

**Development and Characterization of Subgenomic  
and Full-Length Genome Replicons Based  
on the Sequence of HCV AD78 Strain**

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## **Introduction**

Hepatitis C virus (HCV) infection has become one of the most common causes of chronic liver disease in Europe. According to the World Health Organisation (WHO) more than 200 million people worldwide are infected with HCV, of whom probably more than 50% will develop chronic hepatitis, leading to cirrhosis in 10 to 20 % and hepatocellular carcinoma in 1 to 5% of infected individuals. Such life-threatening sequel makes hepatitis C also the major cause of liver transplantation in developed countries. Currently, the only therapy for chronic HCV infection that has a lasting beneficial effect is systemic treatment with pegylated interferon alpha (IFN- $\alpha$ ) in combination with ribavirin, but a sustained response is achieved usually in only 50 to 80% of patients depending on a viral genotype. Thus, it is not likely that the number of HCV infected individuals, who are also the source for new infections, will be significantly reduced in the near future. The situation clearly emphasize the need for novel prophylactic/therapeutic approaches that would prevent spread of HCV and would provide more efficient antiviral therapy of individuals suffering from a chronic hepatitis C. Undoubtedly, the progress in development of HCV vaccine and new therapeutic approaches to a significant extent will depend on our knowledge of biology of HCV and characteristics of its interaction with the human host. In the current section of this thesis an attempt is made to summarise the large bulk of information on the molecular biology of HCV, including organization of its genome, characteristics of virus-encoded proteins, model systems for virus replication, and peculiarities of HCV replication.

## 1. Discovery of HCV and development of diagnostic tests.

The genome of HCV was first cloned in 1989 from the plasma of a chimpanzee that had been experimentally infected with the materials, containing the putative non-A and non-B hepatitis agent (Alter et al., 1975; Prince et al., 1974). Characterization of HCV molecular clones revealed that HCV genome was composed of a positive-stranded RNA of about 10 kb (Choo et al., 1989). Identification of HCV cDNA clones allowed for an expression of recombinant HCV antigens both in *E. coli* and yeast, which in turn, were used for the development of anti-HCV enzyme-linked immunosorbent assay (ELISA) for detection of antiviral antibodies (Kuo et al., 1989). The application of this assay for seroprevalence studies revealed high frequency of anti-HCV detection among patients with post-transfusion non-A and non-B hepatitis in different countries (Kuo et al., 1989; Van der Poel CL et al., 1989; Nishioka et al., 1991). These data clearly indicated that HCV was the major causative agent of parenteral non-A and non-B chronic hepatitis worldwide.

Following the identification of HCV, a number of modifications of ELISA for the detection of anti-HCV were introduced. Nowadays, the so-called “third generation” tests, which include several structural and non-structural HCV proteins, are widely used in medical practice as a primary diagnostic assay (Bresters et al., 1992). To increase the specificity of anti-HCV detection a recombinant immunoblot assay (RIBA) is often used as a supplementary technique to confirm positive ELISA results.

Despite being a very powerful diagnostic tool, the anti-HCV assays provide positive results not for all patients, infected with the virus. First of all, this applies for the so-called “window period”, an interval between exposure and development of antibodies to HCV proteins. Besides that, the anti-HCV production might be low or even absent in immunocompromised patients.

Recent advances in molecular biology have contributed to the development of new powerful tools for the diagnosis of HCV infection. These assays allow for a sensitive and specific detection of HCV RNA genome (up to  $10^2$  copies genome equivalent per ml) in plasma or serum of infected individuals (Pawlotsky; 2002). The first group of methods is based on a combination of a reverse transcriptase reaction and a polymerase chain reaction (RT-PCR)

using the HCV-specific primers. The PCR-based techniques are used both for qualitative and quantitative determination of HCV RNA. Another technique that is currently widely used is transcription-mediated amplification (TMA). In this assay the conserved regions within 5'-UTR of the HCV genome are reverse transcribed into complementary DNA (cDNA) that is then amplified as single-stranded RNA copies by T7 RNA polymerase and the amplicons are detected by hybridization with a complementary RNA labelled probes. Another technique used for quantification of HCV RNA is a branched DNA (b-DNA) assay. In this assay viral RNA is captured by a set of specific synthetic oligonucleotide capture probes, then the signal resulting from a specific hybridization of capture and detection probes with the viral RNA is amplified. In general, nowadays, the diagnostic of HCV infection is based on combination of the assays for the detection of both anti-HCV and HCV RNA.

## **2. Epidemiology of HCV.**

The development of diagnostic tools has facilitated the studies of epidemiology of HCV infection worldwide. The seroprevalence rates of HCV are about 0.5-1% in Western Europe and North America, 3-4% in some Mediterranean and Asian countries and up to 10-20% in parts of Central Africa and Egypt (Wasley et al., 2000; WHO, 2000). Currently, more than 200 millions people worldwide are chronically infected with HCV. According to the WHO report, a chronic liver disease is responsible for about 1.4 millions deaths in 2001, including 796000 due to the cirrhosis and 616000 due to primary liver cancer. At least 20% of these deaths are probably attributable to HCV infection.

HCV is mainly transmitted through contact with blood and blood products (blood transfusion). After introduction in 1990 of anti-HCV screening of blood and blood products into a blood service of the developed countries, post transfusion hepatitis C has virtually disappeared and in most industrialized countries the intravenous drug abuse has become the major identifiable mode of HCV transmission (Moradpour et al., 2001). Unfortunately, the lack of systematic screening of blood donors continues to result in HCV transmission by blood and blood products in many countries with developing or transitional economies.

In addition, large-scale parenteral therapy programs as well as surgical and dental procedures with inadequately sterilized equipment have been important routes of transmission in these countries (Frank et al., 2000). Sexual transmission of HCV between monogamous partners appears to be uncommon.

Detection of HCV-RNA by polymerase chain reaction (PCR) shows that HCV may be present in the saliva of HCV infected patients. Epidemiological studies, however, suggested that the infective capacity of HCV viral particles in saliva is low, and probably transmission of HCV by saliva does not play an important role (Hermida et al, 2002). Finally, a mother-to-infant transmission of HCV has been observed globally, but the risk is probably less than 5% unless the mother is co-infected with human immunodeficiency virus (HIV) (WHO, 1999).

### 3. Taxonomy of HCV.

HCV has been classified as the only member of the *Hepacivirus* genus and belongs to the *Flaviviridae* family, which includes the classical flaviviruses, such as yellow fever virus and dengue virus, the animal pathogenic pestiviruses, such as bovine viral diarrhea (BVDV), and the GB viruses (HGV/GBV-A, HGV/GB-B and HGV/GBV-C). All these viruses share a number of similarities in their genome organization, structure and replication. Thus, all viruses of *Flaviviridae* family have an enveloped particle harbouring a single-stranded RNA genome of positive polarity carrying one long open reading frame (ORF).

HCV isolates demonstrated very high genetic heterogeneity and have been classified into three major categories, depending on the degree of the sequence divergence: genotypes, subtypes, and isolates (Robertson et al., 1998; Simmonds et al., 2005). Existing variants of HCV are classified into six genotypes numbered 1 to 6. Within the genotype, the more closely related variants are classified into subtypes and designated a, b, c, d, etc, in order of discovery. In infected individuals HCV genomes coexist as heterogeneous viral populations closely related designated as quasispecies that result from the accumulation of mutations during viral replication in the host. Genotypes differ from each other by 31% to 33% at the nucleotide level, compared with 20% to 25% between subtypes. Despite the sequence diversity of HCV, all genotypes share an identical complement of collinear genes of similar or identical size in the large open reading frame, and the genetic inter-relationships of HCV

variants are remarkably consistent throughout the genome. This observation served as a basis for the development of different HCV genotyping techniques, which allowed for reliable attribution of the clinical HCV isolates to a particular type/subtype (Robertson et al.1998). Extensive amino acid sequence variability found in both structural and non-structural proteins of HCV leads to antigenic and potentially biological differences between genotypes/subtypes that carry implications for vaccine design and treatment of HCVinfected patients.

