkers demonstrated that NS5A was the only non-structural protein that could be complemented in trans (50). However, it was not clear, whether trans-complementation is also possible with HCV.

3.4.1 Establishment of a trans-complementation assay using selectable replicons

In order to study which components of the HCV RC are exclusively cis-active or can act in trans we first developed a trans-complementation assay that utilizes the transfection of selectable replication-deficient HCV replicons encoding the neomycin-phosphotransferase (neo-replicons) into Huh-7 cells that harbor replicons expressing the hygromycin-phosphotransferase (hyg-replicons) (Fig. 39A).
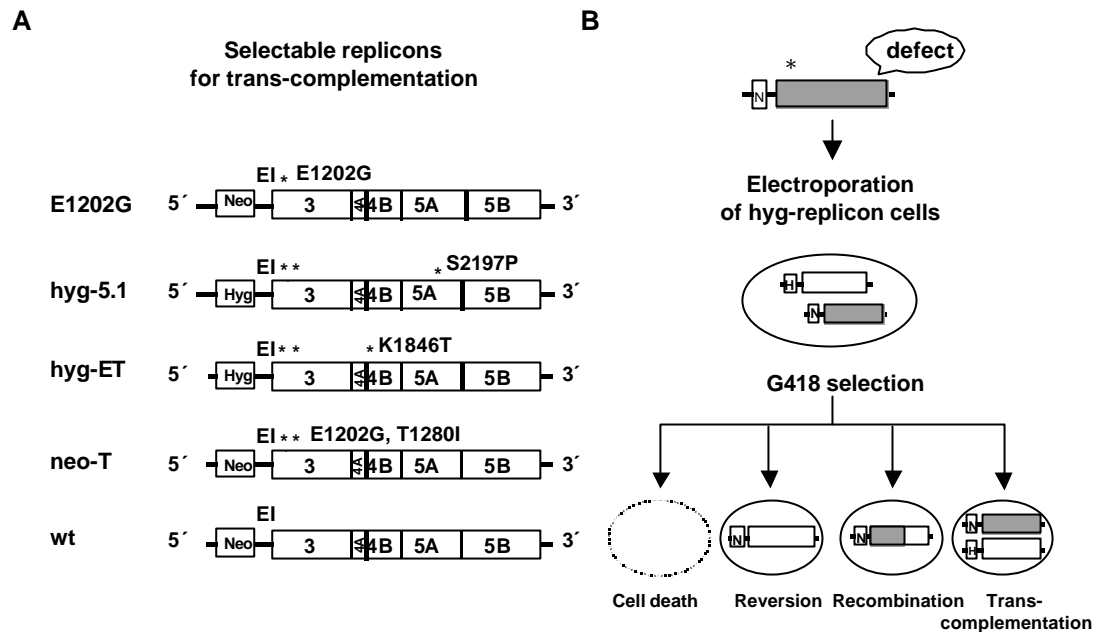
FIG. 39: (A) Structures of replicon RNAs used for trans-complementation assays. Adaptive mutations residing in NS3 (E1202G, T1280I), NS4B (K1846T) and NS5A (S2197P) are indicated by asterisks. Numbers refer to amino acid positions of the HCV Con-1 complete polyprotein. In the case of the selectable replicons expression of the first cistron is mediated by the HCV IRES, whereas translation of the non-structural proteins NS3 to 5B is directed by the EMCV IRES (EI). (B) Schematic drawing of the experimental approach to analyze trans-complementation. Mutations were introduced into a subgenomic neo-replicon carrying one weak adaptive mutation in NS3 (E1202G; indicated by a star). Huh-7 cells containing hyg-helper-replicons were transfected with mutated RNAs and subjected to selection with G418. Possible outcomes are depicted beneath. G418-resistant colonies may arise in case of reversion, recombination, or trans-complementation. Neo or N, neomycin-phosphotransferase; Hyg or H, hygromycin-phosphotransferase; wt, wildtype.

Since the efficiency of trans-complementation can be affected by both the level of HCV proteins expressed from the helper RNA and the compatibility of certain cell culture-adaptive mutations (see below), we used two different helper cell clones: Huh-7-hyg/ET that harbors the hyg-ET-replicon and Huh-7-hyg/5.1 that carries the hyg-5.1-replicon (Fig. 39A). These replicons have two adaptive mutations in NS3 in common but differ with respect to the highly adaptive mutation that resides in NS4B or NS5A (hyg-ET and hyg-5.1, respectively). To increase the efficiency of trans-complementation, transfected cells were cultured in the presence of only G418. Under these conditions, cells carrying only the hyg-helper-replicon are eliminated within about one week. Efficiency of trans-complementation was determined by the increase of G418-resistant cell clones obtained after transfection of the Huh-7-hyg cell clones in comparison to naive Huh-7 cells that lack the hyg-helper RNA.

Three different mechanisms could account for the G418-resistance of a cell clone: (i) reversion or compensatory mutations in the defective neo-replicon RNA, (ii) recombination between mutant and helper RNA, or (iii) trans-complementation (Fig. 39B). To differentiate between these possibilities, RNA present in G418-resistant cell clones was analyzed by Northern-hybridization using *neo-* or *hyg*-specific probes and by sequence analysis of the

mutated neo-replicon RNA after amplification by RT-PCR and subcloning. In the first set of experiments we compared the permissiveness of naive Huh-7 cells and the cells carrying the two different helper RNAs. This comparison was necessary because host cell permissiveness has a strong influence on the efficiency of RNA replication, and therefore, differences in the number of G418-resistant colonies obtained with different Huh-7 cells could merely reflect different levels of permissiveness rather than trans-complementation. To this end, we transfected a wildtype replicon or a replicon that carried two adaptive mutations in NS3 (E1202G and T1280I) enhancing RNA replication about 2 to 5-fold (replicon neo-T; Fig. 40A). After G418 selection for 4 to 5 weeks, cells were fixed onto the plates and stained with Coomassie-blue solution.

	Permissiveness control	
	wt	neo-T
Huh-7		
Huh-7-hyg/ET		
Huh-7-hyg/5.1		

FIG. 40: Analysis of permissiveness of Huh-7 cells carrying stably replicating hygromycin-replicons. Hygromycin-replicon cells (Huh-7-hyg/ET and Huh-7-hyg/5.1) and naive Huh-7 cells were transfected with neo-replicons wildtype (wt) or T (neo-T) expressing non-adapted NS3 to 5B proteins or NS3 to NS5B proteins carrying adaptive mutations in NS3, respectively. After G418 selection for 6 weeks, cells were fixed and colonies were stained with Coomassie blue solution.

As shown in Fig. 40, there was no difference in the number of G418-resistant colonies between naive Huh-7 cells and cells that carry the hyg-helper RNAs, demonstrating the comparable permissiveness of the different Huh-7 cell cultures to be used for the trans-complementation assay.

3.4.2 Trans-complementation analysis of mutations in NS3, NS4B, NS5A and NS5B

To analyze if HCV non-structural proteins could be complemented in trans, we generated a panel of neo-replicons that carried the adaptive mutation E1202G in NS3 and additional inactivating mutations in NS3, NS4B, NS5A or NS5B abrogating RNA replication. The NS3 mutation served as a genetic marker of this RNA and would support RNA replication synergistically in the case of trans-complementation. For the inactivating NS3 mutation P1028H we have recently shown

that it has no influence on proteinase activity and therefore does not interfere with polyprotein processing (76). The exact reasons for the replication defects caused by the various mutations that we introduced into NS4B and NS5A are not known because thus far the exact functions of these proteins have not been firmly established. However, we analyzed all NS4B- and NS5A-mutants for their influence on polyprotein processing and found that in no case it was affected (76, data not shown). It is interesting to note that the amino acid substitutions S2197A, S2201A, and S2204A increased RNA replication when introduced individually into a replicon whereas the combination of two or more of these mutations completely abolished RNA replication (see chapter 2 result section). In the case of NS5B, we inactivated the RdRp activity by deletion of 10 amino acids spanning the active site GDD motif of the polymerase. Equal amounts of in vitro transcripts generated from these mutated template DNAs were introduced by electroporation into naive Huh-7 and the Huh-7-hyg cells. After 4 to 5 weeks of cultivation in medium containing G418 only, cells were fixed, stained with Coomassie-blue and counted. In the case of mutations affecting NS3, NS4B, or NS5B we found no difference in the number of G418-resistant colonies between naive Huh-7 and the two Huh-7-hyg cell clones (Fig. 41).

	NS3	NS4B		NS5A			NS5B
	P1028H	V1897P	Δ1905-1933	S2197A S2201A	S2201A S2204A	Δ2200-2202	ΔGDD
Huh-7							
Huh-7-hyg/ET							
Huh-7-hyg/5.1							

FIG. 41: Representative results of trans-complementation experiments using selectable replicon mutants. Hygromycin-replicon cells (Huh-7-hyg/ET and Huh-7-hyg/5.1) and naive Huh-7 cells were transfected with neo-replicons specified in the upper panel. After G418 selection for 6 weeks, cells were fixed and colonies were stained with Coomassie blue solution.

The fact that a very low number of colonies was obtained after transfection of the NS5B mutant that by definition is unable to replicate argues that these colonies are most likely due to integration of a DNA fragment that encodes the neo-gene. Therefore, we considered this low number of G418-resistant colonies as background. Furthermore, several repetitions of this experiment resulted in no colonies upon transfection of this NS5B mutant and G418 selection of transfected cells. In contrast to these results, an about 50- to 100-fold increase in the number of

G418-resistant colonies was obtained upon transfection of Huh-7-hyg cells with RNAs that carried mutations in NS5A. This result suggested that only mutations in NS5A can be rescued for RNA replication.

To further support this assumption, single cell clones obtained after transfection of Huh-7-hyg cells with NS5A mutants were established and replicon RNAs present in these cells were analyzed by Northern blot hybridization. To discriminate between the mutated and the helper RNA, positive-strand specific riboprobes were used that hybridized to the *neo-* or the *hyg-*gene, respectively. Figure 42A shows the results of an analysis of 9 independently generated cell clones. Excepting for two clones, both types of RNA were consistently detected in the cells. In total, we established 28 independent cell clones of which 25 contained both RNA species, whereas for 3 cell clones we only detected the neo-RNA. This argues that the hyg-replicon indeed complemented the NS5A mutant, which in turn conferred G418 resistance to the cells. Alternatively, the mutated RNAs might have acquired reversions, second site compensatory mutations, or lost the inactivating mutations due to recombination with the helper RNA. In these cases, the neo-replicon would be capable of self-replication. Although this possibility was not likely due to the rapid reduction of the hyg-replicon in the absence of selective pressure (the cells were cultured in the presence of G418 only) we analyzed the sequences of the neo-replicons in more detail.
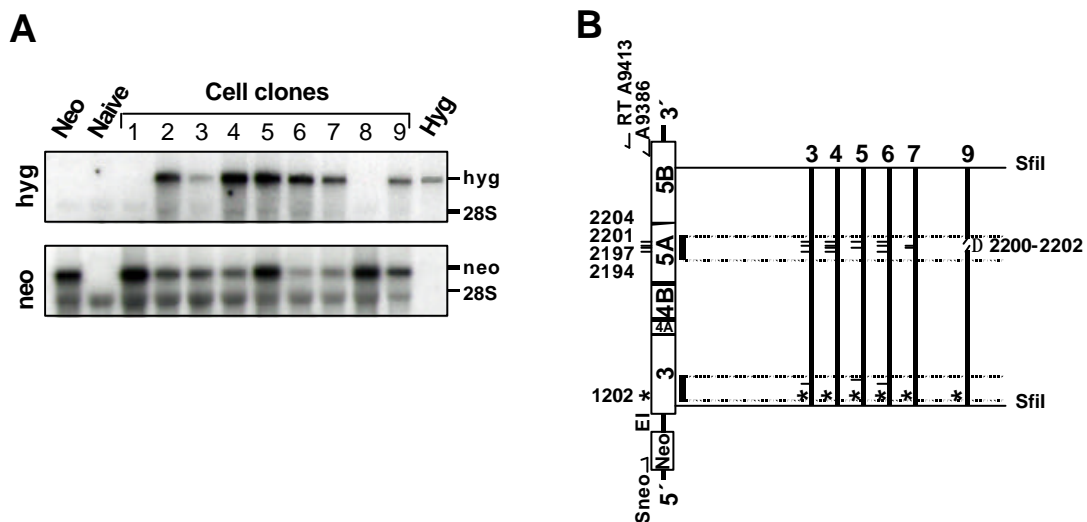
FIG. 42: (A) Analysis of HCV RNA species in G418-resistant cell clones by Northern hybridization. Huh-7 cells carrying stably replicating hyg-helper RNAs (hyg-ET or hyg-5.1) were transfected with neo-replicon mutants carrying lethal mutations in NS5A. After several months of G418 selection single cell clones (1 to 9) were established and analyzed for HCV RNAs by using *neo-* or *hygro-*specific (negative sense) riboprobes. As controls for specificity, neo- and hyg-replicon transcripts (10^8 molecules) as well as total RNA of naive Huh-7 cells were analyzed in parallel. (B) Conserved mutations in neo-replicons isolated from cell clones 3 to 7 and 9. After RT-PCR, two independent DNA clones were sequenced for each given cell clone. Conserved mutations are indicated by aasterisks in the case of the adaptive NS3 mutation and by vertical lines. The minimum region examined by sequence analysis is indicated by dotted lines. In the case of cell clone 4, the entire SfiI fragment was sequenced and no further mutation was detected.

A neo-specific RT-PCR was performed to amplify almost the complete replicon sequence (see Material and Methods, Fig. 42B). After subcloning of a *Sfi*I-DNA fragment that spans most of the HCV coding region, at least two independent clones of each fragment were sequenced in the regions carrying the introduced mutations.

We found that in all sequenced cell clones harboring both replicon RNA species, the inactivating mutations were still present in NS5A (Fig. 42B and Table 4). In addition, all of the replicons had preserved the adaptive mutation in NS3 at amino acid position 1202. Cell clones 3, 5 and 6 obtained one additional conserved mutation in NS3 (Tab. 3), whereas cell clones 4, 7 and 9 had no further mutations in this region. Sequence analysis of the complete *Sfi*I fragment derived from cell clone 4 revealed that no further mutations were present in the HCV coding region, excluding that the NS5A mutations were compensated by amino acid substitutions elsewhere in the polyprotein. Furthermore, in no case we found mutations present in the hyg-replicon in the neo-sequence. For example, in case of the trans-complemented cell clone 4, Huh-7-hyg/5.1 cells were used for super-transfection. The hyg-5.1-replicon contains a total number of 12 nucleotide changes spread over the NS3 to NS5A coding sequence of which 7 lead to an amino acid substitution. Since we found none of the nucleotide exchanges in the sequenced PCR fragments we suppose that our PCR was specific for the amplified neo c-DNA. In the case of the cell clones harboring only the neo-replicons (e.g. cell clones 1 and 8), the inactivating mutations in NS5A were also still present. However, further analysis of cell clone 8 revealed additional conserved mutations shown in Table 4. Currently, it is not clear whether these mutations compensated for the defects in NS5A. Alternatively, these mutants may replicate in a very minor subpopulation of Huh-7 cells. Irrespective of these possibilities, the absence of such mutations in at least one cell clone carrying both mutant and helper replicon RNA strongly suggests that NS5A was complemented in trans. The fact that replication of the RNAs with inactivating mutations in the other non-structural proteins could not be rescued indicated that only mutations in NS5A can be trans-complemented. This result implies that individual NS5A proteins rather than complete replicase protein complexes or RNA templates may become exchanged between helper and mutant RCs. Alternatively, complementing NS5A may provide a function outside the replicase complex and therefore indirectly rescue replication of the NS5A mutants.

Table 4. Sequence analysis of mutated neo-replicon RNAs recovered from G418-resistant cell clones.

Cell clone	Input RNA	Rescued RNA
3	E1202G S2194A S2201A S2204A	E1202G G1388E S2194A S2201A S2204A
4	E1202G S2194A S2197A S2201A S2204A	E1202G S2194A S2197A S2201A S2204A
5	E1202G S2197A S2204A	E1202G S1408P S2197A S2204A
6	E1202G S2194A S2201A S2204A	E1202G G1388E S2194A S2201A S2204A
7	E1202G S2197A S2201A	E1202G S2197A S2201A
8	E1202G Δ2194-2197	E1202G G1388E Δ2194-2197 I2430V
9	E1202G Δ2200-2202	E1202G Δ2200-2202

3.4.3 Establishment of a transient trans-complementation assay

The results described thus far are based on trans-complementation of selectable replicon RNAs after super-transfection of helper-replicon cells. Apart from the requirement to perform cell selection for several months, this approach bears the risk to enrich additional mutations in the non-functional neo-replicon during selection. These mutations may have a compensatory effect

for the inactivating mutation and therefore circumvent trans-complementation by the helper RNA. To overcome these limitations and to screen a broader range of lethal mutations for rescue of RNA replication within a shorter time, we established a transient complementation assay that was based on the co-transfection of mutant and helper RNAs. While the mutated replicon carried the firefly luciferase reporter gene, the replication competent helper RNA carried the *neo*-gene to allow discrimination between both RNA species (Fig. 43B).
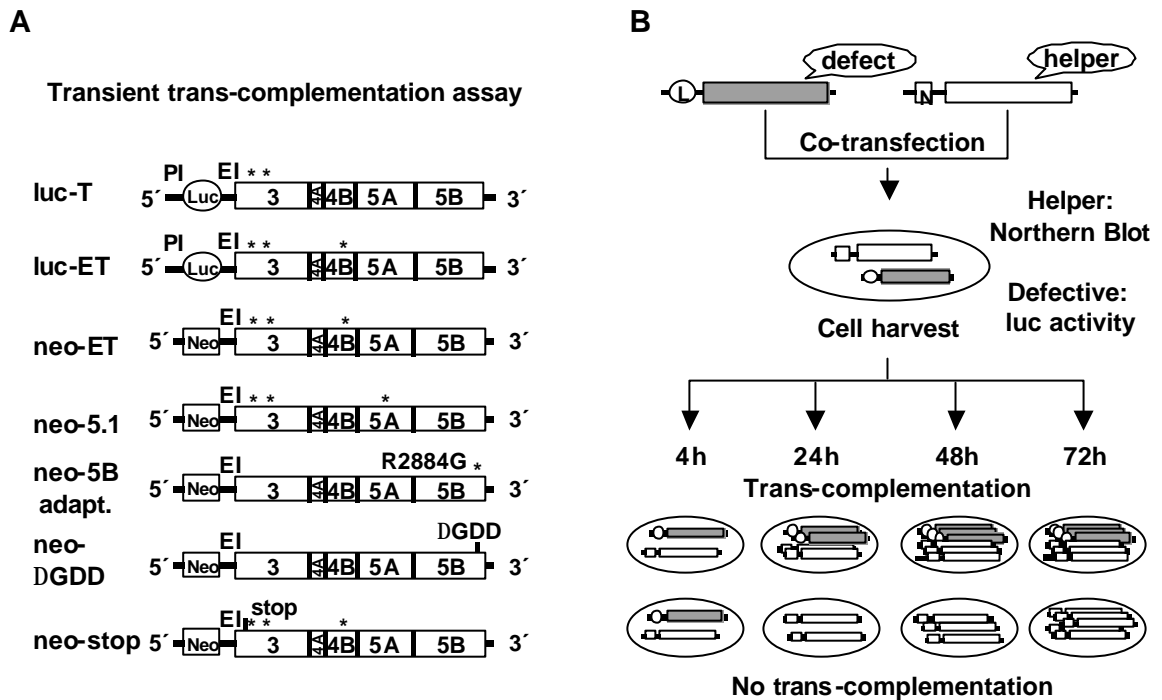
FIG. 43: (A) Structures of replicon RNAs used for transient trans-complementation assay. Adaptive mutations localized in NS3 (E1202G, T1280I), NS4B (K1846T), NS5A (S2197P) and NS5B (R2884G) are indicated by asterisks. Numbers refer to amino acid positions of the HCV Con-1 complete polyprotein. In the case of the luciferase (luc) replicons translation of this reporter is mediated by the PV IRES (PI) that was fused to the 3′end of the HCV IRES. Translation of the HCV replicase is again mediated by the EMCV IRES. (B) A replication-deficient luciferase replicon (striped box) was co-transfected with a neo helper RNA (open box) into cells of a highly permissive Huh-7 cell passage. Equal numbers of cells were seeded and harvested at given time points post transfection. Replication of the mutant was monitored by luciferase assays, whereas replication of the helper RNA was controlled by Northern hybridization. Luc, *Photinus vulgaris* luciferase gene; ΔGDD, deletion of the NS5B GDD motif.