Thus, it is well known that patients infected with HCV genotype 2 or 3 are more likely to have a sustained response to interferon- $\alpha$  therapy than those infected with genotype1 (Zein et al., 1996; Bell et al., 1997).

## **4. HCV genome**

### **4.1. Viral particle of HCV.**

Spherical virus-like particles of 50 to 70 nm in diameter, taken from human serum and liver tissue, were identified as hepatitis virions by immunoelectron microscopy using the antibodies against the HCV envelope protein (Kaito et al., 1994; Shimizu et al. 1996). Recently, Wakita and colleagues reported the production of infectious HCV viral particles in cell culture (Wakita et al., 2005). Via immunoelectron microscopy using an E2-specific monoclonal antibody, the authors succeeded in demonstrating the presence of specifically labelled spherical particles measuring 50 to 65 nm in diameter in the culture supernatant. By analogy with the known 3D structures of closely related flaviviruses and alphaviruses, it is believed that HCV adopt a classical icosahedral scaffold in which its two envelope glycoproteins, E1 and E2, are anchored to the host cell-derived double-layer lipid envelope. Underneath the membrane is the nucleocapsid that likely is composed of multiple copies of the core protein, forming an internal icosahedral viral coat that encapsidates the genomic RNA.

#### 4.2. Organization of HCV genome.

HCV is a positive RNA virus with a genome containing a long open reading frame (ORF) of approximately 9600 nucleotides. This ORF encodes a large polyprotein of about 3000 amino acids, which is cleaved by viral and host enzymes into 10 polypeptides: core, envelope 1 (E1), envelope 2 (E2), p7, non-structural protein 2 (NS2), non-structural protein 3 (NS3), non-structural proteins 4A and 4B (NS4A and NS4B), and non-structural proteins 5A and 5B (NS5A and NS5B) (Fig.1). Besides this ORF, the HCV genome contains a 5' untranslated region (5' UTR) of about 341 base pairs. Downstream to the coding region another untranslated region of approximately 200 nucleotides (3'UTR) is located. Both 5'UTR and 3'UTR bear highly conserved RNA structures essential for polyprotein translation and genome replication.

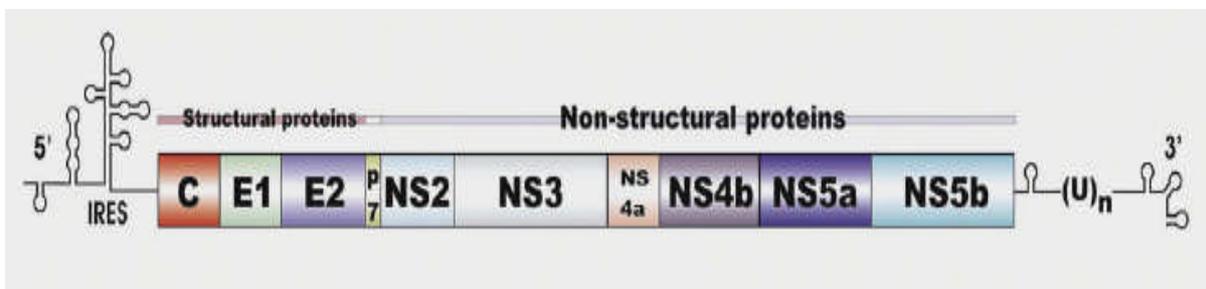
#### 4.3.The 5'untranslated region (UTR)

The 5'UTR of HCV is approximately 341 nucleotides long. This region is highly conserved in terms of nucleotide sequence and secondary structure, although there are a number of genotype-specific variations. The 5'UTR contains four domains (stem-loops) numbered I to IV (Fig.2). Domain III contains a pseudoknot, and the ORF translation initiation codon is located in domain IV. Domains II, III, and IV together with the first 24 to 40 nucleotides of the core-encoding region, constitute the IRES, which mediates viral RNA translation by a cap-independent mechanism (Tchukiyama-Kohara et al., 1992). HCV IRES-mediated translation requires initiation factors such as eukaryotic initiation factor 3 (eIF3), eIF2-GTP-initiator tRNA complex, La auto antigen and polypyrimidine tract-binding protein (PTB) (Ali and Siddiqui 1995, 1997; Sizova et al., 1998; Kruger et al., 2000; Shi and Lai 2001). IRES recruit the 40S ribosomal subunit to an internal initiation codon in the mRNA without scanning phenomenon and without the need for additional canonical or non-canonical translation factors (Pestova et al., 1998). More recently it has been shown that interferons  $\alpha$ ,  $\beta$ , and  $\gamma$  inhibit replication of subgenomic HCV RNA in cell culture model by direct suppression of IRES-mediated translation of viral polyprotein (Dash et al., 2005).

#### 4.4. The 3'untranslated region (UTR).

The 3'UTR, approximately long of 200 nucleotides, is recognized by the viral RNA-dependent RNA polymerase. The 3'UTR has been divided into three regions: a variable sequence of approximately 40 nucleotides, an internal poly (U)/polypyrimidine tract (the poly-U/UC region) of variable length, and a highly conserved region of 98 nucleotides among HCV genotypes containing a stable stem-loop structure which has been termed X-region (Fig.3) (Kolykhalov et al., 1996). The role of the variable region is not clear. The poly-U/UC region and the much-conserved X-region are required for viral replication (Cheng et al., 1999, Oh et al 2000). Genetic studies have shown that a poly (U/UC) tract of at least 25 nucleotides as well as a complete X-tail are required for RNA replication in cell culture and for infectivity of the viral genome in vivo ( Friebe et al., 2002; Gates et al., 2003).

Recently, an additional cis-acting RNA element (CRE) has been identified in the 3' terminal coding region of NS5B (Friebe et al., 2005). This CRE designated 5BSL3.2 forms a long-distance RNA-RNA interaction with SL2 in the X-tail, which is indispensable for RNA replication. La auto antigen and polypyrimidine tract-binding protein (PTB) that bind to 5'UTR of HCV genome have also been identified as cellular factors which bind to the 3'UTR (Tsuchihara et al., 1997; Spangberg et al., 2001), suggesting that La auto antigen and PTB are involved not only in IRES-mediated translation, but also in viral replication.



**Fig. 1. Genome organization of HCV.** The HCV genome is schematically presented. The highly structured 5' and 3'untranslated regions (UTR) are not drawn to scale. The 5' UTR contains an internal ribosomal entry site (IRES). The single HCV open reading frame is shown as a large open box.



## 5. Structure and functions of HCV proteins.

### 5.1. Structural proteins of HCV

#### HCV Core protein.

Localized at the N-terminus of the polyprotein, core is the first structural protein of HCV and results from the cleavage of the polyprotein by cellular and viral proteases. HCV core is rich in basic amino acids and proline residues corresponding to a 20-kDa protein. The major function of HCV core is to encapsidate newly synthesized viral RNA with the formation of the virion nucleocapsid. The full-length core protein has been shown to localize in the cytoplasm on the external membrane (cytoplasmic side) of the endoplasmic reticulum, but some of its truncated forms have been found in the nucleus (Santolini et al., 1994). Because of its cytoplasmic localization, HCV core protein might play a role in transcriptional regulation through its interaction with cytoplasmic proteins and signal transduction pathways (Jin et al., 2000; Otsuka et al., 2000).

The core protein appears to play multiple roles in various cellular signalling pathways, and potentially in oncogenesis (Lai and Ware 2000). HCV core protein has been shown to modify intracellular signalling pathways, which inhibit immune-mediated cell killing (Matsumoto et al., 1997; Kittlesen et al., 2000; Zhu et al., 2001). HCV core inhibits TNF- $\alpha$  mediated apoptosis through a mechanism that involves interaction with the TNF- $\alpha$  receptor (Kittlesen et al., 2000; Tai et al., 2000). HCV core binds to the cytoplasmic domains of tumor necrosis factor receptor 1 (TNFR1), lymphotoxin  $\beta$  receptor, and gC1q receptor and blocks FAS/TNF $\alpha$  receptor signalling (Matsumoto et al., 1997; Kittlesen et al., 2000; Zhu et al., 2001). The core protein has been shown to modulate the activity of transcription factors and cytokines that could promote cellular transformation and hepatocellular carcinoma. More recently it has been shown that HCV core protein induces Huh7 cell proliferation whether alone or in the context of HCV replication, which is at least partly mediated by transcriptional up regulation of growth-related genes, in particular wnt-1 (Fukutomi et al., 2005).

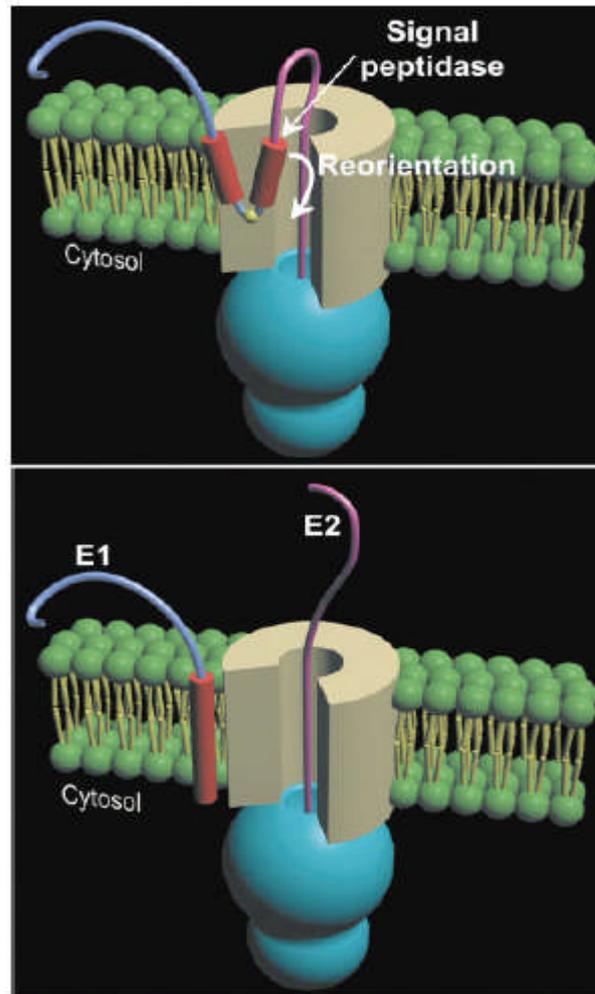
**Alternative reading frame protein or frame shift protein (ARFP/F protein)**

The synthesis of a protein encoded by an alternative reading frame within the core region was reported by several groups (Branch et al., 2005). This protein was designated alternative reading frame protein (ARFP) or frame shift protein (F protein) and comprises up to 160 amino acids. Expression of the ARFP protein of HCV genotype 1a in vitro or in mammalian cells yields a 17 kDa protein. Sequencing of in vitro labelled ARFP protein indicated that the frameshift junction likely occurs at codons 9 to 11 of the core protein sequence (Xu et al., 2001). However, multiple frameshifting events recently have been reported in this region, and a 1.5 kDa protein also could be produced by -1/ +2 frameshifting (Choi et al., 2003). In addition, the frameshift position seems to be genotype dependent, as a +1 frameshift at codon 42 was recently reported for genotype 1b (Boulant et al., 2003). Detection of anti-ARFP antibodies in the serum of HCV-infected patients suggests that ARFP protein is expressed during HCV infection. Functions of ARFP protein are unknown.

**HCV E1 and E2 envelope glycoproteins.**

The two HCV envelope proteins, E1 and E2, are released from the polyprotein by host signal peptidases, and presumably are the key components of the viral envelope, involved in receptor binding and cell fusion (Grakoui et al., 1993; Bartosch et al., 2003). E1 and E2 contain high mannose glycosylation suggesting that they are retained in the endoplasmic reticulum or Golgi, likely due to their C-terminal membrane anchoring sequences (Martire et al., 2001). E1 and E2 are type-I transmembrane (TM) glycoproteins of about 35 kDa and 70 kDa respectively, with a short C-terminal transmembrane domain (TMD) of approximately 30 amino acids and N-terminal ectodomains of 160 and 334 amino acids, respectively. Both E1 and E2 transmembrane domains are composed of two short stretches of hydrophobic amino acids separated by short polar segment containing fully conserved charged residues (Cocquerel et al., 2000). The second hydrophobic stretch acts as an internal signal peptide for the downstream protein (Cocquerel et al., 2002). Before signal-sequence cleavage, the E1 and E2 transmembranes adopt a hairpin structure and after cleavage by a host signal peptidase, the signal-like sequence is reoriented toward the cytosol leading to a single transmembrane passage (Fig.4). E1 and E2 can also form heterodimers of both covalent (disulfide-bonded)

and non-covalent types (Choukhi et al., 1999). The E2 glycoprotein contains a hypervariable region I (HVRI) that presumably contains the major neutralization epitope (Farci et al., 1996). The sequences of HVR differ by up to 80% among HCV genotypes, and even among subtypes of the same genotype. High variability of HVRI during infection is considered as a major mechanism that allows the virus to evade the host humoral immune response (Farci et al., 2000). Another hypervariable region, HVR2, has been described in the E2 glycoprotein of the HCV genotype 1 strains (Kato et al., 2001). HVR2 is a stretch of 7 amino acids (positions 91-97) showing up to 100% sequence diversity. Soluble E2 was shown to bind specifically to hepatocarcinoma cells but also to other cell types, suggesting that the ectodomain of E2 mediates cell attachment (Flint et al., 1999a, 1999b; Heo et al., 2004; Roccasecca et al., 2003; Yamada et al., 2005). Beside its structural role, E2 has been shown to modulate the IFN- $\alpha$  response in vitro (Taylor et al., 1999).

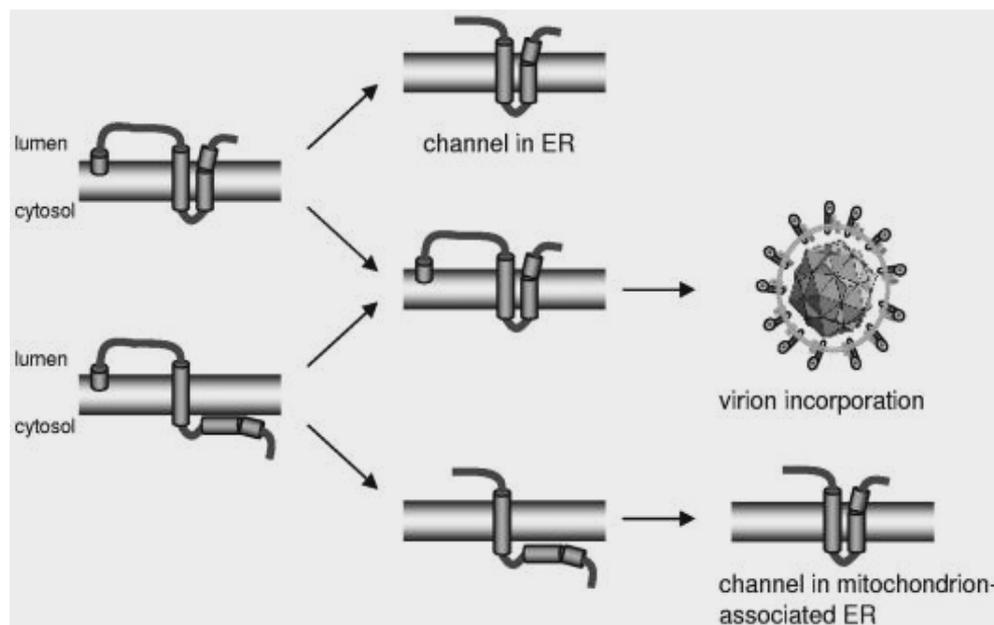


**Fig. 4. Behavior of the transmembrane domains of HCV envelope glycoprotein E1 and E2 during the early steps of their biogenesis. Top**, the N-terminus of E1 is translocated into the lumen of the ER up to the N-terminus of the E1 transmembrane domain, which acts as a stop transfer signal. The C-terminal half of the transmembrane domain of E1 acts as the signal sequence of E2 and has its C-terminus oriented toward the lumen of the ER to allow the translocation of E2. **Bottom**, after cleavage by the signal peptidase, the signal-like sequence is reoriented toward the cytosol, yielding a single transmembrane passage. Adopted from Cocquerel et al., 2002.