After co-transfection into a highly permissive naive Huh-7 cell clone replication of the mutant was determined 4, 24, 48 and 72 h post transfection by measurement of luciferase activity. These cells were chosen since they allow higher replication efficiencies in comparison to even highly permissive naive Huh-7 cell passages (89). In parallel, replication of the helper neo-RNA was determined by Northern blot hybridization. In an attempt to achieve most efficient restoration of RNA replication we used two different helper RNAs designated neo-ET and neo-5.1 (Fig. 43A). These replicons carry the same adaptive mutations in NS3 (E1202G and T1280I), but differ with respect to the additional highly adaptive mutation, which is K1846T in the case of neo-ET and

S2197P in the case of neo-5.1. The inactivating NS5A mutations were introduced into the luc-T replicon that harbors the same two adaptive mutations in NS3 (Fig. 43A). Based on our previous results indicating that only NS5A may be exchanged between different RCs, various combinations of cell culture-adaptive mutations were possible in trans-complemented RCs, depending on which mutant and helper RNA was combined (Fig. 44).
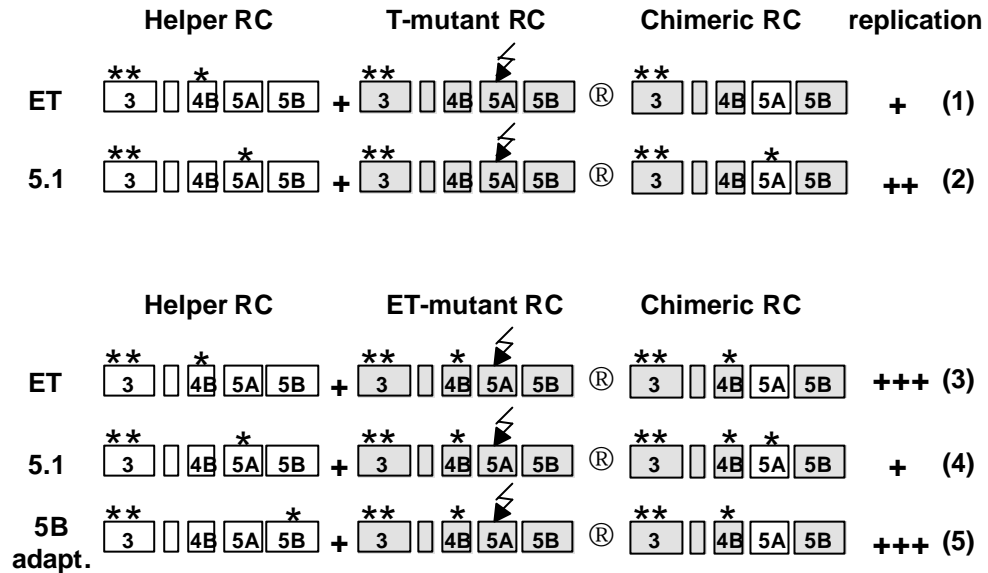
FIG. 44: Possible outcomes of trans-complementation after co-transfection of various helper and mutant replicon RNAs. The wedged arrow indicates inactivating mutations in NS5A. Asterisks refer to the positions of adaptive mutations in NS3, NS4A, NS5A or NS5B. Outcomes of trans-complementation based on the exchange of the NS5A protein and expected replication efficiencies are shown in the right. Numbers in parentheses refer to the text. +, poor replication; ++, moderate replication; +++ high replication. RC, replication complex.

In the first set of experiments we analyzed replication rescue of a luc-replicon carrying the NS5A double mutation S2197A+S2201A by neo-5.1 and neo-ET helper RNAs. To exclude non-specific effects of these helper RNAs, we used as an additional negative control a neo-replicon that carried a stop codon at the beginning of the NS3 coding region. This RNA (designated neo-stop) did not give rise to HCV proteins but otherwise was of the same structure as the other helper RNAs (Fig. 43A). A representative result of such a transient replication assay is shown in Fig. 45. Forty-eight hours after transfection luciferase activity observed with the luc-2197+2201-T mutant was below the detection limit. Co-transfection with the defective helper RNA neo-stop had no effect on replication ability of the mutant. In contrast, co-transfection with either replication competent helper RNA (neo-ET or neo-5.1) rescued the mutant to a level that was comparable to the parental replicon luc-T (Fig. 45A).

A

B

Helper				
neo ET	-		+	+
neo 5.1	-		+	
neo-stop	-	+		

luc 2197+2201T   T

Helper				
neo ET	+			
neo 5.1		+		
neo 5Badapt.			+	
neo-stop				+

luc 2197+2201ET

C
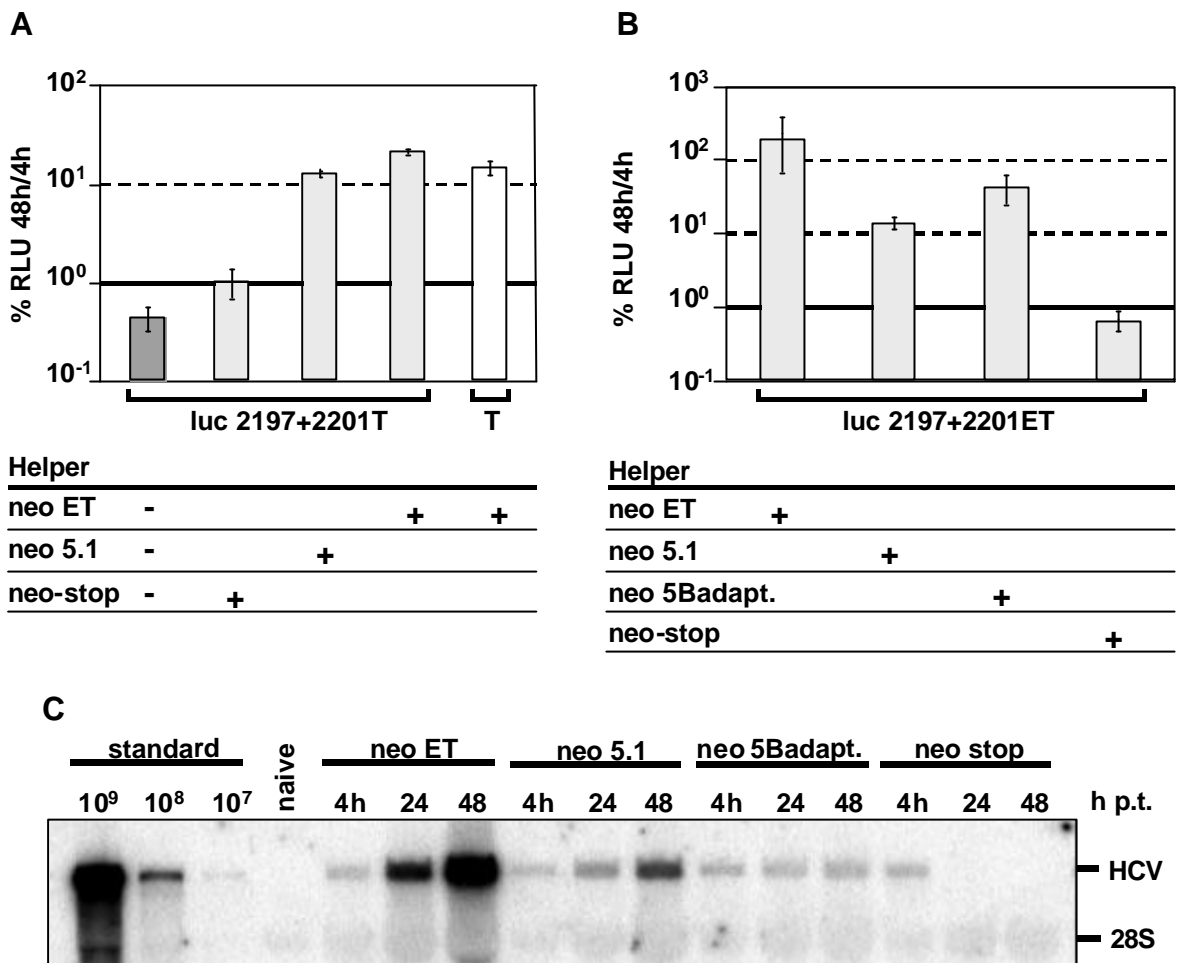
FIG. 45: Trans-complementation of the replicon mutant luc-2197+2201 by different helper RNAs. (A) The inactivating mutations S2197A+S2201A were introduced into the luciferase replicon luc-T (Fig.1A) carrying two weak adaptive mutations in NS3. After cotransfection with given helper RNAs, cells were harvested 4, 24, 48 and 72 h later and luciferase activities were determined. Values are the ratio of the 48h and the 4h values reflecting transfection efficiency. For comparison, the mutant was transfected without helper RNA (shaded box). Luciferase activities measured upon transfection of the parental replicon luc-T served as a positive control (open box). (B) Trans-complementation of mutant luc-2197+2201-ET with given helper RNAs as described in panel A. Representative results of 3 independent experiments are shown. (C) Analysis of helper RNAs specified in (B) by Northern hybridization. Twenty µg of total RNA was analyzed with a neo-specific riboprobe. Total RNA of naive Huh-7 cells served as a negative control. Known amounts of replicon in vitro transcripts were used as positive controls.

Analysis of the neo-helper replicons by Northern-hybridization with a neo-specific probe revealed a much higher level of RNA replication in the case of neo-ET as compared to neo-5.1 replicon (Fig. 45C). Furthermore, using Western blot analysis we found that the neo-ET helper RNA produces the highest levels of NS5A protein, arguing that one should expect a very high replication level of the mutant after cotransfection with this helper. However, the neo-ET helper provides a wildtype NS5A protein in trans and therefore, co-transfection with the luc-2197+2201-T mutant may lead to a RC that carries only a moderately adaptive mutation in NS3 (Fig. 44, example 1). The neo-5.1 helper RNA produces an NS5A protein that carries a highly

adaptive mutation in NS5A. Consequently, co-transfection with the luc-T mutant may restore a much more adapted RC (Fig. 44, example 2) and therefore, lower amounts of NS5A are sufficient to rescue RNA replication of the mutant to the level obtained with the highly replicating neo-ET helper replicon (Fig. 45A and C). In agreement with this assumption, we found that increasing the amount of co-transfected helper RNA from 62 ng up to 5 µg led to an increase of mutant RNA replication (data not shown). Based on these results, the highest level of rescue was expected for a combination of an efficiently replicating helper RNA (neo-ET) and an NS5A mutant that carried the same adaptive mutations (ET) (Fig. 44, example 3). In contrast, the combination of the neo-5.1 helper RNA with the same mutant should lead to a lower degree of RNA replication rescue, because RCs may be generated in which NS4B and NS5A each carry a highly adaptive mutation (Fig. 44, example 4). However, combination of highly adaptive mutations in NS4B, NS5A, or NS5B strongly reduces RNA replication (89, 90).

To further substantiate this assumption, we performed an experiment in which we co-transfected the mutant luc-2197+2201-ET with helper replicons neo-ET, neo-5.1 and neo-5Badapt.. As shown in Fig. 45B, the most efficient rescue was obtained with the neo-ET helper RNA whereas an about 20-fold lower rescue of the same mutant was found upon co-transfection of the neo-5.1 helper RNA. The fact that this helper did not support a higher rescue can be explained by the incompatibility of the highly adaptive mutations in NS4B (present in the mutant) with those in NS5A (present in the helper) (Fig.44, example 4) or by lower replication of the neo-5.1 helper RNA. To differentiate between these two possibilities, we co-transfected the same mutant with the neo-5Badapt. helper RNA. Although replication efficiency of this helper RNA was lower compared to neo-5.1 (Fig. 45C), rescue of RNA replication was 5-fold more efficient than for neo-5.1 (Fig. 45B) due to the provision of wildtype NS5A in trans (Fig. 44, example 5). Therefore, the lower production of NS5A (Fig. 45C) from the neo-5.1 helper plays only a minor role for trans-complementation, whereas the incompatibility of highly adaptive mutations seems to be a major determinant for the efficiency of trans-complementation. These results demonstrate that trans-complementation of NS5A is possible in transient replication assays. Moreover, the data support the notion that either NS5A proteins can be exchanged between different RCs or that NS5A provides a function outside the RC that indirectly rescues replication of the mutant. In the latter case, lower trans-complementation efficiency of neo-5.1 RNA for luc-ET mutants may be due to a competition between the adapted proteins for the same replication supporting function.

3.4.4 Trans-complementation analysis of mutations in the N-terminal amphipathic **a**-helix of NS5A

Having established a sensitive transient trans-complementation assay, we analyzed a comprehensive panel of replicons carrying mutations in different HCV proteins for their ability to be complemented in trans. To this end, a set of mutants was co-transfected with the neo-ET helper RNA. As summarized in Table 5, none of the analyzed mutations in NS3, NS4B, or NS5B that all reduced RNA replication to below the detection limit could be complemented in trans.

Table 5. Trans-complementation of HCV replicon mutants.

Viral protein	Mutation	Complementation
NS3 protease	A1027D	- (t)
	P1028H	- (s)
NS3 helicase, motif II	H1319A	- (t)
NS4B	V1897D	- (s)
	V1897K	-(s)
	V1897P	- (s, t)
	Δ1782-1821	- (s)
	Δ1905-1933	- (s, t)
NS5A	Δ1977-1983	- (s, t)
	Ins. 1980 Ala	- (s, t)
	Ins 1983 Ala	- (s, t)
	Δ2194-2197	+ (s)
	Δ2200-2202	+ (s, t)
	Δ2194-2207	+ (s, t)
	S2197A+S2201A	+ (s ,t)
	S2197A+S2204A	+ (s, t)
	S2201A+S2204A	+ (s, t)
	S2194A+S2197A+S2201A+S2204A	+ (s, t)
	S2194A+S2200A+S2201A+S2202A	+ (s, t)
NS5B	ΔGDD/GND	- (s, t)
	NS5BΔC12	- (s, t)
	NS5BΔC21	- (s, t)
	NS5B-LVL	- (s, t)
	NS5B-R568/570A	- (s, t)

-, no trans-complementation

+, trans-complementation

s, trans-complementation with hyg-replicon helper RNA

t, trans-complementation in transient replication assay

In contrast, all mutations in the central region of NS5A were rescued (Table 5 and Fig. 46). Among these were multiple mutations affecting potential phosphorylation sites as well as small deletions. In striking contrast, all replicons in which we had disturbed the structure of the N-terminal amphipathic a-helix by introducing a twist (ins. 1980Ala, ins. 1983Ala) or shortening its length (Δ1977-1983) could not be trans-complemented (Fig. 46).
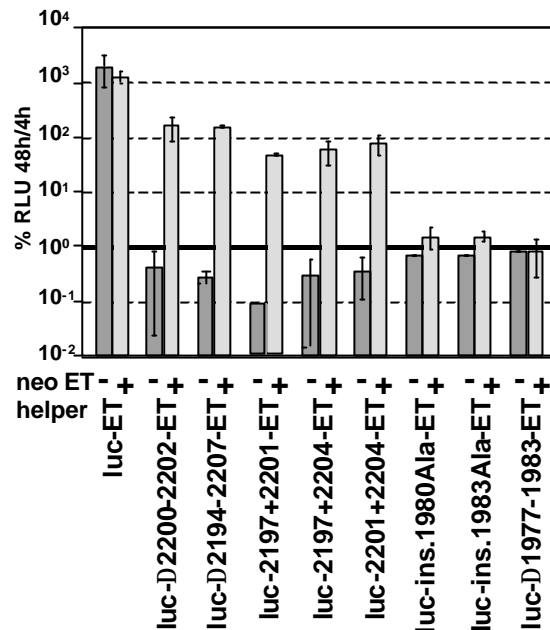
FIG. 46: Transient replication of NS5A mutants after rescue with helper RNA neo-ET (Fig. 43A) in a transient assay. Luciferase activities were determined as described in the legend to Fig.
5. Striped bars represent the replication levels of the mutant in the absence of helper RNA. Bars are means and error ranges of quadruplicate results.

Interestingly, these mutations not only disturb the proper folding of the helix, but also cause a dramatic reduction of NS5A hyperphoshorylation arguing that these mutations had a more general effect on the overall folding of the protein (112). In summary, the data show that neither mutations in NS3, NS4B or NS5B nor mutations affecting the N-terminal membrane anchor of NS5A can be complemented in trans.