## Protein 7

The p7 polypeptide is a small, intrinsic membrane protein of 63 amino acids. The p7 polypeptide has a double membrane-spanning topology, its N- and C-terminal ends face endoplasmic reticulum lumen (Carrere-Kremer et al., 2002). Both transmembrane passages have been predicted to form  $\alpha$ -helices, and the C-terminal transmembrane passage has been shown to function as an internal signal-like peptide. Amino acids variability analysis and helix projections have revealed that each transmembrane passage of p7 has strictly conserved helix faces, suggesting their involvement in specific helix-helix interactions (Carrere-Kremer et al., 2002). Recent data indicate that p7 can mediate ion permeability and can form hexamers (Pavlovic et al.; 2003). Moreover, Griffin and co-workers showed that p7 from genotype 1b HCV forms an oligomeric ion channel in planar lipid bilayers that can be blocked by amantadine at micro molar concentrations (Griffin et al., 2003). These structural and membrane-permeability properties suggest that p7 belongs to the viroporin family and could have an important role in viral particle release and maturation. This is supported by functional data obtained with pestiviruses, indicating that p7 is essential for the production of progeny virus (Harada et al.; 2000). Although p7 seems to be located mainly in endoplasmic reticulum membranes, it can be exported to the plasma membrane and may have functional roles in the secretory pathways (Carrere-Kremer et al., 2002). More recently, Griffin and co-workers have suggested a model for the regulation of p7 localization by combining the role of the signal peptide, its transmembrane topology, and the presence of internal signal sequences (Fig.5) (Griffin et al.2005). Upon translation in the rough ER, a proportion of p7 remains with its C-terminal helix on the cytosolic side of the membrane. As levels of protein increase, a potential signal in the C terminus of the protein binds to a cellular factor that then directs this population of protein to membranes around mitochondria. Conversely, the remaining protein with both termini on the luminal side of the membrane is bound by a factor that causes ER retention. Signal peptide cleavage could theoretically occur in any of the ER-derived membranes, though it is perhaps delayed by binding of an ER retention factor such that a pool of E2-p7 remains in the ER for incorporation into virions. If signal peptide cleavage occurs in the mitochondrial ER cisternae, E2 might be channelled back to the rough ER, whereas p7 remains and adopts its dual-spanning topology. Cleaved p7 in both sets of membranes would then be free to oligomerize and form ion channels in either membrane.

In addition, p7 incorporated into virions as E2-p7 may also be processed during exocytosis to allow the formation of channels that protect E2 from fusogenic change and/or function during virus entry. As more insights into p7 function and behaviour in cells are gained, it is clear that targeting p7 in future antiviral therapies could potentially act by blocking HCV at multiple points in its life cycle, perhaps using compounds based on maintained derivatives. Further experiments on ion channel function, ideally in systems where HCV virions can be produced, will be required to define the precise function of p7 in HCV replication.



**Fig. 5. Hypothetical model for intracellular targeting of p7.** Upon translation, p7 adopts either a single- or double-membrane-spanning topology. A double-membrane-spanning p7 (top left) is more likely to remain in the ER, potentially due to a signal located in the N terminus of the protein. If signal peptide cleavage occurs, double-membrane-spanning p7 will form oligomeric channels, whereas uncleaved E2-p7 is directed into virus particles. A single-membrane-spanning p7 (bottom left) could spontaneously adopt the double-membrane-spanning topology prior to signal peptide cleavage, or by the action of a C-terminal signal, cleaved protein could be targeted to membranes around mitochondria. Upon reaching these membranes, protein would then be free to adopt a double-membrane-spanning topology forming oligomeric channels. Adopted from Griffin et al. 2005.

## 5.2. Non-structural proteins of HCV.

### Non-structural protein 2 (NS2).

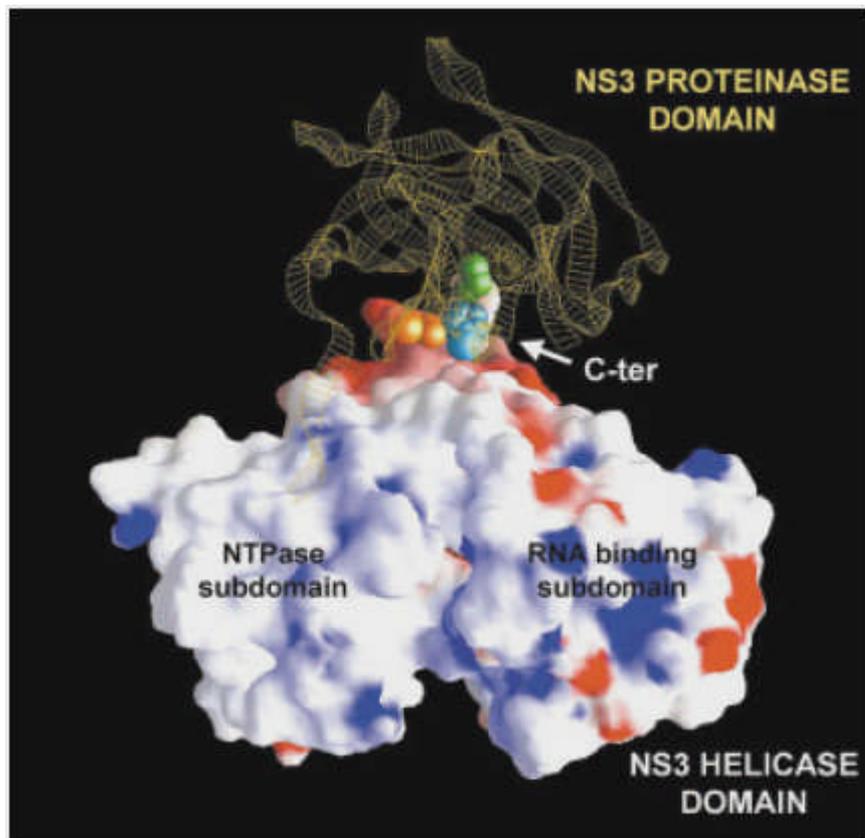
NS2 is a nonglycosylated integral membrane protein rich in hydrophobic amino acids that does not seem to be essential for formation of the replication complex (Lohmann et al., 1999; Blight et al., 2000). The well-known function of NS2 is its participation in proteolytic cleavage at the NS2-NS3 junction of the polyprotein. Most of the NS2 sequence is required for the zinc-dependent proteinase function, which is responsible for the autocatalytic cleavage that separates NS2 from downstream portion of the precursor polyprotein. The autocatalytic cleavage occurs rapidly after translation and involves a conformation-dependent mechanism (Reed et al., 2000). NS2 is a metal-dependent protease, because its activity can be inhibited by EDTA and stimulated by  $ZnCl_2$  (Hijikata et al., 1993; Pieroni et al., 1997). HCV NS2 protein was found to inhibit cell growth and to induce the cell cycle arrest in the S-phase through down-regulation of cyclin A expression, which may be beneficial to HCV viral replication (Yang et al., 2006). HCV NS2 protein was identified as a potent inhibitor of cytokine gene expression suggesting an important role for HCV protease in counteracting host cell antiviral response (Kaukinen et al., 2006). It was suggested that NS2 is a short-lived protein whose degradation by the proteasome is regulated in a phosphorylation-dependent manner through the protein kinase CK2 (Frank et al., 2005). More recently, the role of NS2 protein of HCV in infectious particle production was reported (Yi et al., 2006). By using chimeras' genomes encoding the structural proteins of H77 strain (HCV genotype 1a) within the background of JFH1 strain (HCV genotype 2a), Yi and co workers shown that RNAs encoding polyproteins fused at the NS2/NS3 junction, and at a site of natural, inter-genotypic recombination within NS2 produced infectious virus. Compensatory mutations were observed within E1, P7, NS2 and NS3 genes of produced virions. This finding suggests that interactions between NS2 and E1 and p7, as well as NS2 and NS3, are essential for virus assembly and/or release, and that each of these viral proteins plays an important role in this process.