3.4.5 Trans-complementation analysis of NS5A mutants by non-replicating helper RNA

Encouraged by the successful complementation of replicon RNAs carrying mutations in the central domain of NS5A, we were interested to find out whether production of NS5A in the context of an active RC is required for trans-complementation. In the first set of experiments, we performed a transient complementation assay with a helper RNA that cannot replicate due to a deletion spanning the GDD motif of the NS5B RdRp (neo-ΔGDD). As exemplified by the

co-transfection of the NS5A mutants luc-2197+2201-ET and luc-2201+2204-ET, trans-complementation was possible to a level ~10 fold above background (Fig. 47A).
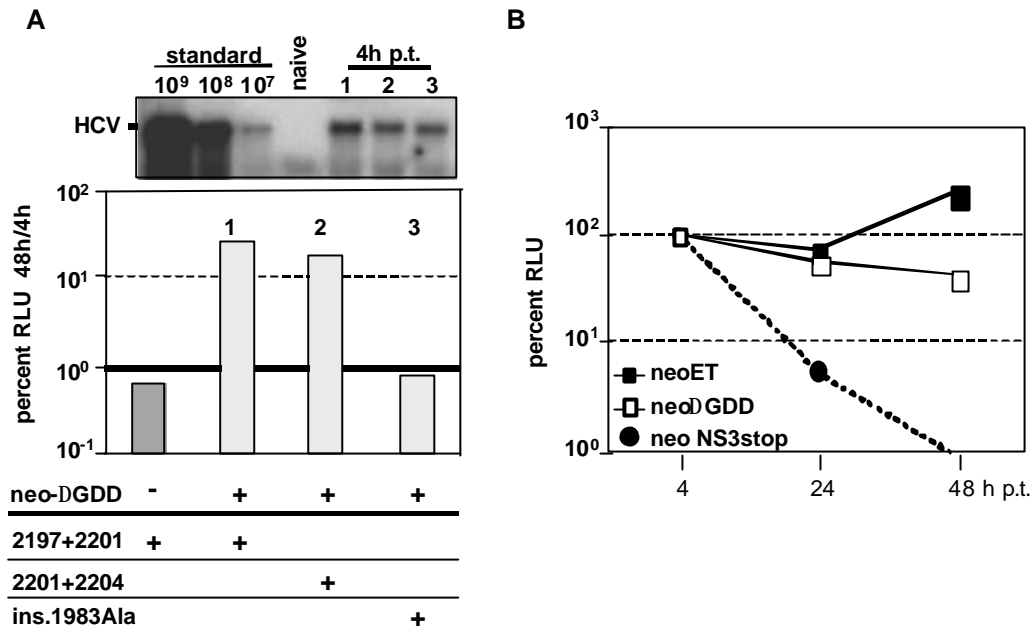
FIG. 47: Rescue of NS5A mutants by a non-replicating helper RNA. (A) Huh-7 cells were co-transfected with given mutants and neo-ΔGDD as helper RNA. Transfection efficiency of the latter was confirmed by Northern hybridization using total RNA prepared from cells harvested 4h after transfection (upper panel). For further details see legend to Fig. 5C. (B) Huh-7 cells were co-transfected with the mutant luc-2197+2201-ET and given helper RNAs. Cells were harvested 4, 24 and 48h after transfection and luciferase activities derived from the mutant were determined. Data were normalized for transfection efficiency using the 4h values. A representative result of 3 independent experiments is shown.

In contrast, the mutant with the altered N-terminal α-helix (ins. 1983Ala) was not rescued in spite of efficient transfection of the helper RNA as shown by Northern-hybridization (Fig. 47A). This result demonstrates that trans-complementation does not require replication of the helper RNA. However, rescue of RNA replication of a mutant replicon was at least 10-fold higher when using a replication-competent helper RNA (neo-ET). In addition, by performing time course experiments we found that after co-transfection of non-replicating helper RNA, luciferase activity reflecting replication efficiency of the trans-complemented luc-mutant steadily decreased, whereas an increase of luciferase activity over time was observed after co-transfection of replication competent helper neo-ET (Fig. 47B). This increase correlates well with the RNA replication of the helper RNA. The 10-fold difference in rescue efficiency 48h after transfection is therefore most likely due to the more continuous and higher expression level of NS5A over time, when a replication competent helper is provided.

3.4.6 Mapping of the minimal sequence necessary and sufficient for trans-complementation

To further confirm that trans-complementation is possible without replication of the helper RNA and to map the minimal sequence sufficient for trans-complementation, we co-transfected the mutant luc-2201+2204-ET with RNAs encoding various fragments of the NS3 to NS5B polyprotein (Fig. 48A).
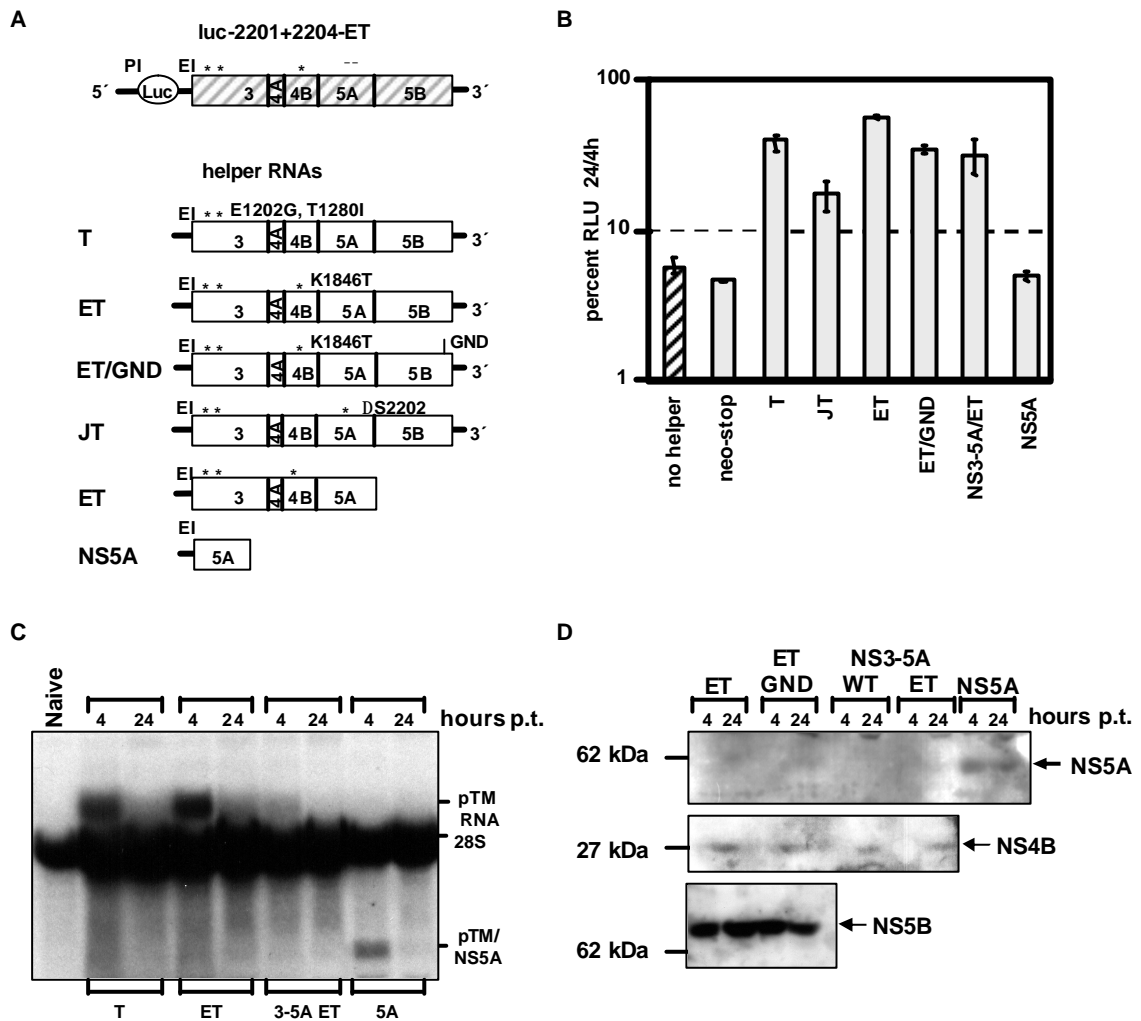
FIG. 48: Rescue of an NS5A mutant by co-expression of NS3 to NS5A in trans. (A) The structure of the mutant is shown in the top, the different helper RNAs are given below. All RNAs carry the EMCV IRES at the 5´end to allow efficient protein expression. Helper RNAs expressing NS3 to NS5B contain the HCV 3´NTR, which is not present with RNAs encoding for NS3 to NS5A or NS5A alone. GND indicates the position of the inactivating amino acid substitution in the NS5B RdRp. Adaptive mutations in NS3, NS4B and NS5A are marked by asterisks. (B) Huh-7 cells were co-transfected with mutant luc-2201+2204-ET and a given helper RNA carrying adaptive mutations as shown in panel A. Cells were harvested 4, 24 and 48h after transfection. Shown are the 24h values normalized to the 4h value. Data are means and standard deviations of at least 3 independent experiments. The striped column represents the replication efficiency of the mutant in the absence of the helper RNA. (C) Detection of helper RNAs by Northern-hybridization. Positions of helper RNAs are indicated on the right. As a negative control total RNA from naive Huh-7 cells was used (left lane). Note that the NS3 to NS5A helper RNA had a size similar to the 28S RNA and therefore could not be detected in this analysis. (D) Western Blot analysis of NS4B, NS5A and NS5B after transfection of given helper RNAs into naive Huh-7 cells. Cells were harvested 4 and 24 h after transfection and HCV proteins were detected in the total lysates using specific anti-NS4B, anti-NS5A and anti-NS5B-antibodies. The position of the protein is indicated by an arrow.

Cells were harvested 4 and 24h after transfection and replication efficiency was determined by measuring luciferase activity. The results in Fig. 48B show that luciferase activity was more than 10-fold increased after co-expression of RNAs encoding an NS3 to NS5B polyprotein compared to the replicon mutant alone. Comparable to the results described above, reduced restoration efficiency was observed in the case of a helper RNA that carried a highly adaptive mutation in NS5A (Fig. 48A, JT). This result is most likely due to the incompatibility of the highly adaptive mutations in NS5A of the helper RNA (Δ2202) and NS4B of the mutant replicon (K1846T). Furthermore, co-transfection of an RNA that encodes only NS3 to NS5A was sufficient to restore replication of the mutant replicon to the same level as with the other helper RNAs. In contrast, when only NS5A was expressed, no rescue of RNA replication was observed. This was not due to low transfection efficiency because the RNA was well-detected 4 h after transfection at a level comparable to those of the other helper RNAs (Fig. 48C) and the same was true for the NS5A protein as detected by Western Blot (Fig. 48D). Taken together, these results show that the minimal region required for trans-complementation is an NS3 to NS5A polyprotein fragment.

3.4.7 Trans-complementation analysis of an NS5A mutant by helper RNAs carrying mutations in NS3, NS4A and NS4B

Since we mapped the minimal sequence sufficient for trans-complementation to the NS3 to 5A region, whereas NS5A alone was not able to restore RNA replication, we were interested to find out the reason for this result. Two possibilities could be envisaged. First, it is known that complete phosphorylation of NS5A depends on its expression in the context of an NS3 to 5A polyprotein. Therefore incompletely phosphorylated NS5A that is generated when NS5A is expressed on its own may not be able to complement in trans. Second, NS5A may only support trans-complementation as part of an RC. To differentiate between these two possibilities, we analyzed a panel of helper RNAs carrying mutations in NS3, NS4A and NS4B for their ability to complement NS5A mutants in trans. The mutations in NS3 and NS4B present in the helper RNAs are described in Table 5. The NS4A mutants were designed not to affect its function as a cofactor of the NS3 protease. One mutation, a leucine substitution for a glycine residue, was located in the central proteinase activation domain (G1678L) and was previously shown not to affect polyprotein processing (76). Furthermore, multiple alanine substitutions were introduced into the C-terminus of the molecule (1690AAA, 1697AAA, 1703AAA and 1707AAAA) that block RNA replication. Thus, the chosen helper RNAs are strongly impaired or incapable of

RNA replication, the inserted mutations have no influence on polyprotein processing and all RNAs express a wildtype NS5A with reduced hyperphosphorylation of NS5A.

To analyze these mutated helper RNAs for their ability to restore RNA replication of a replicon that carried two inactivating mutations in NS5A (S2197A+S2201A), helper and mutant RNAs were co-transfected into Huh-7 cells and replication of the mutant was monitored by luciferase assays at different time points post transfection. As a positive control, we used an RNA expressing wildtype NS3 to 5B.
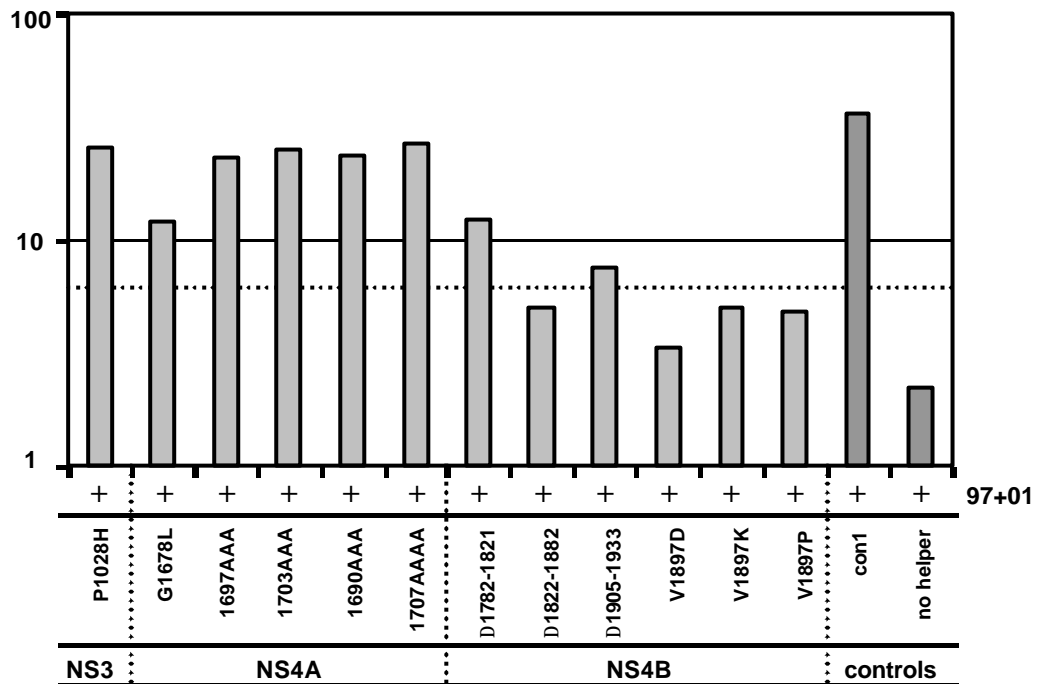
FIG. 49: Trans-complementation of luc-2197+2201-ET mutant by non-replicating helper RNAs carrying the mutations specified below the bars. Inactive replicon luc-2197+2201-ET was co-transfected with given helper RNAs carrying mutations in NS3, NS4A or NS4B. Luciferase activity was determined 4 and 24h after transfection and values were normalized for transfection efficiency. A helper RNA expressing wildtype NS3 to NS5B proteins (Con-1) was used as a positive control. Transfection of the replication deficient replicon without helper RNA served as a negative control (no helper). Values below the dotted line reflect background activity.

The results in Fig. 49 show that trans-complementation of the inactive replicon was possible with helper RNA NS3-5B/P1028H. Furthermore, helper RNAs carrying mutations in the C-terminus of NS4A also restored RNA replication to a level nearly comparable to the positive control (con-1). Interestingly, helper RNAs carrying mutation G1678L (NS4A) or Δ1782-1821 (NS4B) also seemed to restore RNA replication, although the efficiency of trans-complementation was ~3-fold lower compared to the control. All other helper RNAs carrying deletions or mutations in NS4B were dramatically reduced in their capability to restore RNA replication of the NS5A mutant. This result was surprising, because all helper RNAs provided wildtype NS5A in trans. However, as described in chapter 3.2.4, mutations in NS3, 4A and 4B result in a significant

reduction or block of NS5A hyperphosphorylation. Thus, since some of the helper RNAs are able to restore RNA replication, while some of them are not, but all of them have a defect in the phosphorylation of NS5A, there is no correlation between NS5A phosphorylation status and its ability to complement in trans.

To address the question, whether NS5A has to arise from a membrane associated RC, we analyzed the different helper RNAs for their ability to induce membranous web structures, to which the RCs are tightly associated. Therefore, Huh-7 cells stably expressing T7 RNA-polymerase were seeded on cover-slips and transfected with T7-based expression plasmids encoding NS3 to 5B polyproteins. Eight hours after transfection cells were fixed, permeabilized and stained for DNA with DAPI (blue) and NS5A (green) by immunofluorescence. Representative results are shown in Fig. 50, a summary of all data is given in Table 6. In case of the wildtype protein that was used as a positive control, we observed the typical dot like staining for NS5A. These dot like structures correspond to the vesicular membrane rearrangements that are induced by NS4B and that represent the membranous web. Save for one, similar distribution of NS5A was found with the NS4A mutants, although the morphology differed slightly from the wildtype web. In fact, in case of the C-terminal alanine insertions the dot like structures appeared larger and the number of smaller dots was reduced. The exception was the G1678L mutant where nearly no dot like structures were visible within the cells. The NS5A protein was diffusely spread throughout the cytoplasm of the cell. The same picture was observed for the deletions in NS4B and the point mutations at position V1897 that abrogated RNA replication (V1897D, K, and P). Interestingly, conservative alterations at the same position to alanine or leucine resulted in an enhanced RNA replication and the normal wildtype distribution pattern for NS5A was found. In summary, we found a correlation between the ability of NS5A to complement in trans and its subcellular distribution into the membranous webs, suggesting that NS5A must be part of the viral RC to achieve full functionality. However, no correlation was found between the phosphorylation status of NS5A and its ability to act in trans, since mutations that strongly reduced NS5A hyperphosphorylation in one or the other case supported trans-complementation (compare e.g. V1897A and V1897D in Table 6).

Table 6: Characterization of helper RNAs used for trans-complementation

Mutation	Replication	p58	Membranous web/ 5A colocalization	Trans-complementation
NS3				
P1028H	+	red.	+	+
NS4A				
G1678L	-	-	red.	red.
1690AAA	-	-	+	+
1697AAA	-	-	+	+
1703AAA	-	-	+	+
1707AAAA	-	-	+	+
NS4B				
Δ1782-1821	-	-	red.	red.
Δ1822-1882	-	-	red.	-
Δ1905-1933	-	-	red.	-
K1846T	+	red.	+	+
V1897A	+	-	+	+
V1897L	+	-	+	+
V1897M	+	-	+	+
V1897D	-	-	red.	-
V1897K	-	-	red.	-
V1897P	-	-	red.	-

A
Membrane Topology of NS4A

5A

| G1678L | 1690AAA | 1697AAA | 1703AAA | 1707AAAA | wildtype |

B
Membrane Topology of NS4B

5A

| D1782-1821 | D1822-1882 | D1905-1933 | K1846T | Mock |

5A

| V1897A | V1897L | V1897D | V1897K | K1897P | Wildtype |
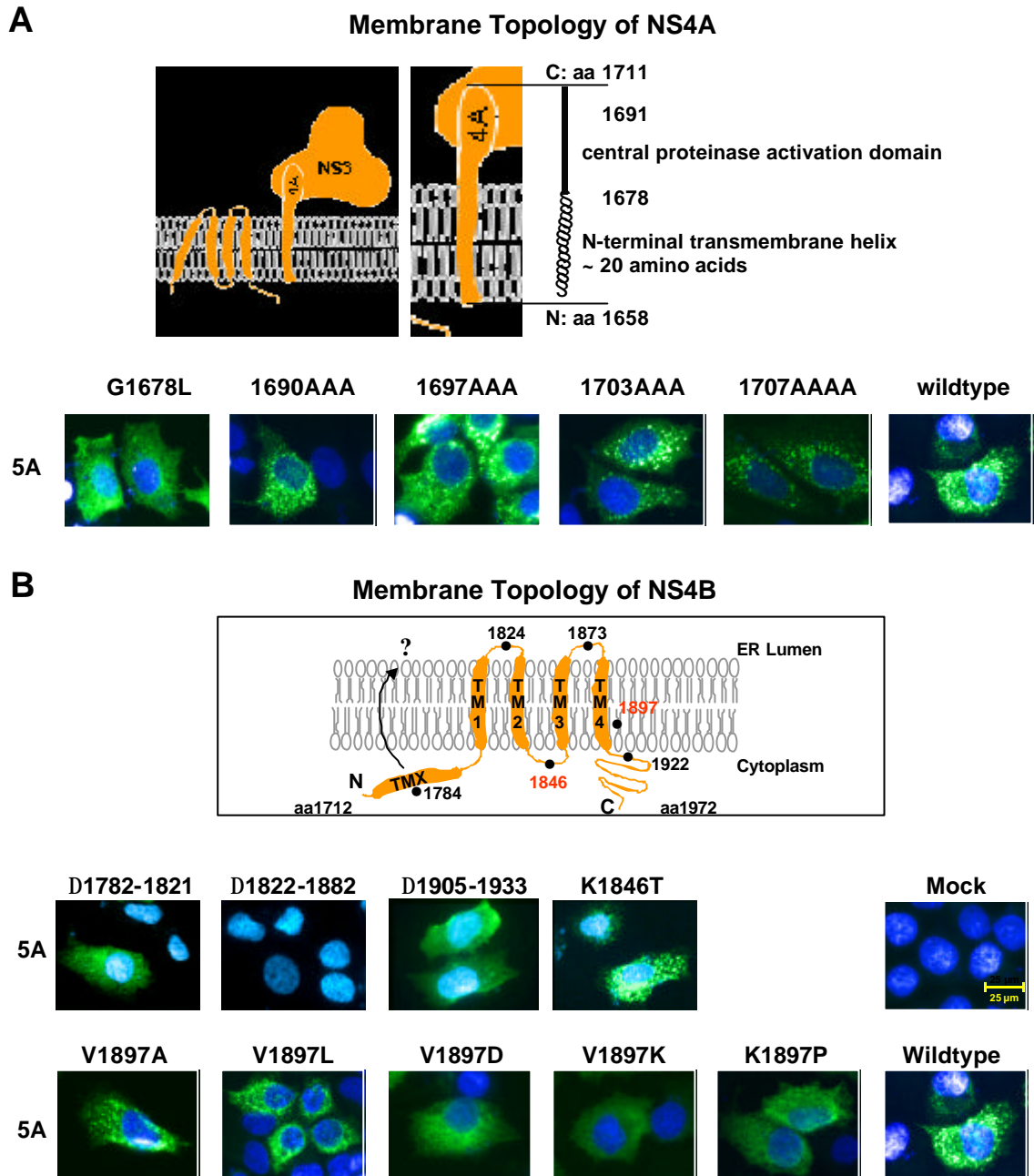
FIG. 50: Immunofluorescence analysis of NS5A proteins from different helper RNAs. (A) Putative membrane topology of NS4A. NS4A is targeted to intracellular membranes *via* an N-terminal transmembrane helix. Mutations or alanine insertions of NS4A are indicated above the immunofluorescence pictures. For immunofluorescence analysis Huh-7/T7 cells were seeded on glass cover slips, transfected with the NS3 to NS5B expression vectors carrying the indicated mutations and fixed 8h after transfection. For detection of NS5A a monoclonal anti-NS5A antibody was used that in turn was stained with an Alexa 488 secondary antibody. Nuclei were counterstained with dapi. (B) Schematic presentation of the putative membrane topology of NS4B. Amino acid numbers refer to the position in the complete polyprotein of the Con-1 genome. Adaptive mutations are given in red color. For immunofluorescence analysis cells were treated as described in (A). Pictures show the intracellular localization of NS5A proteins. Mock transfected cells served as a negative control, whereas cells expressing wildtype NS3 to NS5B proteins were used as positive control.

3.5 Identification and characterization of a novel cellular interaction partner of the hepatitis C virus NS5A protein

NS5A became the focus of studies concerning cellular binding partners when it was reported to be involved in HCV resistance to IFN-α. As deduced from sequence analysis of HCV genomes isolated from Ifn-treated patients, a stretch of 40 amino acids in the center of NS5A was implicated in determining Ifn-α sensitivity. Hence, this region was designated IFN-sensitivity determining region (30). Two years later, Gale and coworkers proposed that NS5A counteracts Ifn-α induced antiviral state by complex formation with IFN-induced double-stranded RNA-activated protein kinase (PKR) and inhibition of PKR activity (40, 41). In addition, mechanisms for PKR-independent repression of IFN action by NS5A, as well as interference with multiple other cellular pathways have been reported (117). While for most of these interactions no functional relevance has been described, one interesting interaction partner in terms of RNA replication is the human SNARE-like protein, human vesicle-associated membrane-protein associated protein A (h-VAP-A), which is involved in intracellular vesicle transport (151, 157). RNAi-mediated knockdown of hVAP-A expression as well as overexpression of a dominant-negative mutant reduced HCV RNA replication suggesting that the hVAP-A/NS5A interaction plays a very important role for RNA replication. NS5A is a highly phosphorylated protein that is phosphorylated by not yet defined cellular kinase(s) (32, 43). Several potential interaction sites with such kinase(s) reside in NS5A. Most obvious are three proline rich motifs (Pro Xaa Xaa Pro), which are present in many signaling proteins and that bind to Src homology-3 (SH3) domains. Two of these motifs are near the C-terminus of NS5A and predicted to be surface-exposed. Interaction of NS5A *via* these C-terminal class II polyproline motifs with Grb2 and Src-family kinases Fyn, Lck and Hck by their SH3 domain has been reported, however, the physiological significance of nearly all cellular NS5A-binding proteins for the HCV infectious cycle remains unclear (92, 93).

To address the question of how NS5A might interact with cellular kinase signaling networks, in close cooperation with a group at the Axxima Pharmaceuticals AG in Munich, we set out to isolate cellular phosphoproteins that could specifically associate with NS5A.

3.5.1 Identification of NS5A-interacting phosphoproteins

For the identification of phosphorylated interaction partners of NS5A, a recombinant glutathione *S*-transferase (GST)-NS5A fusion protein was expressed in *E. coli* and subjected to *in vitro*

association by using total lysates from Huh-7 cells that had been metabolically labeled with
[^{33}P]orthophosphate. For this purpose, GST-NS5A fusion protein immobilized on glutathione-
sepharose was incubated with the radiolabeled cellular proteins and bound proteins were
analyzed by 2-D gel electrophoresis. For the first dimension, isoelectric focusing was performed
using linear pH 4-7 gradient strips and after second dimension by SDS 10% PAGE, proteins
were detected by Coomassie staining and autoradiography.
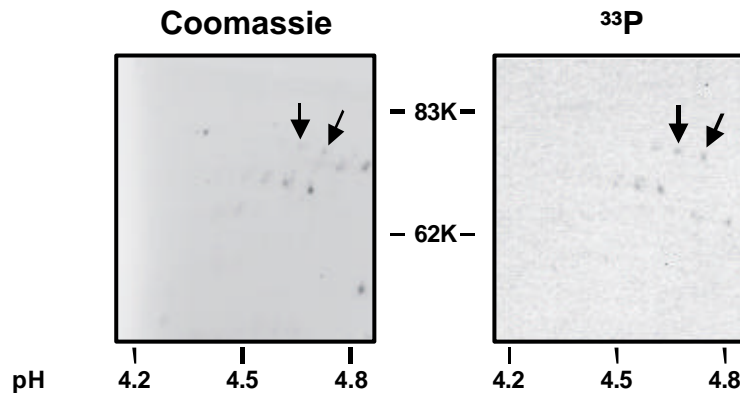
Fig. 51: Purification of NS5A-interacting phosphoproteins. After metabolic labeling of Huh-7 cells with [^{33}P]
orthophosphate, cell lysates were used for in-vitro association with GST-NS5A. Bound proteins were resolved by
2D gel electrophoresis and then visualized by Coomassie-staining (left panel) and autoradiography (right panel).
Arrows indicate the positions of two NS5A-interacting phosphoproteins identified as amphiphysin II by mass
spectrometry. The phosphoprotein spots assembled in a row might represent differentially phosphorylated
amphiphysin II species.

As shown in Fig. 51, several cellular ^{33}P-labeled protein species were detected in the acidic
region of the 2-D gel and these proteins specifically associated with GST-NS5A and precisely
co-migrated with Coomassie-stainable protein spots. Corresponding spots were subjected to mass
spectrometry analysis by nano-electrospray ionization on a Q-TOF mass spectrometer (10). The
spectra generated allowed sequencing of four different peptides, which were all identical to
sequences found in human amphiphysin II. The human amphiphysin II (also referred to as Bin1)
gene encodes various different isoforms, which result from alternative splicing (150, 156).
Unfortunately, none of the peptide sequences mapped to alternatively spliced regions of
amphiphysin II, therefore it was not possible to discriminate between different isoforms based on
the mass spectrometry analysis. Interestingly, amphiphysin II contains a SH3 domain at its
C-terminus that may interact with the class II polyproline motif of NS5A. Amphiphysin II is
ubiquitously expressed and supposed to be implicated in clathrin-mediated endocytosis by
interaction with dynamin, clathrin and the clathrin adaptor protein (AP2) (159, 160). These
interactions are phosphorylation dependent. In addition, amphiphysin II was found to interact

with the *myc* oncogene and implicated in *myc*-induced apoptosis (25, 125). In summary, the phosphoprotein amphiphysin II was identified as a novel interaction partner of NS5A in vitro.

3.5.2 Identification of the amphiphysin II binding site in NS5A

To confirm amphiphysin II as a novel NS5A-interacting protein and to characterize the domains of NS5A required for amphiphysin II binding, immunoblot analysis using different GST-NS5A fusion proteins were performed. Amphiphysin II contains at the N-terminus a BAR (Bin/amphiphysin/RVS) domain, which is supposed to be a crescent-shaped dimer that binds preferentially to highly curved negatively charged membranes. Localized in the center of the molecule is a CLAP domain, which is supposed to bind clathrin and adaptor proteins and at the C-terminus it contains an SH3-domain, binding proline-rich sequences (113). Since NS5A carries two proline-rich sequences at its C-terminus, a mutant was generated in which three out of five prolines from the classical class II proline-rich region (aa P350A, P353A and P354A) at the C-terminus were changed to alanines (NS5A polyP$^-$). In addition, a mutant lacking the last 169 amino acids of NS5A including both polyproline motifs was made (NS5AΔC). After in vitro association of Huh-7 total cell lysates with either GST or the different GST–NS5A fusion proteins, bound proteins were resolved by 10 % SDS-PAGE followed by immunoblotting with anti-amphiphysin II antibody. As shown in Fig. 52, two splice variants of amphiphysin II of approximately 66 and 73 kDa interacted with the GST–NS5A fusion protein, but not with GST alone.
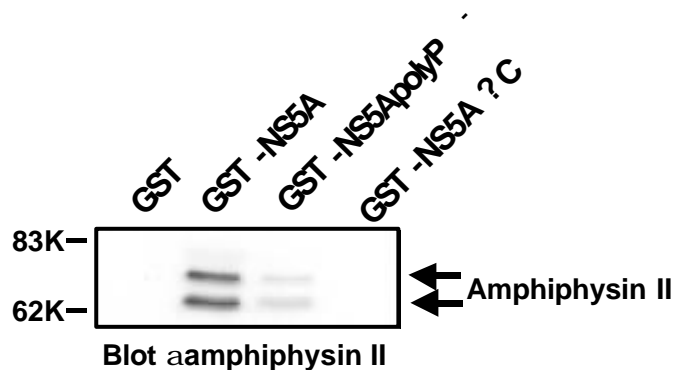
FIG. 52: Specific in-vitro association of endogenous amphiphysin II with GST-NS5A fusion proteins. Total cell lysates prepared from Huh-7 cells were subjected to in-vitro association with either GST or different GST-NS5A fusion proteins containing full length NS5A; NS5A, that carried three alanine substitutions for proline (P350A, P353A, and P354A) designated NS5Apoly$^-$, or truncated NS5A terminating at amino acid 277 (NS5A?C). Bound proteins were resolved by SDS 10 % PAGE followed by immunoblotting with anti-amphiphysin II / Bin1 antibody. Molecular mass is indicated on the left and amphiphysin II isoforms are marked by arrows.

Binding of endogenous amphiphysin II to the GST–NS5ApolyP$^-$ mutant fusion protein was strongly reduced but not completely abrogated. No detectable amphiphysin II binding was

observed when the C-terminal part of NS5A was deleted in the GST fusion protein, arguing that the amphiphysin II binding site is located at the C-terminus of NS5A.

Furthermore, two amphiphysin II splice variants were PCR-amplified from human cDNA libraries and cloned into a CMV-driven expression vector. Sequencing revealed that one construct represented the amphiphysin II2 splice variant of 482 amino acids (GenBank accession no. AF001383.1), whereas the other clone was a shorter version of amphiphysin II2 harboring a deletion of amino acid 304–346. In addition, a vector was generated expressing only the FLAG-tagged SH3 domain of amphiphysin (residues 393–482 of amphiphysin II2).

All three plasmids were then used for transient transfection of Huh-7 cells and subsequent *in vitro* association with GST, GST-NS5A or GST-NS5ApolyP$^-$ fusion proteins. Protein complexes were analyzed by immunoblotting using monoclonal anti-FLAG antibody. Both amphiphysin II2 and amphiphysin II2 short interacted with GST-NS5A fusion protein but not with GST itself, confirming the specific NS5A-amphiphysin complex formation observed for endogenous amphiphysin II (data not shown). Interaction of both amphiphysin II variants with the GST-NS5ApolyP$^-$ fusion protein was somewhat weaker, although the reduction in affinity was not as pronounced as observed with endogenous amphiphysin II, most likely due to the high amphiphysin II expression levels obtained in the transient expression experiments. Specific association with GST–NS5A was also observed when only the SH3 domain was expressed, but this interaction depended on the presence of the class II proline-rich region of NS5A (data not shown). Thus, the class II proline-rich region comprising amino acids 350–356 of NS5A significantly contributes to binding through interaction with the amphiphysin II SH3 domain, however other determinants residing in the C-terminal part of NS5A play a role in NS5A-amphiphysin II complex formation too.

3.5.3 Interaction of NS5A and amphiphysin II in Huh-7 replicon cells

To address the important issue of the physiological significance of the NS5A-amphiphysin II interaction, the Huh-7 replicon cell clone 5-15, that contains a stably replicating subgenomic neo-replicon was analyzed by immunoprecipitation for the NS5A/amphiphysin II interaction. As a negative control Huh-7 cells expressing only the neo-resistance gene were used. Cell lysates were subjected to immunoprecipitation with a monoclonal antibody directed against NS5A, followed by Western Blot analysis for amphiphysin and NS5A.
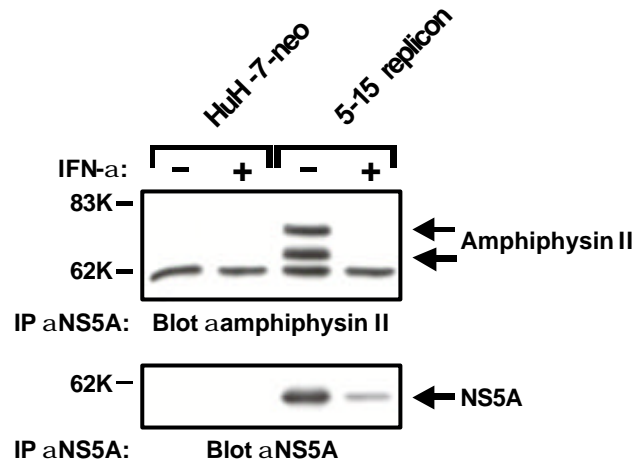
Fig. 53: Co-precipitation of NS5A with endogenous amphiphysin II from HCV replicon cells. Huh-7-neo control cells or 5-15 replicon cells were pretreated with 1000 units/ml IFN-a prior to cell lysis where indicated. Lysates were subjected to immunoprecipitation with anti-NS5A antibody. After gel electrophoresis and transfer onto nitrocellulose membrane, immunoblotting was performed with either anti-amphiphysin II antibody (upper panel) or anti-NS5A antibody (lower panel). Amphiphysin II isoforms and NS5A are marked by arrows on the right.

As shown in Fig. 53, two variants of amphiphysin II of identical molecular mass, as found in our *in vitro* association experiments, were specifically detected in anti-NS5A immunoprecipitates from 5-15 replicon cells. Immunoprecipitation of NS5A protein from the 5–15 replicon cells, but not from control cells (Huh-7-neo), was confirmed by parallel immunoblotting with anti-NS5A antibody. Moreover, pretreatment of cells with 1000 units interferon alpha (Ifn-α) per ml, which leads to a rapid degradation of HCV RNA not only strongly reduced NS5A expression but also abrogated amphiphysin II co-precipitation. Thus, endogenous amphiphysin II specifically binds NS5A under physiologically relevant conditions and can be isolated from 5-15 replicon cells in a stable complex with NS5A. Similar results were obtained with other replicon cell clones like 9-13 and 11-7 (data not shown).