**Non-structural protein 3 (NS3).**

NS3 is a bifunctional molecule carrying at its N-terminal part a serine protease domain and at the carboxyl-terminal part an NTPase/helicase domain (Fig.6). The NS3 serine protease comprises the 189 N-terminal amino acids of the 70 kDa NS3 protein. It is part of the NS2-NS3 protease responsible for autocatalytic cleavage at the NS2-NS3 site. The NS3 serine protease domain associates with the NS4A cofactor (54 amino acids), stabilizing the protease and activating it to cleave sites 4A/4B, 4B/5A, and 5A/5B. The 3D structure of NS3 serine protease, both free (Love et al., 1996) and complexed with NS4A (Kim et al., 1996; Yan et al., 1998) shows that the central part of NS4A is mandatory for proper NS3 folding, though the formation of a  $\beta$ -strand inserted into the N-terminal  $\beta$ -barrel of NS3 (Penin et al., 2004). The hydrophobic N-terminal part of NS4A appears to form a transmembrane segment required for NS3 targeting and anchoring to the endoplasmic reticulum membrane (Wolk et al., 2000). The structure of the NS3 serine protease domain demonstrated a chymotrypsin-like folding, with two six stranded  $\beta$ -barrel subdomains of identical topology (Penin et al. 2004) that are believed to have arisen from an ancient gene duplication event (Mc Lachlan et al., 1972). The catalytic triad is formed by residues from the same loops of the two  $\beta$ -barrels: His 57 and Asp81 in the N-terminal  $\beta$ -barrel and Ser 139 in the C-terminal  $\beta$ -barrel. Beside its proteinase activity, the NS3 serine proteinase domain contributes to the helicase RNA substrate binding sites (Gallinari et al., 1998; Urvil et al., 1997; Kumar et al., 1997). It was shown that the NS3/4A serine protease of HCV causes specific proteolysis of Toll-IL-1 receptor domain-containing adaptor inducing IFN-beta, an adaptor protein linking TLR3 to kinases responsible for activating IFN regulatory factor 3 (IRF-3) and NF-kappaB, transcription factors controlling a multiplicity of antiviral defenses (Li et al., 2005). Recent data suggest that HCV NS3 serine protease inactivates Cardif, a protein central to innate immunity (Evans et al., 2006). The possibility that NS3 plays an important role in the hepatocarcinogenesis of HCV by interacting differentially with p53 in an NS3 sequence-dependent manner was reported (Deng et al., 2006). Moreover, single-point mutations L106A and F43A within HCV NS3 serine protease were found to impair p53 interaction and anti-apoptotic activity of NS3 protein (Tanaka et al., 2006).

The NS3 helicase-NTPase domain consists of the 442 C-terminal amino acids of NS3. The crystal structure has been determined for this domain, both free (Yao et al., 1997; Cho et al., 1998) and complexed with DNA (Kim et al., 1998), and is representative of superfamily-2 helicase (Kwong et al., 2000). It contains two structurally related subdomains folded with  $\beta$ - $\alpha$ - $\beta$  topology, and a third C-terminal subdomain containing seven  $\alpha$ -helices and three  $\beta$ -strands. The first  $\beta$ - $\alpha$ - $\beta$  subdomain carries the NTPase activity and the second the RNA-binding function. The three domains are separated by deep clefts. The conserved helicase motifs all lie in regions that face the clefts. The NS3 helicase-NTPase domain probably has multiple functions, including RNA-stimulated NTPase activity, RNA binding, and unwinding of RNA regions with extensive secondary structure by coupling unwinding and hydrolysis. This enzyme acts as an ATP-driven motor and is thought to switch between alternative conformations during active unwinding of the double-stranded RNA. Despite abundant structural data obtained by X-ray crystallography, the mechanism of action of the helicase domain and its precise role during the replication are not fully understood (Penin et al., 2004). However, it was recently demonstrated that the RNA-unwinding activity of the HCV NS3 helicase is needed for RNA replication (Lam et al., 2006).

Additionally to its role as multifunctional enzyme, NS3 interacts directly with NS5B (Ishido et al., 1998), and also with NS4B and NS5A via NS4A (Lin et al., 1997) within the replication complex. Numerous NS3 interactions with cellular components, including protein kinases A and C, p53, and histones H2B and H4 also have been reported, but their significance is unclear (Tellinghuisen et al., 2002). Recent data suggest that HCV NS3 protein promotes caspase-8 induced apoptosis at a pathway site distal to FADD, and that flaviviruses NS3 may represent a new class of pro-apoptotic proteins (Prihod'ko et al., 2004).



**Fig. 6. Structure of full-length NS3 protein** (PDB accession code 1CU1). The C-terminus of helicase domain (indicated in white) lies in the active site of the proteinase domain. Adopted from Penin et al.,2004.

**Non-structural proteins 4A (NS4A) and 4B (NS4B).**

NS4A, which is a small protein of 54 amino acid residues, forms a stable complex with the NS3 protein as an essential cofactor of the NS3 proteinase and this complex is required for the efficient processing of NS proteins (Bartenschlager et al., 1994; Failla et al., 1994; Lin et al., 1994; Chung et al., 2000). Stable NS4A-NS3 complex requires the 22 amino acid residues at the N-terminal of the NS3 protein, suggesting that the interaction between NS3 and NS4A is primarily important for the NS4A dependent processing of NS proteins (Failla et al., 1995; Satoh et al., 1995). Recent data suggests that NS4A protein inhibits host and viral translation though interacting with eEF1A, implying a possible mechanism by which NS4A is involved in the pathogenesis and chronic infection of HCV (Kou et al., 2006).

NS4B protein (261 amino acid residues for HCV-1b) is rich in hydrophobic amino acid residues and has been detected primarily in the membrane fraction (Hijikata et al., 1993; Selby et al., 1993). The function of the NS4B protein remains unknown, although it has been recently demonstrated that the NS4B protein in association with the Ha-ras gene played an important role in the malignant transformation of NIH3T3 cells (Park et al., 2000). It was recently found that expression of NS4B induces the formation of a seemingly ER derived membranous web that harbours all HCV structural and non-structural proteins (Egger et al., 2002), as well as replicating viral RNA (Gosert et al., 2003). Thus, one function of NS4B may be to induce a specific membrane alteration that serves as a scaffold for the formation of the HCV replication complex. Recent data suggest the possibility that NS4B and NS4A play an important role in inducing the IL-8 gene expression under certain cellular conditions, which might be one of the strategies to establish persistent HCV infection (Kadoya et al., 2005).

**Non-structural protein 5A (NS5A).**

NS5A is a membrane-associated phosphoprotein, which has been predicted to be predominantly hydrophilic and contains no transmembrane helices.

NS5A protein is composed of an N-terminal amphipathic  $\alpha$ -helix (Brasset et al., 2002; Penin et al., 2004) and three distinct domains separated by the low-complexity sequences (LCS) I and II (Tellinghuisen et al., 2004).

The N-terminal  $\alpha$ -helix is an membrane anchor carrying a tryptophan-rich hydrophobic side embedded in the cytosolic leaflet of the phospholipid bilayer and a fully conserved polar side that define a unique platform probably involved in specific protein-protein interactions essential for the formation of a functional HCV replication complex (Brasset et al., 2002; Penin et al., 2004). Domain I (amino acid 32-213 of NS5A) appear to be involved in RNA binding. Domain II may be involved in inhibition of IFN-induced dsRNA activated protein kinase PKR (Gale et al., 1998). Domain III is only poorly conserved between different genotypes can in part be deleted with only moderate reductions of RNA replication in cell culture and tolerates the insertion of rather large heterologous sequences (Appel et al., 2005, Moradpour et al., 2004).

NS5A is found in a basally phosphorylated form of 56 kDa (p56) and in a hyperphosphorylated form of 58 kDa (p58) with phosphorylation being mediated by unknown cellular kinase(s) at serine, and to a much lesser extent, threonine residues (Bartenschlager et al., 2004). The major phospho- acceptor sites of NS5A appear to be genotype- or isolate-specific. The serine residues S2194 and S2321 have been identified as major phosphorylation sites of NS5A, respectively in an HCV 1b isolate in an HCV1a isolate (Katze et al., 2000; Reed et al, 1999). Hyperphosphorylation of NS5A is dependent on the presence of other non-structural proteins. Thus far the functional relevance of different phospho forms of NS5A is unknown. However, phosphorylation of NS5A is a conserved feature among hepaci- and pestiviruses, and also found with flavivirus NS5, arguing that phosphorylation plays an important role in the HCV life cycle.