To exclude the possibility that the interaction of amphiphysin II and NS5A as described above in stable replicon cells occurred during cell lysis, but is not present in living cells, we performed immunofluorescence analysis. Therefore, Huh-7 cells from the replicon cell clone 9-13 were fixed and co-stained with a monoclonal anti-amphiphysin II antibody and a rabbit polyclonal NS5A-specific antibody. Bound primary antibodies were detected with Cy3-conjugated anti-rabbit or FITC-conjugated anti-mouse antibodies, respectively. The cell nucleus was counterstained using bis-benzimide.

9-13

NS5A   Amph II   merge

Naive Huh-7
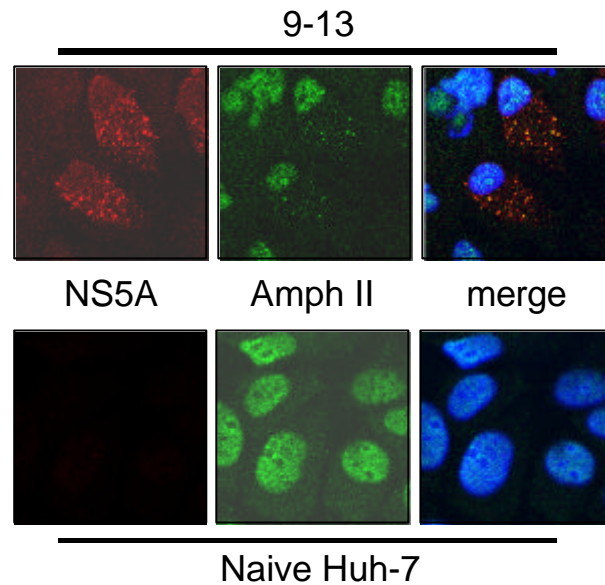
FIG. 54: Colocalisation of endogenous NS5A and amphiphysin II in Huh-7 replicon cells. NS5A was detected with a Cy3 labeled (left upper panel) and AmphiphysinII/Bin1 with a FITC labeled secondary antibody (middle upper panel) in cell clone 9-13. Both proteins colocalized in distinct granules that probably correspond to the membranous web (right upper panel). Naive Huh-7 cells stained for amphiphysin II / Bin1 served as a reference and the same cells stained for NS5A were used as a negative control (lower panels).

By using confocal microscopy we found that in naive Huh-7 cells, the majority of amphiphysin II resided in the nucleus. However, in cells carrying a replicon (9-13) amphiphysin II and NS5A co-localized to distinct granules (Fig. 54), which correspond to the membranous web. These results show a co-localization of NS5A and amphiphysin II not only in vitro, but also in Huh-7 replicon cells.

3.5.4 Influence of NS5A-amphiphysin II interaction on RNA replication

We next investigated whether the NS5A-amphiphysin II interaction is essential for HCV RNA replication in cell culture. For this purpose, we generated recombinant adenoviruses for ectopic expression of the N-terminal FLAG-tagged amphiphysin II (both splice variants) and the SH3 domain and infected replicon cells at an m.o.i. of 3000. The replicon cell clone contained a bicistronic replicon stably expressing luciferase allowing easy determination of RNA replication by measurement of luciferase activity. Cells were harvested 4, 24, 48 and 72 h after infection and analyzed by luciferase assay and Western Blot.
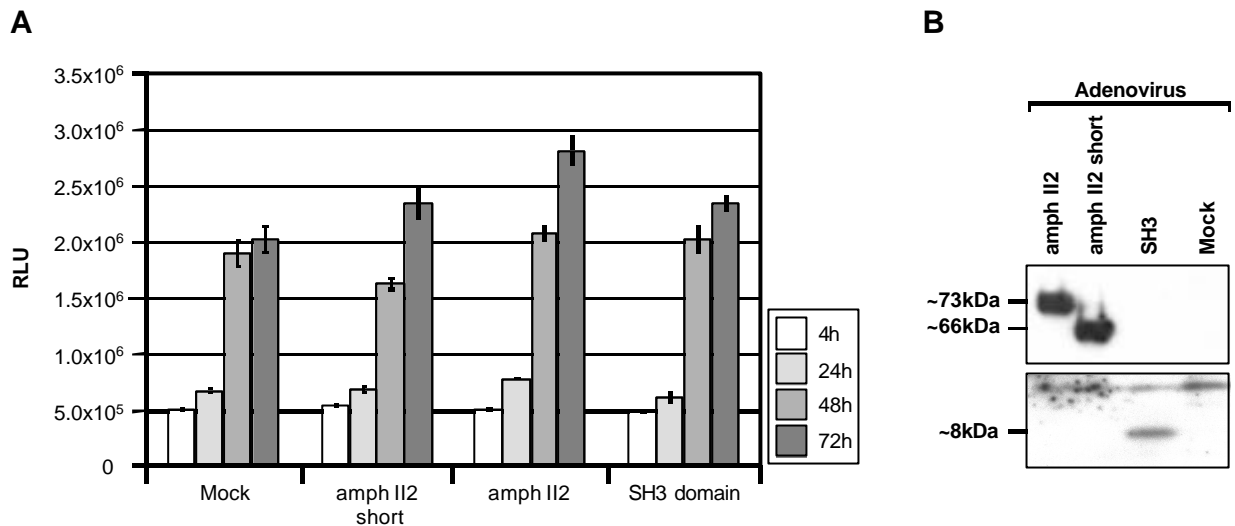
FIG. 55: Influence of overexpression of amphiphysin II or its SH3 domain on HCV RNA replication. (A) Huh-7 cells containing persistently replicating subgenomic luc-replicons were infected with recombinant adenoviruses expressing the two splice variants of amphiphysin II (amph II2 short and amph II2) or its SH3 homology domain. As a negative control, cells were infected with the same m.o.i. of 3000 with an adenovirus carrying no heterologues sequences. Luciferase activities were determined 4, 24, 48 and 72 h after infection and means of absolute relative light units of two independent experiments are shown. (B) Western Blot analysis of recombinant proteins. Cells were harvested 48h after adenovirus infection and proteins of total lysates were separated by SDS 10% (upper panel) and 16% (lower panel) PAGE. Recombinant proteins were detected by using an anti-Flag antibody.

As shown in Fig. 55B, expression of recombinant proteins was detectable 48h after infection by Western Blot analysis. However, expression of the different amphiphysin II variants, as well as the SH3 domain had no significant influence on RNA replication (Fig. 55A). Likewise, infection of 5-15 replicon cells with an adenovirus expressing the amphiphysin II SH3 domain specifically disrupted endogenous NS5A-amphiphysin II complexes, but had no significant effect on RNA replication (data not shown). Thus, expression of the amphiphysin II SH3 domain appears to negatively interfere with the NS5A–amphiphysin II interaction, but this interaction seems to be dispensable for HCV RNA replication in replicon cells.

These results were further confirmed by an additional approach where a subgenomic replicon was created expressing a luciferase-ubiquitin-amphiphysin or luciferase-ubiquitin-SH3-domain fusion protein from the first cistron (Fig. 56).
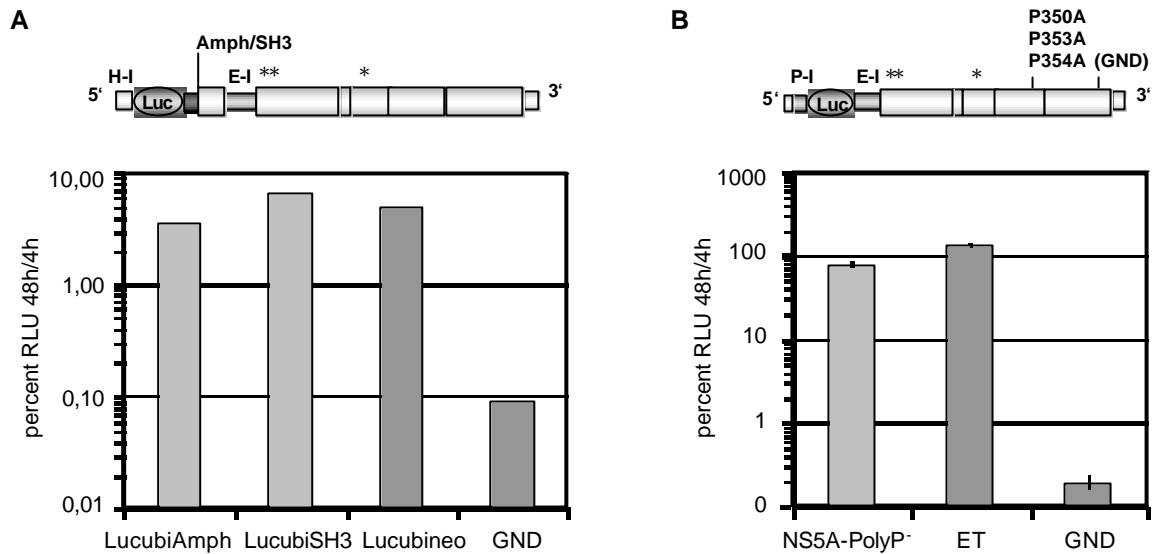
FIG. 56: Disturbance of the NS5A-amphiphysin II interaction has no significant effect on RNA replication. (A) Bicistronic reporter replicons were generated expressing amphiphysin II (both splice variants) or the SH3 domain from the first cistron as shown in the upper panel. To guarantee cleavage between luciferase and amphiphysin/SH3-domain, the ubiquitin sequence was inserted between the two genes. After cleavage, ubiquitin stays at the C-terminus of the luciferase. Huh-7 cells were transfected with the given replicons and luciferase activity determined 4 and 48h after transfection. Values were normalized for transfection efficiency by using the 4h values. A replicon expressing a luc-ubi-neo cassette from the first cistron was used as a positive control. GND replicon served as negative control. (B) Huh-7 cells were transfected with replicons carrying the indicated mutations. Replicon NS5A-PolyP$^-$ carries three alanine substitutions for proline residues that are located in the C-terminal proline rich motif of NS5A and affect the major interaction site for amphiphysin II binding. As positive and negative control replicons luc-ET and luc-GND were used, respectively. Adaptive mutations of the replicons are indicated by stars. Transient replication assay was performed as described above.

As described in chapter 3.3.4, this approach allows co-expression of the fusion protein with the HCV replicase at the same time and the same site within the cells. However, even under these conditions, we observed no influence of amphiphysin or the SH3 domain on RNA replication (Fig. 56A). Finally, we generated luciferase replicons carrying mutations in the NS5A proline-rich region that disturb interaction with amphiphysin II. As shown in Fig. 56B, HCV RNA replication was not significantly reduced by the P350A, P353A and P354A substitutions in NS5A. The modest reduction of RNA replication may result from changes in the NS5A secondary structure due to substitution of three adjacent alanine residues for proline rather than from the reduced interaction of NS5A with SH3-containing proteins. Taken together, these data suggest that the interaction between NS5A and amphiphysin II is dispensable for RNA replication.

4. Discussion

4.1 Identification of adaptive mutations in NS5A and mechanism of adaptation

The first aim of this PhD thesis was to improve the subgenomic replicon system essentially by developing a transient replication assay using reporter-gene replicons. Based on the observation by Blight et al. (2000) and Lohmann et al. (2001) who identified cell culture adaptive mutations that enhanced colony formation efficiency, we focussed our study on the identification of even more efficient adaptive mutations. To achieve this goal, we used a functional screening assay of 82 replicons that were isolated from a given replicon cell clone as described in chapter 3.1. We identified a replicon, designated neo-5.1, with an ECF of ~5x10^{5}cfu/μg RNA, which is ~20-fold higher than the best-adapted replicon, available at that time. This enhancement of colony formation was due to two amino acid substitutions in NS3 (E1202G and T1280I) and one in NS5A (S2197P). Using these highly adaptive mutations, we were able to develop a transient replication assay, based on the transfection of subgenomic replicons carrying the luciferase gene of the firefly (*Photinus vulgaris*) instead of the neomycin-phosphotransferase. Determination of the luciferase activity in cell lysates allowed monitoring of the transient RNA replication. For the mechanism of adaptation two possibilities could be envisaged. The first possibility was that the parental wildtype replicon exerted a cytopathic effect, leading to a low number of G418 resistant colonies after transfection. By the accumulation of adaptive mutations this cytopathic effect was reduced resulting in a higher number of G418 resistant colonies. The second possibility might be that adaptive mutations enhanced replication efficiency of the parental RNA and therefore led to a higher number of G418 resistant colonies after transfection. Cytopathogenicity has also been described for replicons of other HCV related viruses like Sindbis virus or BVDV (1, 6, 37, 100, 145). In both cases, cythopathogenicity correlated with an enhanced RNA replication i.e. exceeding of a defined replication level caused cell death. In the case of HCV replicons one would expect a reduced replication level for the adapted replicons, if cytopathogenicity was the reason for adaptation. To differentiate between these two possibilities, we performed a transient replication assay with a series of reporter-replicons varying in their ECF. We observed an increase in luciferase activity for the most adapted replicon luc-5.1 in a time-course from 24 to 72h after transfection. In addition, we found a clear correlation between the ECF and the replication levels determined by the luciferase activity of different adapted replicons in a way that the higher the ECF, the higher the luciferase activity was. Furthermore, by using the transient replication assay we were able to follow replication of the wildtype replicon for the first

time (Fig. 10). These results lead to the conclusion that wildtype RNA replicates within transfected cells at a very low level that is insufficient to confer G418 resistance to these cells. To allow generation of a G418 resistant colony, the viral RNA polymerase must generate mutations that in a few instances are adaptive. By increasing the level of RNA replication, cells harboring such a replicon develop G418 resistance. In fact, the identified adaptive mutations were conserved in all replicons present in this particular cell clone (5-15-9-2-3). Since most mutations do not increase but rather reduce replication or are inactivating (69 out of 82 analyzed clones abrogated RNA replication), development of an adaptive replicon is a rare event, explaining why the numbers of G418-resistant colonies that are obtained with a non-adapted RNA are very low. These results are also in line with our observation that the RNA copy number within selected cell clones differed only by a factor of two, arguing that the level of cell culture adaptation is determined by the initial replication level, which also determines the level of G418 transduction efficiency. Hence, the high genetic variability of the virus that ensures its survival under natural conditions can be used in our experimental system to develop highly adapted replicons.

Currently, we can only speculate about the molecular mechanism of cell culture adaptation. In a previous study we had shown that a single amino acid substitution in NS5B increased the ECF ~500-fold compared to that of the parental RNA (90). During the course of this work, we identified two substitutions in NS3 and one in the center of NS5A. Three-dimensional structure modeling experiments suggest that the substitution at the carboxy terminus of NS3 (position 1202) is located on the surface of the molecule and probably has no effect on its various enzymatic activities (Neera Borkakoti, personal communication). The same is probably true for the mutation in domain 1 of the NS3 helicase at position 1280. We therefore favor the hypothesis that these amino acid changes affect an interaction site with a cellular or viral protein important for RNA replication. With NS5A, the substitution affected serine 2197, and this residue was shown to be important for hyperphosphorylation. The loss of this serine residue in the adapted replicon RNA may suggest that phosphorylation at this position is inhibitory for RNA replication. However, it is not known whether serine 2197 is a phosphoacceptor site or plays some other role in hyperphosphorylation. As deduced from phosphorylation analysis, the NS5A in cells harboring replicon 5.1 is still hyperphosphorylated. However, the appearance of the typical p56/58 double band (p56 corresponds to the basal phosphorylated form of NS5A and p58 presents its hyperphosphorylated variant) in conventional sodium dodecyl sulfate-polyacrylamide gel electrophoresis may not be sufficient to reveal subtle differences in the phosphorylation patterns between the parental NS5A and the adaptive mutation.

By using highly adaptive mutations, subgenomic replicons carrying the hygromycin-phosphotransferase- or the zeocin-resistance gene were generated. In contrast to neo-replicons transfection of high amounts of RNA was necessary to confer drug resistance to selected cells and even then colony formation efficiency was still reduced more than 10,000 fold. This result may be due to the properties of drug resistance proteins and their mode of action. First, half-life of the neomycin-phosphotransferase appears to be much longer as compared to the hygromycin-phosphotransferase or the zeocin-resistance protein. Second, hygromycin- and neomycin-phosphotransferase detoxify the corresponding drug by an enzymatic reaction, whereas resistance against zeocin is caused by a stoichiometric binding of the resistance protein to zeocin, thereby disturbing its DNA cleavage activity. Consequently, much lower amounts of neomycin- and hygromycin-phosphotransferase are required. Another possibility for the low ECF of the hyg- and zeo-replicons may be that further adaptive mutations were necessary to confer drug resistance to transfected cells. Since the overall replication level within selected cell clones was comparable to cell clones carrying neo-replicons, it may be that adaptation in this case increased activity or stability of the resistance proteins. If this hypothesis was true, one would expect that transfection of replicon RNA present in these cell clones should lead to higher numbers of resistant cell clones compared to transfection of in vitro transcripts. However, this was not the case (data not shown), arguing that additional adaptive mutations did not accumulate in hygromycin- or zeocin-replicons. It is therefore more likely, that we selected for particular cell clones that supported higher levels of hygromycin- or zeocin-replicon RNA replication. By using subgenomic hygro- or zeo-replicons it is now possible to analyze G418 resistant cell lines such as PH5CH, that have been shown to be susceptible for HCV infection, for their ability to support HCV replication (63).

Furthermore, using highly adaptive mutations, we generated cell culture-adapted authentic and selectable full-length HCV genomes (sfl genomes) as a first step towards the development of a fully permissive HCV cell culture system. Both constructs were tested for their replication ability in cell culture and infectivity in chimpanzee experiments (data not shown). In spite of efficient RNA amplification and expression of all viral proteins in transfected cells, no clear evidence for the production of virus particles in cell culture has been obtained. Furthermore, the adapted genome failed to establish a productive infection in chimpanzees (12). Attempts using a genomic RNA harboring only the highly adaptive mutation in NS5A (S2197P) resulted in a delayed infection, but all recovered virus genomes were revertants and carried the original wildtype sequence instead of the adaptive mutations. The reason for the attenuation of the adapted genome is not known yet, since neither the mechanism of adaptation nor the function of NS5A is known.

Nevertheless, many examples exist of other viruses in which cell culture adaptive mutations result in attenuation *in vivo*. Among members of the related flaviviruses, the yellow fever 17D vaccine and candidate vaccines for Japanese encephalitis virus or dengue virus represent such attenuated viruses. Among the known hepatitis viruses only HAV routinely grows in cell culture. However, adaptive mutations are required for efficient growth in vitro, but these mutations attenuate the virus for in vivo replication (29).