The function of NS5A in the HCV replication cycle is unknown. A cluster of mutations, which enhanced the replication of HCV RNA, has been identified in the central region of NS5A in the replicon system (Blight et al., 2000; Bartenschlager et al., 2002). These findings suggested that NS5A is involved in the viral replication process either directly and/or by interaction with cellular proteins and pathways. This together with the modulation of NS5A hyperphosphorylation by the nonstructural proteins 3, 4A and 5B (Koch et al., 1999; Neddermann et al., 1999) supports the view that NS5A is an essential component of the HCV replication complex. However, adaptative mutations found to confer a significant advantage in the replicon system failed to initiate productive infection after inoculation of *in vitro*-transcribed RNA into the liver of chimpanzees (Bukh et al., 2002). During the last few years, NS5A has attracted considerable interest because of its potential role in modulating the response to interferon alpha therapy (Pawlotsky et al., 2000; Katze et al., 2002). Japanese studies reported the existence of a conserved region of 40 amino acids in the center of NS5A in IFN- $\alpha$ -resistant HCV isolates, and HCV variants with mutations within this region appeared to be more sensitive to interferon alpha therapy (Enomoto et al., 1996), suggesting that NS5A played a role in conferring IFN- $\alpha$  resistance, thus this region was termed the interferon sensitivity determining region (ISDR). NS5A has been reported to interfere with the activity of the double-stranded-RNA-activated protein kinase *in vitro* and the interaction of NS5A with PKR required the ISDR and an additional 26 residues C-terminal to PKR (Gale et al., 1998). However, intragenotypic replicon chimera variants harbouring NS5A genes amplified from HCV-1b infected patients, which were responders or non-responders to IFN- $\alpha$  therapy, were more recently used to elucidate the role of NS5A protein in the resistance of HCV to IFN- $\alpha$ . Unfortunately, it was observed that replicon chimeras bearing NS5A of HCV sequences from IFN- $\alpha$  therapy responders as well as from non-responders patients were susceptible to IFN- $\alpha$  treatment, suggesting that NS5A does not contribute to the resistance of HCV subgenomic replicon to IFN- $\alpha$  *in vitro* (Aus dem Siepen et al., 2005). These findings on IFN- $\alpha$  resistance are controversial, and the role of NS5A in the viral resistance to IFN- $\alpha$  remains unclear (Podevin et al., 2001; Ezelle et al., 2001; Pawlotsky et al., 2002). Many other potential functions recently have been attributed to NS5A, including transcriptional activation and involvement in the regulation of cell growth and cellular signalling pathway (Tan et al., 2001; Tellinghuisen et al., 2002). However, the role of NS5A in HCV replication and in the pathogenesis of HCV infection remains to be established.

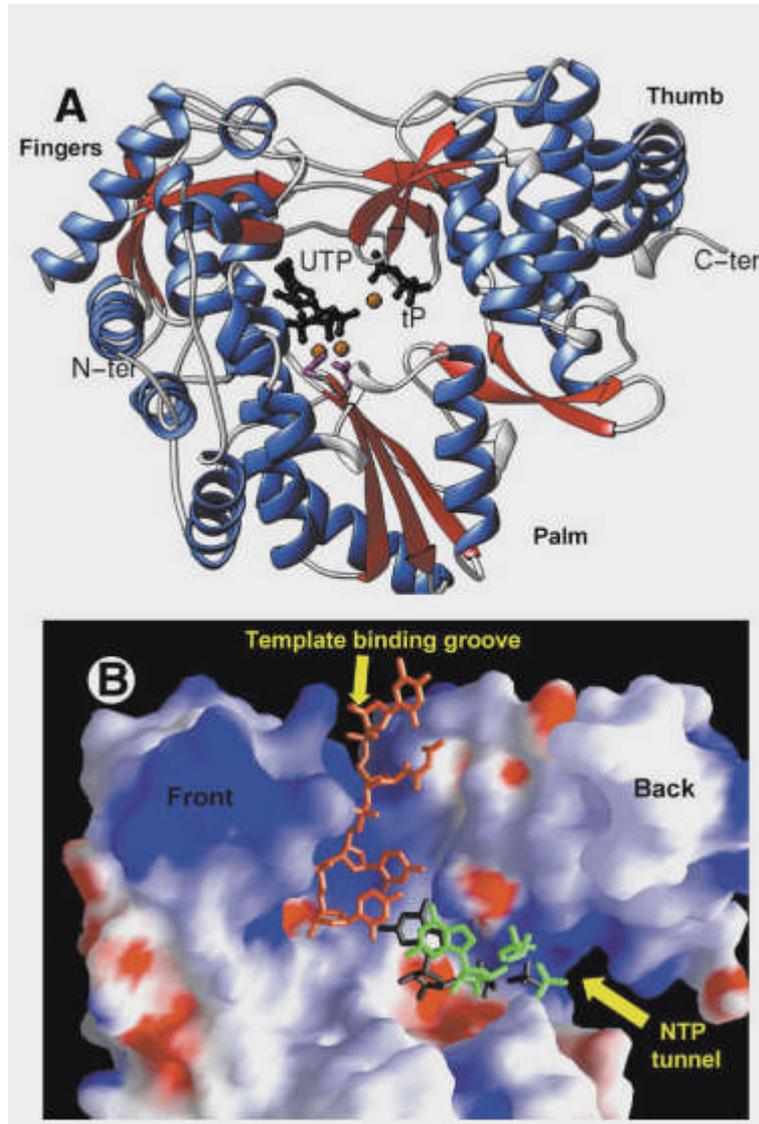
### Non-structural protein 5B (NS5B)

NS5B is the RNA dependent RNA polymerase (RdRp) and, therefore, the catalytic centre of the HCV replication complex. HCV replication proceeds via the synthesis of a complementary minus-strand RNA using the genome molecule as template, and subsequent synthesis of genomic plus –strand RNA from this minus-strand RNA template. RdRp activity of NS5B appears to be modulated by interaction with cyclophilin B as well as by interaction with the viral factors NS3 and NS5A. NS5B protein binds to homopolymeric RNAs with the preference for poly (U). In addition, a specific binding of NS5B to stem loop 5BSL3.2 has been described (Lee et al., 2004). This binding may recruit the enzyme to 3' end of the positive strand RNA for which the cis acting regions 5BSL3.2 and SL2 RNA-RNA interaction may be required.

The HCV RdRp belongs to a class of membrane proteins termed tail-anchored proteins (Schmidt-Mende et al., 2001). The crystal structure of the NS5B catalytic domain revealed a structural fold comparable to other polymerases with palm, finger and thumb subdomain (Fig. 7A). A special feature of NS5B protein is that the finger subdomain contains an extension that interacts with the thumb subdomain and restricts the mobility of one subdomain with respect to the other. The catalytic aspartic acids (Asp 220 and Asp 318) are located in the palm subdomain and chelate two catalytic metal ions that are responsible for the polymerisation reaction. The RNA template binds in a groove that leads directly to the active site and the NTPs access this site through an NTP tunnel (Fig 7B). Binding of the RNA template and initiation of RNA synthesis are supposed to be regulated by a highly flexible  $\beta$ -hairpin loop located in the thumb domain and pointing towards the active site (Lesburg et al., 1999; Zhong et al., 2000). The  $\beta$ -hairpin generates a narrow gate that ensures initiation of RNA synthesis from 3' end of the template. Initiation of RNA synthesis by NS5B has been found to proceed through a *de novo* mechanism in the absence of primer.

Interestingly, an overall structural similarity of NS5B and the RNA dependent RNA polymerase of bacteriophage  $\phi$  6 was found and common model for *de novo* synthesis by these two enzymes has been proposed (Butcher et al., 2001). Another interesting feature of NS5B is a low-affinity GTP binding site located at the interface of the thumb and finger subdomain and residing on the surface of the molecule (Bressanelli et al., 2002).

Binding of GTP to this site may induce a conformational change resulting in efficient initiation of RNA synthesis. The catalytic domain of NS5B protein is membrane associated *via* C-terminal transmembrane domain, which is essential for HCV RNA replication (Moradpour et al., 2004). The structure of NS5B complexed with two different non-nucleoside inhibitors was reported (Bressanelli et al., 2002). These drugs were found to bind at a surface site in the thumb approximately 30 Å from the active site and closely to the allosteric GTP site. Identification of these structures suggests that non-nucleoside inhibitors may act by blocking the enzyme in the initiation mode though inhibition of a conformational change need to proceed with elongation.



**Fig. 7. Crystal structure of the catalytic domain of NS5B, the HCV RNA-dependent RNA polymerase (PDB accession code 1GX6).** (A) Ribbon diagram of the complexed with UTP and Mn<sup>2+</sup>. (B) Superimposition of HCV NS5B with the bacteriophage 6 polymerase, showing the path of the template strand and the NTP tunnel. The thumb domain is omitted from this image for clarity. Adapted from Penin et al., 2004.

## 6. Life cycle of HCV.

### 6.1. Attachment and cell entry of HCV.

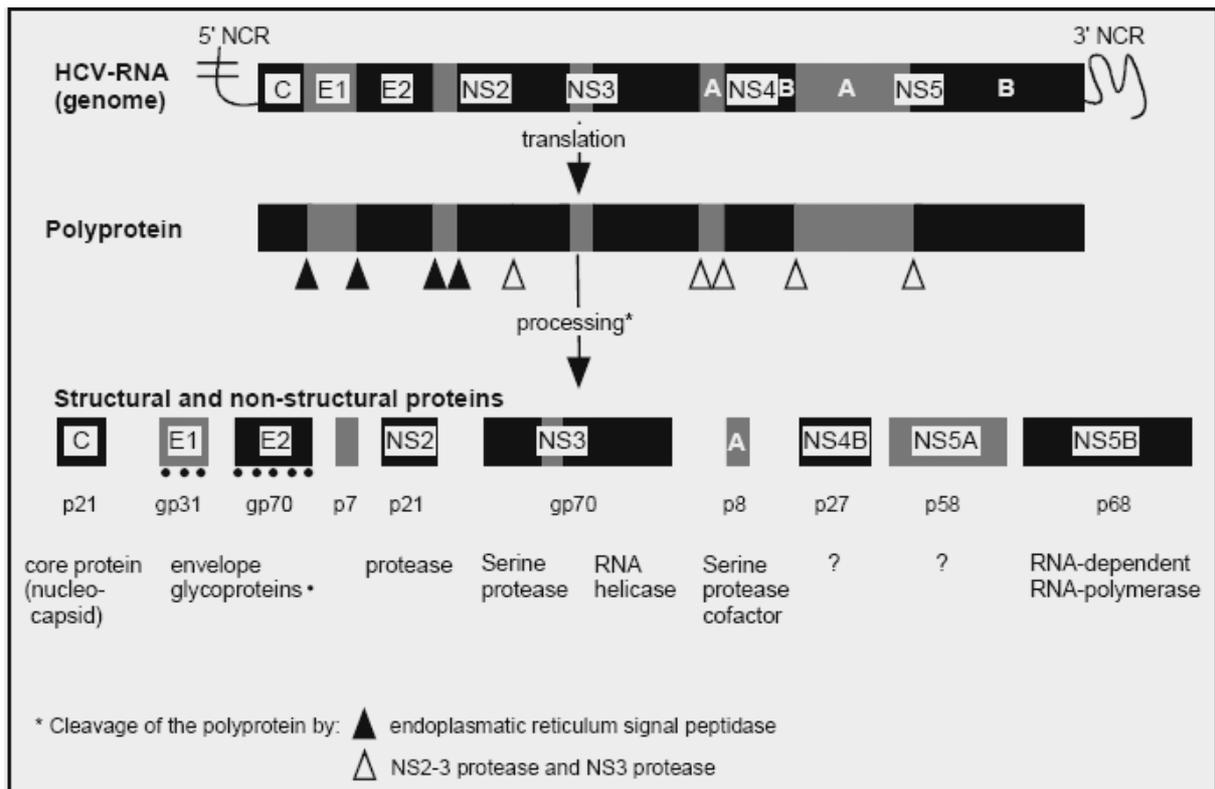
For long time studies of HCV life cycle were hampered by the lack of efficient cell culture systems. Many attempts have been made to identify cell lines that allow efficient infection with HCV and virus production, but these systems suffered from very low virus yield and poor reproducibility. Albeit this limitation, a significant number of model systems have been developed and the route of cell entry as well as capture molecules involved in low-affinity interactions for the initial contact of HCV with target cells and potential high-affinity receptor candidates that may mediate HCV trafficking and fusion has been described (Bartosch et al., 2006).

Soluble E2 was shown to bind specifically to hepatocarcinoma and other cell types, suggesting that the ectodomains of E2 mediates cell attachment (Flint et al., 1999a, 1999b; Heo et al., 2004; Higginbottom et al., 2000; Michalak et al., 1997; Yagnik et al., 2000; Yamada et al., 2005). Infectious HCV pseudoparticles that display E1 and E2 glycoprotein complexes have been generated (Bartosch et al., 2003). Using HCV pseudoparticles it has been demonstrated that HCV uptake is a pH-dependent event, indicating that HCV is internalized in cells via receptor-mediated endocytosis. Once within an endocytic vesicle, HCV requires exposure to low pH in order to fuse with the cellular target membrane. But how and at what stages of its entry HCV needs to bind to cellular receptors with high affinity in order to convert into a fusogenic conformation remain unclear. A considerable number of receptors have been proposed for HCV. Potential receptors include the low-density lipoprotein receptor (Angello et al., 1999; Monazahian et al., 1999), the human tetraspanin CD81 (Pileri et al., 1998), the human scavenger receptor class B type I (SR-BI) (Scarselli et al., 2002), the mannose binding lectins DC SIGN and L SIGN (Gardner et al., 2003; Lozach et al., 2004; Lozach et al 2003; Pohlmann et al., 2003), the asialoglycoprotein receptor (ASGPr) (Saunier et al., 2003), glycosaminoglycans (Barth et al., 2003; Germi et al., 2002), and Claudin-1 von Hahn et al., 2006). Experimental data using HCV pseudoparticles have confirmed functional roles for CD81 and SR-BI in HCV entry, and a requirement for CD81 in cell entry has recently been confirmed with cell culture produced HCV (HCVcc) (Lindenbach et al., 2005; Wakita et al., 2005).

It is important to note that, neither CD81 nor SR-BI has a liver specific expression profile. Curiously, these molecules are expressed on a number of cell lines of hepatic and non-hepatic origin. Because hepatic cell lines expressing CD81 and SR-BI are permissive to HCV pseudoparticles as well as to cell culture produced HCV, while the non-hepatic cell line expressing CD81 and SR-BI are not or only to very low levels, it is tempting to speculate that the missing entry factors for HCV are indeed liver-specific molecules (Bartosch et al., 2003a, 2003b, 2003c, Lindenbach et al., 2005; Zhang et al., 2004).

## **6-2. Polyprotein translation and processing.**

Following entry of virus into the cell, viral RNA is directly translated. Translation of the HCV genome, which lacks a 5' cap, depends on IRES within the 5'UTR. The IRES binds 40S ribosomal subunits directly and avidly, bypassing the need for pre-initiation factors, and inducing an mRNA-bound conformation in the 40S subunit (Spahn et al., 2001). The IRES-40S complex then recruit eukaryotic initiation factor (eIF) 3 and the ternary complex of Met-eIF2-GTP to form a non-canonical 48S intermediate, before a kinetically slow transition to the translationally active 80S complex (Ji et al., 2004; Otto et al., 2004). As has been already mentioned, the translation of the HCV genome produces a large polyprotein of 3010-3011 amino acids, which undergoes cotranslational and post-translationally proteolytic processing in the cytoplasm or in the endoplasmic reticulum of the infected cell to give rise to four structural (core protein, envelope glycoproteins E1 and E2 and small hydrophobic polypeptide p7) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Hijikata et al., 1991) (Fig.8). The structural proteins mature by signal peptidase cleavages between C/E1, E1/E2, and E2/p7. In addition, signal-peptide peptidase releases core from the E1 signal peptide. Within the non-structural region, the p7/NS2 junction is also cleaved by signal peptidase. Further proteolytic processing within the non-structural region occurs through the action of two viral enzymes, the NS2 auto protease, which cleaves at the NS2/3 junction; and the NS3-4A serine protease, which cleaves at all downstream sites (Fig.8).