4.2 Role of phosphorylation for NS5A function

By performing an extensive mutation analysis of 3 serine clusters implicated in the phosphorylation and hyperphosphorylation of NS5A, we analyzed the role of these NS5A modifications for RNA replication (chapter 3.2). In most cases, alanine substitutions for serine residues in cluster 1 that enhanced RNA replication to highest levels, led to a reduction of NS5A hyperphosphorylation. Interestingly, most of the adaptive positions (e.g. S2197, S2201/2202 and S2204) have been identified in replicons isolated from Huh-7 clones, too. One exception is amino acid S2207 that has not been described as a site for adaptive mutations, although it enhances RNA replication as efficiently as substitutions affecting S2204. This fact can not be ascribed to the codon usage because a single nucleotide substitution is sufficient to alter the UCU serine codon into the GCU alanine codon. In general, transitions occur more frequently than transversions, but in the case of the adaptive nucleotide changes both reactions were found. Like the adaptive alanine exchanges in NS5A, adaptive mutations in NS4B that is also part of the RC resulted in a reduction of NS5A hyperphosphorylation. This result leads to the conclusion that adaptive mutations in NS4B and NS5A enhance RNA replication by the same mechanism. In agreement with this assumption, all combinations of highly adaptive mutations in NS5A, e.g. S2197A + S2201A, resulted in a complete loss of RNA replication. Likewise, combination of highly adaptive mutations in NS4B and NS5A dramatically reduced or abrogated RNA replication (89). Thus, the incompatibility of highly adaptive mutations may be ascribed to the fact that they act *via* the same or a very similar mechanism. In addition, it may be that a minimum of p58 is required for efficient RNA replication.

Recently, Neddermann et al. reported an enhanced replication of the non-adapted Con-1 wildtype RNA in the presence of compounds that are supposed to be kinase inhibitors (oral presentation, 11th international symposium on HCV and related viruses). This is the first demonstration that the presence of high amounts of p58 causes inhibition of HCV RNA in cell culture. Interestingly, they also observed that the same compounds that enhanced replication of

the wildtype replicon inhibited replication of already adapted replicons supporting our hypothesis that a minimum of p58 is required for efficient RNA replication.

Several models can be envisaged how adaptive mutations mutations exert their adaptive function. For NS4B no enzymatic function has been found yet, however it plays an important role in the induction of intracellular membrane alterations. These membrane alterations termed membranous web are a necessary prerequisite for the formation of a functional RC. One possibility could be that by introduction of an adaptive mutation in NS4B the kinetic of the web formation was enhanced leading to a more rapid formation of functional RCs. Alternatively, adaptive mutations in NS4B may lead to conformational changes within the RC enhancing replication, but limiting phosphorylation of NS5A. In this case, hyperphosphorylation would be an epiphenomenon that reflects a different conformation of NS5A suggesting that not the hyperphosphorylation itself affects RNA replication but rather the structure of NS5A and in this respect its accessibility for the cellular kinase. In agreement with earlier reports, such structural changes could be induced by substitutions within NS5A or by mutations affecting other HCV proteins within the RC, most importantly NS4B (76, 103). Another possibility may be that adaptive mutations may modify the interaction with a cellular protein. Although adaptive mutations are located in different regions of the polyprotein, they could be in close proximity in the RC and simultaneously or sequentially interact with this cellular factor. Finally, adaptive mutations in NS4B may cause an enhanced dephosphorylation of NS5A and thereby reduce hyperphosphorylation of NS5A. In contrast, adaptive mutations in NS5A may counteract hyperphosphorylation in a more direct way, by hiding potential phospho acceptor sites. Nevertheless, we also found a highly adaptive mutation that did not affect hyperphosphorylation of NS5A and this mutant (S2201E) replicated most efficiently in spite of unaltered levels of p58. This result suggests that the loss of a phospho group rather than the loss of the negative charge at position 2201 is sufficient for the enhancement of RNA replication. Furthermore, it is tempting to speculate that S2201 of the wildtype-replicon is not phosphorylated *in vivo*, since otherwise the wildtype-replicon should replicate to much higher levels. For the other serine residues involved in cell culture adaptation (S2197, S2202, S2204, and S2207) the loss of a negative charge seems to enhance RNA replication. One explanation of this contradictory result may be that the negative charge at position 2201 may mimic a phospho group in a way that the kinase still can bind to and phosphorylate NS5A, but since position 2201 itself cannot be phosphorylated other serines residues in close proximity serve as phospho acceptor sites. In conclusion, formation of hyperphosphorylated NS5A *per se* does not block high level RNA replication.

Interestingly, the combination of S2201E with S2194E had no negative influence on RNA replication, whereas it has been reported that combination of the adaptive mutation S2204I and S2194D dramatically reduced RNA replication. Considering the missing correlation between adaptation and hyperphosphorylation in case of the S2201E mutant, it is tempting to speculate, that the mechanism of adaptation for S2201E may be different to all other NS4B and NS5A adaptive mutations that also reduce hyperphosphorylation of NS5A. Unfortunately, all attempts to analyze phosphorylated NS5A species by 2 dimensional gel electrophoresis, which would allow a more refined analysis of the phosphorylation variants failed and therefore, currently we can not distinguish between different phosphorylated NS5A proteins.

For the basal phosphorylation of NS5A two distinct regions (amino acid 2200-2250 and amino acid 2350-2419) were shown to be important. Whether these domains have an influence on the phosphorylation of each other is not known. Deletion or substitution of highly conserved serine residues in cluster 3 caused a reduced basal phosphorylation of NS5A, but had no effect on RNA replication. These results lead to the conclusion that basal phosphorylation of NS5A plays no important role for the function of NS5A during RNA replication. In addition, it seems that low amounts of phosphorylated NS5A are sufficient for RNA replication.

Considering our mutation analysis of the C-terminus of NS5A two models for NS5A phosphorylation can be envisaged. In the first model, phosphorylation in the central- and C-terminal domain would occur independently. The C-terminal domain of NS5A may be the major site for basal phosphorylation, which would lead to the formation of p56, whereas production of p58 would only be due to phosphorylation in the center of NS5A. In this case, alteration of potential phosphorylation sites at the C-terminus would reduce p56 amounts without affecting formation of p58. In the second model, phosphorylation in the central domain of NS5A depends on prior phosphorylation at the C-terminus. In this case, decreased phosphorylation at the C-terminus would lead to lower amounts of p56 that in turn may result in lower amounts of p58. Upon radioactive labelings of C-terminal NS5A mutants we observed formation of p56 and p58, however only p58 was phosphorylated. This result rises evidence that phosphorylation of p58 is independent from prior phosphorylation at the NS5A C-terminus and therefore supports the first hypothesis. However, further pulse chase experiments are required to clarify this conclusion.

Finally, we analyzed the C-terminus of NS5A for its ability to tolerate insertions of heterologous sequences. The fact that only insertions of the eGFP or dsRed genes allowed efficient RNA replication may be due to the particular structures of the fluorescent reporter proteins. In the three-dimensional structure of GFP and dsRed the N- and C-termini of these proteins are in close

proximity with the majority of the polypeptide forming an almost perfect cylinder (106, 167). Therefore these insertions probably form a well-separated extra-domain in which the NS5A junctions are still kept together in close proximity by the N- and C-termini of eGFP or dsRed. This would explain why there is no major effect on conformation, and function of NS5A. Unfortunately the crystal structure of renilla luciferase is not known and therefore, we can only speculate about the inhibition of replication by its insertion into NS5A. The most likely explanation is that this luciferase has a more extended conformation which is preserved in the fusion protein as deduced from high level renilla luciferase activity, but disturbs NS5A folding and thereby its function for RNA replication.

4.3 Membrane association of NS5A and its role for RNA replication

Using three-dimensional NMR structure analyses of the NS5A[1-31] peptide, Penin and colleagues reported, that the N-terminal membrane anchor domain of NS5A has the potential to form a long amphipathic α-helix (amino acid 5-25). This helix seems to be divided into two portions separated by a putative flexible region located in the center of the helix (amino acid 15-17; see Fig. 33) (112). The hydrophobic amino acids were found to be mainly buried in the hydrophobic core of the detergent-peptide micelles. It is therefore likely, that the NS5A membrane anchor domain is embedded in-plane in the cytosolic leaflet of the ER membrane, with a hydrophobic side buried in the membrane and a polar/charged side accessible from the cytosol while the polar and charged amino acid were mainly accessible at their surface. The location of the Trp residues at the interface between the hydrophilic and hydrophobic sides of the amphipathic helix is a very typical feature. Since the Trp residues are highly conserved among various HCV genotypes, they are supposed to participate in specific protein-protein interactions at the level of the membrane interface, possibly with other components of the HCV RC, besides their function to ensure membrane association. The polar side of the α-helix exhibits a striking asymmetry in charge distribution with positively charged residues (Arg6, Lys20, and Lys24) placed in-line along one border and negatively charged residues (Asp7, Asp10, Glu14, and Asp18) along the other. This particular arrangement of these remarkably conserved charged residues suggests that the polar side of the helix forms a specific interaction site. Hence, the helix appears as a multi-interaction platform with both the hydrophilic side and the borders of the hydrophobic side likely involved in specific protein-protein interactions. Based on the above structural features and the observation that NS5A expressed alone behaves as an integral membrane protein (11), a model has been proposed, where the rest of NS5A folds onto the polar side of the N-terminal amphipathic α-helix membrane anchor. The 3D structures of two other

monotopic membrane proteins with a similar organization have been described, namely prostaglandin H synthase and squalene cyclase. In both cases, integral membrane association is mediated by in-plane amphipathic α-helices. Furthermore, interactions between the membrane anchor and the rest of the protein are mainly ensured by charged and polar residues, which define interaction sites on both part of these proteins (105, 158). A similar arrangement likely occurs in NS5A, as suggested by the platform of hydrophilic residues and the asymmetric distribution of charged residues on the cytosolic side of the N-terminal a-helix. Predicted amphipathic α-helices mediating membrane association have been described in other positive-strand RNA viruses too. For example, an amphipathic helix was predicted in the N-terminal region of PV and HAV 2C proteins (26, 79, 109, 147). However, no structural data have been reported for these picornaviral proteins. Investigation of Semliki Forest virus mRNA-capping enzyme nsp1 by NMR spectroscopy revealed a short amphipathic α-helix mediating monotopic membrane association (81). Sequence alignments suggested that a similar amphipathic α-helix is present in nsp1 of other alphaviruses. By analogy to HCV NS5A, it is reasonable to speculate that these amphipathic helices constitute the main structural determinants of membrane association and are essential for replication of the corresponding viruses. In conclusion, the NS5A N-terminal in-plane amphipathic α-helix is not only a structural determinant for ER membrane targeting and binding *via* protein phospholipid interactions, but is also a multi-interaction binding platform thought to ensure the stabilization of NS5A folding and/or its positioning in the HCV RC *via* intermolecular interactions.

It was recently reported that disruption of the amphipathic nature of the N-terminal helix of NS5A by introduction of several charged residues into the hydrophobic side abolished membrane association of NS5A and, by consequence, HCV RNA replication (28). The more subtle mutational analysis reported here revealed that our NS5A membrane anchor mutants, including the 8+A and 11+A mutants defective in RNA replication, show proper membrane association, with only slightly reduced strength of membrane binding. However, incorporation of the mutant NS5A proteins into the dot-like structures, which represent membranous webs harboring HCV RCs (5, 6), were affected when they were expressed in the context of a NS3-5B polyprotein. Structure-function analyses of these mutants, demonstrated that perturbation of the relative positioning of conserved residues by Ala insertion at position 8 or 11, leading to a twist of the α-helix by 110° (see Fig. 5 B and C), completely abrogated RNA replication without significantly affecting membrane association. In contrast, Ala replacement of selected residues (R6A/D7A, W9A or C13A) had no or only moderate effect on HCV RNA replication. The absolutely conserved Cys13 could be either involved in a disulfide bond or form a link to a lipid.

In both cases, replacement by Ala should result in only a limited destabilization of protein folding and/or protein-protein or protein-membrane interactions. Similarly, replacement of either Arg6 and Asp7 or Trp9 by Ala may be to subtle to destabilize essential interactions and may be compensated by the remaining residues involved in these interactions. Alternatively, the unimpaired replication of the R6A/D7A, W9A and even C13A mutants raises the possibility that the N-terminal portion of the helix may not be required for an intramolecular interaction, but may be involved in intermolecular interactions that are not essential for replicon activity. Interestingly, when the NS5A membrane anchor mutants were expressed in the context of a NS3-5B polyprotein and analyzed with respect to their phosphorylation state, it was found that the replication-defective mutants 8+A, 11+A and 5-11 were not hyperphosphorylated in contrast to the mutants R6A/D7A, W9A and C13A. This concordance between preserved hyperphosphorylation and RNA replication suggests that the kinase binding or accessibility of phosphoacceptor sites is depending on a proper incorporation of NS5A into the RC. Clearly, resolution of the 3D structure of the entire NS5A protein will shed further light on these mechanisms.

Recently a synthetic peptide representing the N-terminal amphipathic α-helix of NS5A was found to block membrane association of NS5A *in vitro* in a dose-dependent and sequence-specific fashion (28). The mechanism of inhibition remains to be determined, but it is very likely that such an effect also causes a reduction of RNA replication since membrane association of NS5A is an important step for the integration of NS5A into the HCV replicase complex. All attempts to study such an effect in cell culture by using the subgenomic replicon system failed so far. Expression of the wildtype NS5A amphipathic helix, as well as mutated helices in cis from the same RNA molecule as the HCV replicase complex showed no effect on RNA replication. In addition, expression of the wildtype NS5A amphipathic helix from a co-transfected DNA plasmid also had no effect. However, this may be due to insufficient expression levels or short half-live of the peptides. Alternatively, low amounts of membrane associated NS5A may be sufficient for RNA replication. Finally, it is also possible that overexpression of the amphipathic helix has no effect on RNA replication. However, the fact that membrane association is necessary to allow efficient RNA replication argues against this possibility. In conclusion our study revealed an essential role of the N-terminal NS5A membrane anchor for RNA replication.

4.4 Trans-complementation of NS5A

With the aim to study the role of NS5A for RNA replication in more detail, we established two alternative assays that allow rescue of RNA replication for NS5A mutants by trans-complementation (chapter 3.4). The first assay is based on the super-transfection of non-replicating selectable neo-replicons into Huh-7 cells that carry a functional helper replicon. The disadvantage of this assay is the length of time required to establish cell clones, the low success in recovery of viable cell clones and the possibility for accumulation of second-site compensatory mutations or reversions in the replicon mutant. On the other hand, this approach has the advantage that stable cell clones can be examined in detail for the replicating RNA species. In this way we found that only mutations in NS5A could be rescued for RNA replication and in all analyzed complemented cell clones the mutants still carried the inactivating substitutions in NS5A. Complete sequence analysis of the HCV coding region of one cell clone revealed no further mutations, suggesting that trans-complementation took place. In 3 out of 28 cases, only the mutated neo-replicons were detected. Analysis of one of these clones revealed that the neo-RNAs had acquired additional mutations that may have compensated for the defects. Another possibility is that we selected by chance for a more permissive host cell that supports replication of this RNA. Further studies will be required to differentiate between these two possibilities. Surprisingly, in no case was RNA recombination observed, although naturally occurring recombinant HCV isolates have been described (18, 67). Very recently, a model was proposed, in which homologous recombination occurs during negative-strand synthesis by template switching of the polymerase that is facilitated by conserved hairpin structures (66). For members of the *Picornaviridae* family recombination is a well-known phenomenon. For instance, in the case of PV it was reported that up to 79% of virus strains secreted after vaccination are recombinants (19). RNA recombination was supposed to occur by a copy-choice mechanism. More recently, two independent groups reported the generation of recombinant genomes of PV (46) and BVDV (5, 42) by a nonreplicative recombination mechanism in vivo.

The second assay allowed us to monitor transient replication of an inactivated replicon after co-transfection with a helper RNA. This assay has two advantages over the selection approach. Firstly, trans-complementation can be measured within a very short time, does not depend on the selection of cell clones and circumvents the problem of accumulation of compensatory mutations. Secondly, non-replicating helper RNAs can be used, which is not possible with the co-selection approach. In fact, by using this assay we found that expression of an NS3 to NS5A polyprotein was sufficient to rescue replication of an NS5A replicon mutant, whereas NS5A expressed on its own did not support trans-complementation. These data suggest that the other

non-structural proteins NS3, NS4A and NS4B affect production of a functional NS5A protein. This result is in remarkable agreement with the previous observation that proper phosphorylation of NS5A requires expression of this protein in the context of the same polyprotein (76). However, the phosphorylation status of NS5A does not reflect its ability to perform trans-complementation. In fact, this process requires NS5A that is a part of RCs. Mutations in NS4A or NS4B interfering with localization of NS5A into RCs also interfere with trans-complementation. Whether the induction of the membranous web or the recruitment of NS5A into the RCs is affected by these mutations is not clear and must be clarified by further immunofluorescence analysis. Nevertheless, this observation may explain, why mutations affecting the overall folding or the length of the N-terminal amphipathic helix can not be trans-complemented. As described in chapter 3.3.1, amino acid substitutions in the amphipathic helix affect the incorporation of NS5A into RCs and therefore not only abrogate RNA replication, but also disturb proper formation of the RCs. As a consequence, incorporation of wildtype NS5A from functional RCs into these misfolded RCs may be impossible.

Our results for HCV are in line with a recent report by Grassmann and co-workers who studied the conditions for trans-complementation of the pestivirus BVDV (50). It was found that only mutations in NS5A, but not in NS3, NS4A, NS4B, and NS5B can be rescued by trans-complementation. However, in this study complementation was only possible when mutants were transfected into cell clones that carried stably replicating replicons, whereas co-transfection of helper RNAs or infection with helper viruses did not restore replication of the mutants. In contrast, we achieved trans-complementation also with non-replicating helper RNAs. The reason for this discrepancy is not known, but may be due to a higher sensitivity of our transient trans-complementation assay.

Trans-complementation has also been described for the two flaviviruses *Yellow fever virus* (YFV) and KV. In both cases, trans-complementation of NS1 was possible after co-expression of this protein by noncytopathic Sindbis virus replicons with the NS1 mutant (72, 84). For KV, it was also possible to complement NS5 polymerase mutants by expression of isolated NS5, although rescue in trans was more efficient when NS5 was expressed as part of a NS1 to NS5 polyprotein (71, 72). Furthermore, trans-complementation was demonstrated for the NS3 helicase region of KV (87). In contrast, for both HCV and BVDV only mutations in NS5A can be complemented in trans. This result suggests that NS3 (presumably as a NS3/4A complex), NS4B, and NS5B are strictly cis-acting within a higher order complex. This result is in agreement with the general notion that the RC of HCV has a rather closed conformation. For instance, it was found that in vitro, isolated RCs are unable to switch to an exogenous template

(80). Furthermore, RNA present within this complex is fairly resistant to nucleases, as are the viral proteins actively engaged in RNA replication (80, 98). Like for virtually all other positive-strand RNA viruses, HCV RNA replication takes place within a membranous compartment that probably limits access for exogenous RNAs and proteins, but on the other side protects these viral factors from degradation and reduces the induction of antiviral defense mechanisms in the host cell. The formation of such a compartment may limit the possibility for trans-complementation.

In view of a model for trans-complementation three possibilities can be envisaged. In the first model, expression of functional NS5A may confer an increased permissiveness to the host cell, for instance by interfering with a cellular inhibitor, which would result in an overall enhancement of HCV RNA replication. In this case functional NS5A would have the capacity to support the defective replicon by performing a function outside of the RC. However, we found that replication of low-efficiency replicons like wildtype or a replicon carrying two weakly adaptive mutations in NS3 was not enhanced in trans-complementation assays. Neither in the co-selection approach, nor in the transient assay was an increase in replication of such RNAs found, suggesting that an indirect function of NS5A that enhances host cell permissiveness and/or RNA replication in general is less likely. A second model is based on the exchange of the complete NS3 to NS5B polyprotein complex between the functional and the inactive RC. This model seems to be less likely since trans-complementation due to a template switch should also be possible for proteins other than NS5A. In the third model, trans-complementation is mediated by an exchange of the functional NS5A protein into the altered RC. Three lines of evidence support this notion. First, trans-complementation was only observed for NS5A, but not for the other NS proteins. Second, complementation efficiency was significantly reduced with mutants carrying a highly adaptive mutation in NS4B upon co-transfection with a helper RNA that delivered an adapted NS5A protein. This result suggests that a chimeric RC was generated carrying two proteins with highly adaptive mutations. In this case the reduction of replication is due to the combination of proteins with highly adaptive mutations that are antagonistic when present in the same RC (89). Third, NS5A is anchored to ER membranes by an amphipathic α-helix but not by a trans-membrane domain, as is the case for NS4B, NS5B and, *via* NS4A, for the NS3/4A complex. It is therefore tempting to speculate that NS5A is less tightly associated with membranes that provide the scaffold of the RC. This looser association may facilitate trans-complementation.

Durin the course of this work, Graziani et al. (51) described a dominant negative effect of wt NS5A on subgenomic replicons carrying adaptive mutations in NS5A. They found that

expression of wildtype NS5A in the context of a NS3 to NS5A polyprotein or from a wt- or NS5B-adapted replicon reduced replication of an NS5A-adapted replicon to about 10%. However, this dominant negative effect of wt NS5A did not affect replication efficiency of replicons carrying wildtype NS5A. The authors proposed two models to explain their results. In one of these, NS5A is engaged in protein-protein interactions e.g. between NS5A and NS5B. This hypothesis implies that the dominant negative effect of wt NS5A is due to its interaction with NS5B, encoded by another (the NS5A-adapted) replicon. In fact, our observation that NS5A mutants can be complemented in trans is compatible with this exchange of proteins encoded by different replicons. Another similarity is the observation that a dominant negative effect is exerted only, when NS5A is expressed as part of a NS3 to NS5A polyprotein, whereas expression of NS5A alone or together with other individual non-structural proteins has no effect. Likewise we found that efficient trans-complementation depends on the expression of NS5A as part of an NS3 to NS5A polyprotein. Finally, Graziani and Paonessa observed that adapted NS5A protein has neither a dominant negative phenotype nor does it enhance replication of wt replicons. We also did not observe enhanced replication of wt or weakly adapted replicons in our trans-complementation assays. This result is difficult to envision, because an exchange of proteins or an indirect function of NS5A outside the RC should increase RNA replication of weakly replicating RNAs. Further studies will be required to clarify the underlying mechanism.

In summary, our results constitute the first report describing the trans-complementation of an HCV non-structural protein. The assays reported here may help for better understanding of the role of NS5A in the viral life cycle.

4.5 Interaction of NS5A with amphiphysin II

With the aim to study how NS5A might interact with the cellular signaling network we identified amphiphysin II as a new interaction partner of NS5A (chapter 3.5). Amphiphysin II is a phosphoprotein whose function is regulated by its phosphorylation status. Two isoforms of amphiphysin are known, designated amphiphysin I and II with an amino acid sequence identity of 49%. While amphiphysin I is primarily expressed in the brain, amphiphysin II is ubiquitously expressed with a wide range of splice variants that in some case share a very similar structure with amphiphysin I and therefore bind the same proteins (13). Amphiphysins are rather well characterized molecules that contain distinct functional domains. At the N-terminus they carry a so called BAR domain, named for the Bin-Amphiphysin/Rvs proteins in which it was first identified. The Bar domain is a dimerization, membrane binding, curvature-sensing module that

in the case of amphiphysin also contains an N-terminal amphipathic α-helix (119, 159). This helix enhances the ability of amphiphysin to deform membranes into tubes (113, 138). The central domain, termed CLAP domain, interacts with clathrin and the clathrin adaptor protein 2 (AP-2), whereas the C-terminal SH3 domain binds to dynamin and presents an interaction site for NS5A binding (21). Due to these structural features, amphiphysin I seems to function in synaptic vesicle endocytosis, whereas amphiphysin II is supposed to play an important role during clathrin-mediated endocytosis. This process plays an important role in the life cycle of viruses such as alphaviruses or adenoviruses that enter the cells by receptor mediated endocytosis. In this case, clathrin is the main component of budding vesicles but it offers only a mechanical scaffold and alone is incapable of selecting cargo. The clathrin adaptor protein 2 (AP-2) is responsible for loading cargo protein into the vesicle, while dynamin drives scission of clathrin-coated pits from the plasma membrane. Amphiphysin II interacts with clathrin, AP-2 and dynamin in a phosphorylation dependent manner (132). Phosphorylation of amphiphysin inhibits binding to clathrin and AP-2, while phosphorylation of dynamin prevents its binding to amphiphysin. Expression of isolated SH3 domains of amphiphysin disrupts endocytosis by preventing multimerization of dynamin (107, 130, 160).
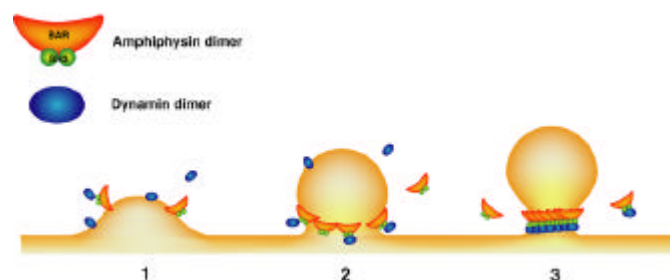
FIG.57: Possible function of amphiphysin in the fission process of endocytic pit (adapted from Yoshida et al., 2004, EMBO J. **23**, 3483-91). (1) Amphiphysin is recruited to the membrane initiating invagination either via its BAR domain or via clathrin/AP-2-binding domain. (2) The BAR domain may help generating the bud neck of the appropriate dimensions. (3) As the pit has been matured, amphiphysin could be more efficiently recruited to the neck due to its curvature-sensing properties. Dynamin also assembles to form a ring at the bud neck, and its GTPase is fully activated so that vesicle fission is achieved. Coat components are omitted.

Recently, a novel isoform of amphiphysin, designated amphiphysin IIm has been identified in macrophages where it was associated with phagosomes (47). Expression of a dominant negative amphiphysin IIm mutant, lacking the SH3 domain, blocks phagocytosis by preventing membrane extension around bound particles. Interestingly, treatment of macrophages with a phosphoinositide 3-kinase (PI3K) inhibitor prevents recruitment of amphiphysin and dynamin to the site of particle binding and thereby disturbs particle internalization. Therefore, it was proposed that PI3K recruits amphiphysin to the phagosome, which in turn is necessary for the recruitment of dynamin. PI3K also has been shown to interact with NS5A in Huh-7 replicon

cells (55). This interaction augmented lipid kinase activity 10-fold both in vitro and in vivo (135).

Our results suggest that NS5A recruits a certain fraction of cellular amphiphysin II to the sites of viral RNA replication, which is mediated through NS5A-containing HCV protein complexes tightly associated to membranous web structures. It is therefore tempting to speculate that the NS5A amphiphysin II interaction may play a role for alterations of the vesicular web structure or for particle assembly, when viral RNA and structural proteins need to be merged at the same location to produce progeny virus. Another possibility may be that the interaction is important for the entry or uncoating process. However at the moment it is not known if NS5A or any other non-structural protein is incorporated into the virus particle and what entry mechanism HCV follows.

Taken together, our data argue against a major role for the NS5A-amphiphysin II interaction in HCV RNA replication in the replicon cell culture model. However, due to the specificity of the interaction, we propose that amphiphysin II might be an essential host-cell factor during natural HCV infection, whose function cannot be studied in replicon cells. With the recent development of a system that supports the production of infectious HCV particles, this possibility can now be addressed.

4.6 Perspectives

The improvements of the HCV replicon system as described in this study will greatly facilitate future genetic studies and contribute to the development of a fully permissive cell culture system that allows efficient propagation of virus particles. Highly adapted HCV replicons permit transient replication assays that are superior to selectable neo-replicons for two reasons. First, the analysis of engineered mutants can be performed within a few days rather than several weeks or months; second, transient replication assays circumvent the problem that during selection of cells, the effect of a mutation to be studied is masked by second-site reversions or compensatory mutations. Furthermore, many cellular interaction partners of HCV non-structural proteins were identified in the past by using yeast two hybrid screenings or other in vitro assays. With the advent of transient replication assays, the importance of these interactions for RNA replication can now be studied in much greater detail in a nearly authentic cell culture system. For instance, the system can be used to clarify the mechanism of adaptation. In fact, as described in chapter 3.2 we obtained evidence that a reduction of NS5A hyperphosphorylation may be one mechanism of adaptation, arguing that NS5A hyperphosphorylation is a critical determinant regulating RNA replication. However, the molecular details are currently unclear. One possibility may be that hyperphosphorylation of NS5A modulates interaction of NS5A with a host cell factor. An interesting candidate identified by a yeast two hybrid screen with NS5A is the t-SNARE human vesicle-associated membrane protein-associated protein A (hVAP-A) (151). RNAi-mediated knockdown of hVAP-A expression as well as overexpression of a dominant-negative mutant reduced HCV RNA replication suggesting that the hVAP-A/NS5A interaction plays a very important role (43). The ability of NS5A to associate with hVAP-A is supposed to depend on NS5A phosphorylation. A model has been proposed by Evans and co-workers (32) in which hyperphosphorylated NS5A presents a "closed" conformation that is unable to associate with h-VAP-A or other proteins required for viral RNA replication, whereas hypophosphorylated NS5A presents an "open" conformation that is able to interact with h-VAPA, which in turn modulates RNA replication. In this model, hyperphosphorylation could serve as a switch to disassemble or otherwise inactivate a particular replicase by inducing a closed conformation of NS5A. This event would release viral proteins and RNA for subsequent steps in the viral life cycle, such as virion assembly or RNA packaging.

In agreement with this model, we found that NS5A adaptive mutations dramatically reduce the production of virus particles (Pietschmann et al., submitted for publication). Taking into account

that these mutations on one hand enhance RNA replication, but on the other hand reduce particle production, and often NS5A hyperphosphorylation, it is tempting to speculate that phosphorylation of this protein is involved in regulating a switch from replication to assembly. If this assumption is correct, a reduction of NS5A phosphorylation or hyperphosphorylation, as achieved by highly adaptive mutations, would enhance RNA replication at the expense of assembly. This model would also explain our observation that genomes carrying adaptive mutations are unable to establish a robust infection in vivo. In collaboration with the group of Dr. Jens Bukh at the NIH, we tested the infectivity of a genome carrying the adaptive mutations in NS3 (E1202G and T1280I) and NS5A (S2197P), identified during the course of this PhD thesis (see chapter 3.1). Upon intrahepatic inoculation of chimpanzees, this genome was not viable. In contrast, when only the mutation in NS5A was introduced, the animal became viremic, but infection was delayed. Sequence analysis of circulating viruses demonstrated that the adaptive mutation in NS5A that enhances RNA replication but reduces virus production had reverted to wildtype (12). This observation underscores the important role of NS5A for RNA replication and it suggests that this protein is a key regulator of replication versus assembly that is most likely regulated by its phosphorylation status.

Recently, a new HCV genotype 2a isolate (JFH1) has been identified that efficiently replicates in Huh-7 cells in the absence of adaptive mutations. Moreover, this genome supports assembly and allows production of infectious virus particles (T. Pietschmann, submitted). The availability of such a cell culture system that recapitulates the complete HCV life cycle should facilitate to unravel the functions of NS5A.

5. Abbreviations

2-ME:	2-Mercaptoethanol
Ab:	Antibody
aa:	amino acid
bp:	base pair
cfu:	colony forming units
CRE:	cis-acting RNA element
BVDV:	Bovine Viral Diarrhea Virus
DMEM:	"Dulbeccos minimal essential medium"
DNA:	desoxyribonucleic acid
dNTP:	desoxyribonucleosidtriphosphat
DTT:	dithiothreitol
ECF:	Efficiency of colony formation
EDTA:	ethylendiamintetraacetic acid
EMCV:	Encephalomyocarditis Virus
ER:	endoplasmic reticulum
FITC:	fluoresceinisothiocyanat
FCS:	fetal calf serum
h:	hour
HBV:	Hepatitis B Virus
HCV:	Hepatitis C Virus
HDV:	Hepatitis Delta Virus
Hepes:	4-(2-Hydroxyethyl)-1-piperazin-ethansulfonacid
Hygro:	Hygromycin-phosphotransferase
IF:	Immunfluorescence
IFN-a:	Interferon alpha
Ig:	Immunoglobulin
IRES:	internal ribosome entry site
kB:	kilobases
kD:	kilodalton
min.	minute
MOPS:	3-morpholino-1-propansulfonacid
NaAc:	sodium acetate

NANBH:	Non-A Non-B Hepatitis
neo:	Neomycin-phosphotransferase
nt.:	nucleotide
NTP:	ribonucleosidtriphosphate
NTR:	non translated region
ORF:	open reading frame
PAA:	polyacrylamide
PBS:	Phosphat buffered saline
PCR:	polymerase chain reaction
PMSF:	phenylmethylsulfonylfluorid
PVDF:	polyvenylidenedifluoride
PV:	Poliovirus
RC:	replicase complex
RNA:	ribonucleic acid
RT:	reverse transcriptase
RT-PCR:	reverse transcription followed by polymerase chain reaction
SDS:	sodium-dodecyl-sulfate
SDS-PAGE:	SDS-polyacrylamide-gel electrophoresis
sec.:	second
Temed:	N,N,N',N'-Tetramethylethylendiamin
Tris:	Tris(hydroxymethyl)aminomethan
RC:	Replicase complex
RpM:	Rounds per minute
ON:	over night
vol.:	volume
VV:	Vaccinia virus
wt:	wildtype
zeo:	zeocin

6. References

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7. Appendix

The name of the replicon constructs is based on the nucleotide and amino acid sequence of the HCV Con-1 genome. Three fragment ligation means ligation of one vector with two insert fragments. Restrictions are indicated by "x". PCR fragments are generated as described in chapter 2.2.8 and 2.2.9 of the Materials&Methods section. Primers and template DNA are given in the description.

7.1 Basic constructs

Replicons pFK1-389neoEI3420-9605/wt, pFK1-389LucEI3420-9605 and pFK1-341spacerPILucEI3420-9605 often served as template DNA for the generation of new constructs.
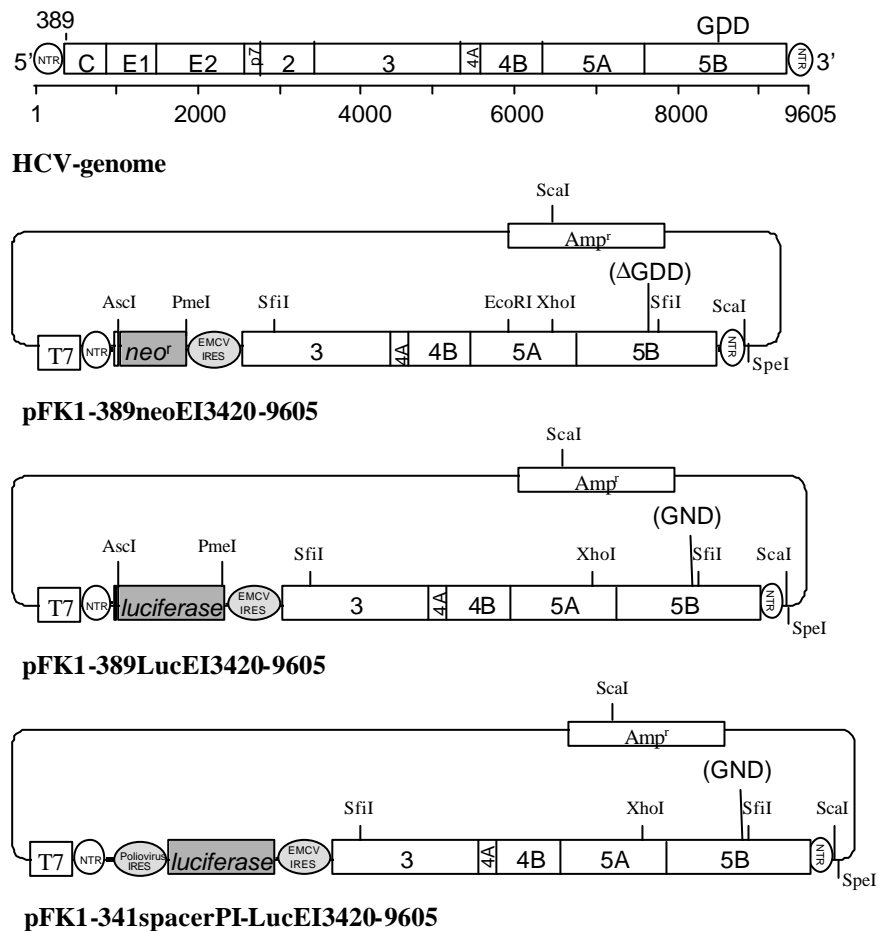
FIG. 58: Basic constructs for the construction of adapted HCV replicons, further selectable replicons and full-length replicons. Shown in the upper panel is the HCV Con-1 genome. Numbers refer to the nucleotide position within this genome. Below this genome, three plasmids that were used for the construction of other replicons are depicted.

7.2 Construction of adapted HCV-replicons

pFK1-389neoEI3420-9605/NK4.1-4.3,   **pFK1-389neoEI3420-9605/NK5.1-5.16,**
pFK1-389neoEI3420-9605/NK1-66: SfiI-fragments (4,8kb) generated by Long-PCR were
inserted into pFK1-389neoEI3420-9605/Wt x SfiI (6,1 kb)

pFK1-389neoEI3420-9605/NK5.1/4180 T? I: 3-fragment ligation with PmeI-BstXI-fragment
of pFK1-389neoEI3420-9605/NK5.1 and BstXI-Fragment of pFK1-389neoEI3420-9605/wt into
pFK1-389neoEI3420-9605/Wt x PmeI x BstXI

pFK1-389neoEI3420-9605/NK5.1/5610 L? I: 3-fragment ligation with PmeI-SalI-fragment of
pFK1-389neoEI3420-9605/Wt and SalI-MluI-fragment of pFK1-389neoEI3420-9605/NK5.1
into pFK1-389neoEI3420-9605/Wt x PmeI x MluI

pFK1-389neoEI3420-9605/NK5.1/6666 N? D: MluI-EcoRI-fragment   of
pFK1-389neoEI3420-9605/Wt   was   exchanged   against   MluI-EcoRI-fragment   of
pFK1-389neoEI3420-9605/NK5.1

pFK1-389neoEI3420-9605/NK5.1/6930 S? P: EcoRI-XhoI-Fragment   of
pFK1-389neoEI3420-9605/Wt   was   exchanged   against   EcoRI-XhoI-Fragment   of
pFK1-389neoEI3420-9605/NK5.1

pFK1-389neoEI3420-9605/NK5.1/7320 P? S: 3-fragment ligation with AscI-XhoI-fragment of
pFK1-389neoEI3420-9605/Wt and XhoI-PshAI-fragment of pFK1-389neoEI3420-9605/NK5.1
into pFK1-389neoEI3420-9605/Wt x AscI x PshAI

pFK1-389neoEI3420-9605/NK5.1/7389 K? E: 3-fragment   ligation   with
MluI-PshAI-fragment   of   pFK1-389neoEI3420-9605/Wt   and   PshAI-SpeI-fragment   of
pFK1-389neoEI3420-9605/NK5.1 into pFK1-389neoEI3420-9605/Wt x MluI x SpeI

7.3 Construction of replicons carrying different selection markers

pFK1-389HygroEI3420-9605/9-13F: Amplification of the hygromycin-phosphotransferase gene by PCR (ShygroAscI + AhygroPmeIStop, template pTKhyg); AscI-PmeI fragment was inserted into pFK1-389neoEI3420-9605/9-13F x AscI x PmeI

pFK1-389HygroEI3420-9605/NK5.1: SfiI-fragment of pFK1-389HygroEI3420-9605/9-13F was exchanged against SfiI-fragment of pFK1-389neoEI3420-9605/NK5.1

pFK1-389HygroEI3420-9605/ET: SfiI-fragment of pFK1-389HygroEI3420-9605/9-13F was exchanged against SfiI-fragment of pFK1-389neoEI3420-9605/ET

pFK1-389HygroEI3420-9605/D*: XhoI-SpeI-fragment of pFK1-389HygroEI3420-9605/9-13F was exchanged against XhoI-SpeI-Fragment of pFK1-389neoEI3420-9605/ΔGDD

pFK1-389zeoEI3420-9605/9-13F: Amplification of the zeocine resistance-gene by PCR (SZeoAscI + AzeoPmeIStop, template pCRScriptzeocinA); AscI-PmeI fragment was inserted into pFK1-389neoEI3420-9605/9-13F x AscI x PmeI

pFK1-389zeoEI3420-9605/NK5.1: SfiI-fragment of pFK1-389zeoEI3420-9605/9-13F was exchanged against SfiI-fragment of pFK1-389neoEI3420-9605/NK5.1

pFK1-389zeoEI3420-9605/D*: XhoI-SpeI-fragment from pFK1-389zeoEI3420-9605/9-13F was exchanged against XhoI-SpeI-Fragment of pFK1-389neoEI3420-9605/ΔGDD

7.4 Construction of reporter-replicons

pFK1-389lucEI3420-9605/NK5.1: Amplification of the firefly luciferase-gene by PCR (SLucAscI + ALucPmeIStop, template pCMVLuc); AscI-PmeI fragment was inserted into pFK1-389neoEI3420-9605/9-13F x AscI x PmeI ; SfiI-fragment of

pFK1-389lucEI3420-9605/9-13F   was   exchanged   against   SfiI-fragment   of
pFK1-389neoEI3420-9605/NK5.1

7.5 Construction of selectable HCV full-length genomes

pFK1-389neo342-9605/NK5.1: SfiI-fragment of pFK1-389neo342-9605/9-13F was exchanged against SfiI-Fragment of pFK1-389neoEI3420-9605/NK5.1

pFK1-9605/S2197P: SfiI-fragment of pFK1-9605/Con-1 was exchanged against SfiI-fragment of pFK1-389neoEI3420-9605/6930S**?** P

7.6 Construction of NS5A mutants

PCR based mutagenesis: N-terminal mutations xMluI x HpaI

Central mutations x EcoRI x XhoI

C-terminal mutations x XhoI x BclI

7.7 Result of the sequence analysis of the adapted HCV replicons

Table 7: Mutations found in adaptive replicons NK5.1, NK19, NK5.2 and NK5.16. Shown are the nucleotide exchanges within the SfiI-fragment. Nucleotide exchanges that did not result in an amino acid substitution are indicated by a minus. If the nucleotide exchange resulted in an amino acid substitution, position and region within the HCV genome were given.

Replicon	Nucleotide position[1]	Nucleotide exchange	amino acid substitution	amino acid position[2]	HCV protein
NK5.1	3946	A? G	Glu? Gly	1202	NS3
	4180	C? T	Thr? Ile	1280	
	4679	C? T	-		
	4682	T? C	-		
	5610	C? A	Leu? Ile	1757	NS4B
	6437	A? G	-		
	6666	A? G	Asn? Asp	2109	NS5A
	6842	C? T	-		

	6926	C? T	-		
	6930	T? C	Ser? Pro	2197	NS5A
	7320	C? T	Pro? Ser	2327	NS5A
	7389	A? G	Lys? Glu	2350	NS5A

Replicon	Nucleotide position[1]	Nucleotide exchange	amino acid substitution	amino acid position[2]	HCV protein
NK19	3946	A? G	Glu? Gly	1202	NS3
	4078	C? G	Ala? Gly	1246	NS3
	4180	C? T	Thr? Ile	1280	NS3
	4682	T? C	-		
	5610	C? A	Leu? Ile	1757	NS4B
	5958	A? T	Met? Leu	1873	NS4B
	6170	T? A	-		
	6596	G? A	-		
	6598	C? G	Ala? Gly	2086	NS5A
	6833	C? T	-		
	6842	C? T	-		
	6930	T? C	Ser? Pro	2197	NS5A
	7141	A? G	Glu? Gly	2267	NS5A
	7320	C? T	Pro? Ser	2327	NS5A
	7389	A? G	Lys? Glu	2350	NS5A
	7735	G? A	Ser? Asn	2463	NS5B

Replicon	Nucleotide position[1]	Nucleotide exchange	amino acid substitution	amino acid position[2]	HCV protein
NK5.2	3946	A? G	Glu? Gly	1202	NS3
	4115	C? T	-		
	4180	C? T	Thr? Ile	1280	NS3
	4682	T? C	-		
	4979	A? G	-		
	5610	C? A	Leu? Ile	1757	NS4B
	5663	T? C	-		

	5730	A→ G	Ile→ Val	1797	NS4B
	6410	C→ T	-		
	6842	C→ T	-		
	6930	T→ C	Ser→ Pro	2197	NS5A
	7045	A→ G	Gln→ Arg	2235	NS5A
	7161	A→ G	Ile→ Val	2274	NS5A
	7320	C→ T	Pro→ Ser	2327	NS5A
	7389	A→ G	Lys→ Glu	2350	NS5A
	7584	G→ A	Asp→ Asn	2415	NS5A
	8348	A→ G	-		

Replicon	Nucleotide position[1]	Nucleotide exchange	amino acid substitution	amino acid position[2]	HCV protein
NK5.16	3946	A→ G	Glu→ Gly	1202	NS3
	4180	C→ T	Thr→ Ile	1280	NS3
	4601	C→ T	-		
	4682	T→ C	-		
	4774	C→ T	Pro→ Leu	1478	NS3
	5474	C→ T	-		
	5610	C→ A	Leu→ Ile	1757	NS4B
	6842	C→ T	-		
	6930	T→ C	Ser→ Pro	2197	NS5A
	7320	C→ T	Pro→ Ser	2327	NS5A
	7389	A→ G	Lys→ Glu	2350	NS5A
	7489	A→ G	Gly→ Glu	2383	NS5A
	7939	A→ G	Lys→ Arg	2533	NS5B
	8126	C→ T	-		

[1] Nucleotide position within the HCV Con-1 genome

[2] Amino acid position within the HCV Con-1 polyprotein

7.8 Generation of hygromycin- and zeocin-replicon cell clones

Table 8: Overview of replicon cell clones carrying subgenomic hygromycin- or zeocin-replicons

Replicon	Cell-clone	Selection	Northern-Blot	reporation total RNA	Cell-clone	Selection	Northern-Blot
Hyg-5.1	000120/28-1	50µg/ml	20.04.00 +	03.08.00	000803/25-1 000803/25-2 000803/25-3	25µg/ml	06.12.00
	000120/28-2	50µg/ml	20.04.00 +	03.08.00	000803/27-1	25µg/ml	06.12.00
	000120/28-3	50µg/ml	20.04.00 +	03.08.00	000803/28-1	25µg/ml	06.12.00
Hygubi -5.1	000411/43-1	25µg/ml	18.08.00 +	11.09.00	000911/11-1	25µg/ml	14.12.00*
	000411/43-2	25µg/ml	16.08.00 +				
	000411/45	25µg/ml	11.07.00 -				
	000803/37-1	25µg/ml	06.12.00 +				
	000803/37-2	25µg/ml	06.12.00 +				
	000803/37-4	25µg/ml	06.12.00 +				
	000803/37-6	25µg/ml	06.12.00 +				
	000803/38-2	25µg/ml	06.12.00 +				
	000803/38-3	25µg/ml	06.12.00 +				
	000803/38-4	25µg/ml	06.12.00 +				
	000803/38-5	25µg/ml	06.12.00 +				
	000803/38-7	25µg/ml	06.12.00 +				
	000803/38-8	25µg/ml	06.12.00 +				
Hyg-ET	011010/3N**	50µg/ml					
Zeo-5.1	000215/63cplt	10µg/ml	11.07.00 +				
	000215/65-I	10µg/ml	04.05.00 +	15.05.00	000515/35-1	10µg/ml	28.09.00
	000215/65-II	10µg/ml	11.07.00 +				
	000215/65-III	10µg/ml	04.05.00 +				
	000215/65-IV	10µg/ml	11.07.00 +				
	000215/65-V	10µg/ml	11.07.00 +				
	000215/65-VI	10µg/ml	11.07.00 +				
	000215/65-VII	10µg/ml	11.07.00 +				
	000323/7-1	5µg/ml	11.07.00 +				
	000323/7-2	5µg/ml	11.07.00 +				
	000323/7-3	5µg/ml	11.07.00 +				
	000323/7-4	5µg/ml	11.07.00 +				
	000323/7-5	5µg/ml	11.07.00 +				
	000323/7-6	5µg/ml	11.07.00 +				

Zeoubi-5.1	000803/31-1	10µg/ml	06.12.00 +				
	000803/32-1	5µg/ml	11.10.00 +				
	000803/32-2	5µg/ml	11.10.00 +				
	000803/32-3	5µg/ml	06.12.00 +				

7.9 Generation of trans-complemented replicon cell clones

Table 9: Overview of trans-complemented replicon cell clones

Cell-clone	Replicon[1]	Northern Blot	Hygro RNA	Neo RNA
010723/4-2-1	E1202G+Δ2194-2197	21.12.01	-	+
010723/4-2-2	E1202G+Δ2194-2197	21.12.01	+	+
010723/5-2-1	E1202G+Δ2200-2202	21.12.01	+	+
010723/6-1-1	E1202G+Δ2200-2202	28.11.01	+	+
010723/6-1-2	E1202G+Δ2200-2202	21.12.01	+	+
010723/7-3-1	E1202G+Δ2194-2207	21.12.01	+	+
010723/8-2-1	E1202G+Δ2194-2207	28.11/21.12	-	-
010830/6-1-1	E1202G+Δ2194-2207	21.12.01	+	+
010830/6-1-2	E1202G+Δ2194-2207	21.12.01	+	+
010830/9-2-1	E1202G+S2201A+S2204A	21.12.01	+	+
010830/9-2-2	E1202G+S2201A+S2204A	21.12.01	+	+
010830/11-2-1	E1202G+S2197A+S2201A	21.12.01	+	+
010830/11-2-2	E1202G+S2197A+S2201A	21.12.01	-	+
010830/(1)4-2-1	E1202G+Δ2200-2202	21.12.01	+	+
010830/(1)4-2-2	E1202G+Δ2200-2202	21.12.01	-	+
010830/(1)4-2-3	E1202G+Δ2200-2202	21.12.01	+	+
010830/(1)4-2-4	E1202G+Δ2200-2202	21.12.01	+	+
010913/7A	E1202G+Δ2200-2202	21.12.01	-	+
010913/7B	E1202G+Δ2200-2202	21.12.01	+	+
010913/8A	E1202G+S2194A+S2200A+S2201A+S2202A	21.12.01	+	+
010913/8B	E1202G+S2194A+S2200A+S2201A+S2202A	21.12.01	+	+
010913/9A	E1202G+S2194+S2201A+S2204A	21.12.01	+	+

010913/9B	E1202G+S2194+S2201A+S2204A	21.12.01	+	+
010913/10A	E1202G+S2194+S2197A+S2201A+S2204A	21.12.01	+	+
010913/11A	E1202G+S2201A+S2204A	21.12.01	+	+
010913/11B	E1202G+S2201A+S2204A	21.12.01	+	+
010913/12A	E1202G+S2197A+S2204A	21.12.01	+	+
011023/23 pool	E1202G+S2194A+S2201A+S2204A	21.12.01	+	+
011023/27 pool	E1202G+S2197A+S2201A	21.12.01	+	+

[1]: selectable neo-replicon pFK1 -389neo/EI3420-9605/E1202G carrying inactivating mutations in NS5A

8. Publications

Krieger, N V.Lohmann and R. Bartenschlager. 2001. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations.J Virol. 75(10):4614-24

Friebe, P., V. Lohmann, N. Krieger, and R. Bartenschlager. 2001. Sequences in the 5' nontranslated region of hepatitis C virus required for RNA replication. J. Virol. 75:12047-12057.

Bukh, J., T. Pietschmann, V. Lohmann, N. Krieger, K. Faulk, R. E. Engle, S. Govindarajan, M. Shapiro, M. St Claire, and R. Bartenschlager. 2002. Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. Proc. Natl. Acad. Sci. U. S A 99:14416-14421.

Frese M, V. Schwarzle, K. Barth, N. Krieger, V. Lohmann , S. Mihm, O. Haller and R. Bartenschlager. 2002. Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. Hepatology. 35(3):694-703

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Zech B, A. Kurtenbach, N. Krieger, D. Strand, S. Blencke, M. Morbitzer, K. Salassidis, M. Cotten, J. Wissing, S. Obert, R. Bartenschlager, T. Herget and H. Daub. 2003. .Identification and characterization of amphiphysin II as a novel cellular interaction partner of the hepatitis C virus NS5A protein. J Gen Virol. 84(Pt 3):555-60.

Penin F, V. Brass, N. Appel, S. Ramboarina, R. Montserret, D. Ficheux, HE. Blum, R. Bartenschlager and D. Moradpour. 2004. Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A.. J Biol Chem. 279(39):40835-43. Epub 2004 Jul 07

Appel N, U. Herian and R. Bartenschlager. 2004. Efficient rescue of hepatitis C virus RNA replication by trans-complementation with nonstructural protein 5A. J. Virol. in press

Appel N, T. Pietschmann and R. Bartenschlager. 2004. Mutation analysis of hepatitis C virus nonstructural protein 5A: Potential role of differential phosphorylation for RNA replication and identification of a genetically flexible domain. J. Virol. in press.

Abstract:

Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus that belongs to the family *Flaviviridae*. Its genome encodes a polyprotein with a length of about 3000 amino acids. This polyprotein is cleaved into 10 different products. One of these is NS5A, a highly phosphorylated protein that contains an amphipathic α-helix at the N-terminus responsible for membrane association of the protein. NS5A is implicated in counteracting the antiviral state that is induced by interferon-α. In addition, it is assumed to be involved in RNA replication.

It was the overall aim of this PhD thesis to gain insight into the role of NS5A for RNA replication. A large panel of mutations affecting potential phospho acceptor sites were designed and analyzed for their replication ability and phosphorylation status. We found that certain serine substitutions in the center of NS5A enhanced RNA replication concomitant with a reduction of NS5A hyperphosphorylation. Second, we studied the impact of mutations in the N-terminal amphipathic α-helix of NS5A on membrane association, RNA replication and NS5A phosphorylation. Structural changes of the α-helix were found to disturb incorporation of NS5A into the HCV replicase complex, concomitant with a block of RNA replication. Furthermore, these mutations interfered with hyperphosphorylation of NS5A arguing that the amphipathic α-helix is required to ensure proper folding and phosphorylation of NS5A, which in turn is required for RNA replication. Third, we wanted to know whether components of the HCV replicase complex (RC) are strictly cis-acting or can be complemented in trans. To address this question, two alternative trans-complementation assays were established. We found that NS5A is the only non-structural protein that can be complemented in trans. In the last chapter of this thesis, we aimed at the identification of cellular phosphoproteins that interact with NS5A. In the course of this study, amphiphysin II was identified as a novel NS5A interaction partner.

In summary, these data show that NS5A plays a very important role for RNA replication that is most likely regulated by its phosphorylation status. Based on most recent data, showing that adaptive mutations in NS5A enhance RNA replication, but reduce virion formation, we speculate that NS5A may be a key regulator of the switch from RNA replication to virus assembly. This switch may be regulated by differential phosphorylation of this important protein.