**Fig. 8. Translation and processing of hepatitis virus polyprotein.** The 9.6 kb plus strand RNA is genome is represented at the top. Internal ribosomal entry site-mediated translation yields a polyprotein precursor of about 3010 amino acids that is processed into the mature structural and non-structural proteins.

### 6.3. Replication of HCV.

Replication of HCV RNA proceeds via synthesis of a complementary minus-strand RNA using the genome as a template and the subsequent synthesis of genomic plus-strand RNA from this minus-strand RNA intermediate (Fig.9).

As in all positive-strand RNA virus, HCV forms a membrane-associated replication complex, composed of viral proteins, replicating RNA, altered cellular membranes and additional host cell factors (Egger et al., 2002; Gosert et al., 2003). This strategy may offer multiple advantages, including: compartmentalization and local concentration of viral products, physical support and organization of the RNA replication complex, tethering of viral RNA during unwinding, supply of lipid constituents important for replication and protection of viral RNA from double-strand RNA-mediated host defences and RNA interference. For HCV, physical interactions among non-structural proteins, for example, between NS5A and NS5B (Shirota et al., 2002), have been described. In addition, the determinants of membrane association of HCV non-structural proteins have been mapped (Schmidt-Mende et al., 2001; Brass et al., 2002). A candidate HCV replication complex, named “membranous web”, recently was identified in tetracyclin-regulated cell lines inducibly expressing the entire HCV polyprotein (Egger et al., 2002). The membranous web harboured all the viral proteins and was very similar to the sponge like inclusions previously revealed by electron microscopy in liver cells of HCV-infected chimpanzees. It was recently shown that the membrane web is indeed the viral replication complex in Huh7 cells harbouring autonomously replicating HCV RNAs (Fig.9) (Gosert et al., 2003).

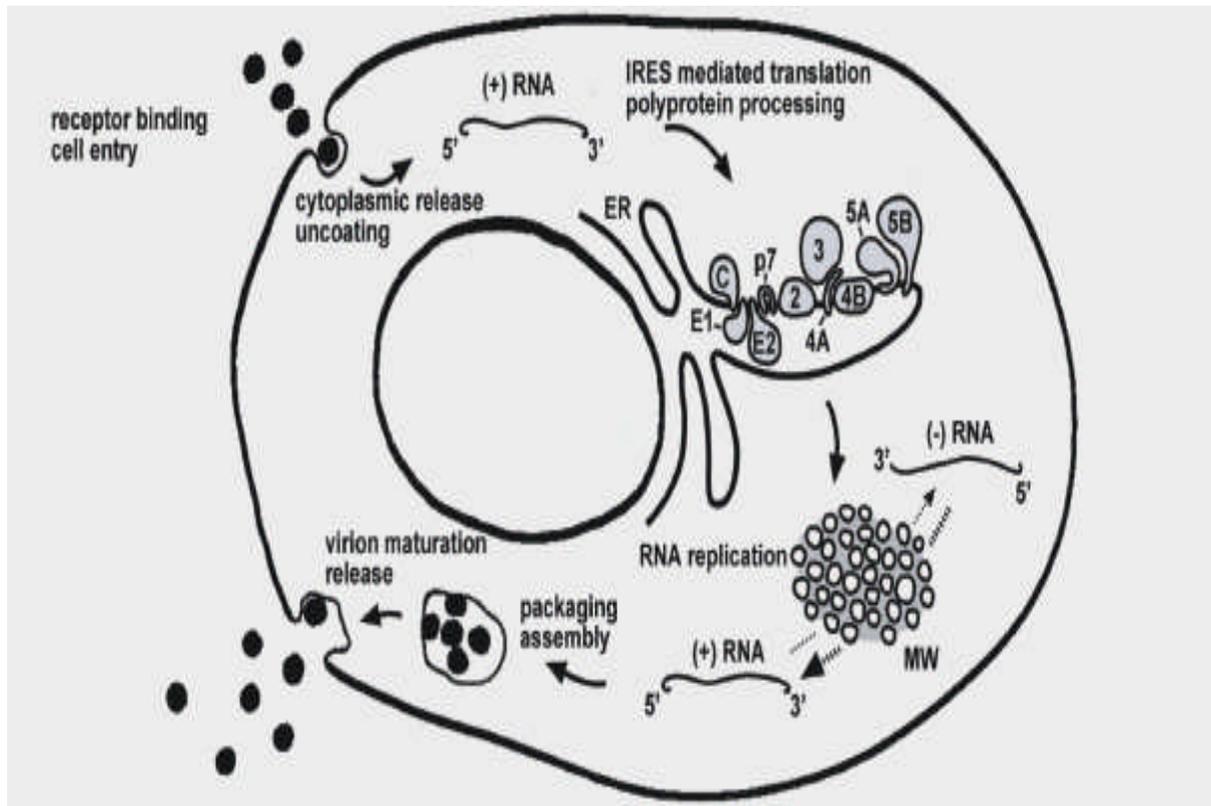
#### 6.4. Assembly and release of HCV virions.

The mature HCV virion is thought to possess a nucleocapsid and outer envelope composed of a lipid membrane and envelope proteins. Virion assembly presumably begins with the interaction of capsid proteins and genomic RNA to form a nucleocapsid. The nucleocapsid acquires an envelope and the mature virion is released from the infected cell.

The mechanism of HCV RNA packaging has not been determined, but data available for other RNA viruses suggests that the packaging reaction specifically incorporates viral RNA into the capsid with the total exclusion of cellular RNAs and negative-strand viral RNA. To achieve this specificity the positive-strand RNA is thought to possess an encapsidation signal, which exhibits a specific binding affinity for the capsid protein.

Enveloped viruses acquire their envelope by the process of budding, either through intracellular membranes or through the plasma membrane. Electron microscopy studies with flaviviruses have shown that nucleocapsid form in the cytoplasm and bud into intracellular vesicles derived from the ER, acquiring envelopes in the process (Hase et al., 1987). The assembled virions are then released from the cells via the exocytosis pathway.

Because HCV glycoprotein complexes are mostly retained in the ER, it is thought that HCV budding may occur in the ER or in ER-like structures (Duvet et al., 1998). In this case the virus may be exported via the constitutive secretory pathway. In agreement with this assumption, complex N-linked glycans were found on the surface of partially purified virus particles, suggesting virus transit through the Golgi (Sato et al., 1993). However, since HCV particles tend to associate with cellular components it remains to be determined whether these glycans are present on the E proteins or on the cellular proteins associated with HCV particles.



**Fig. 9. Life cycle of HCV.** The steps of the viral life cycle are depicted schematically. The topology of HCV structural and non-structural proteins at the endoplasmic reticulum (ER) membrane is shown. HCV RNA replication occurs in a specific membrane alteration, the membranous web (MW). IRES-mediated translation and polyprotein processing as well as membranous web formation and replication, illustrated here as separate steps for simplify, may occur in a tightly coupled manner. Adapted from Volker Brass et al., 2006.

## 7. Model systems to study HCV replication.

### 7.1. In vivo models.

The only animal that can be infected with HCV reproducibly is the chimpanzee, but its use is limited by ethical reasons, its scarcity and high maintenance costs. In an attempt to establish a small animal model Xie et al., (1998) experimentally inoculated tupaia (*T. belangeri chinensis*), a species shown to be susceptible to infection with hepatitis B virus (Walter et al., 1996; Yan et al. 1996). However, only about one-quarter of the animals became infected with HCV and developed either transient or intermittent viraemia with rather low titres.

Recently, an *in vivo* model of HCV infection was developed involving a SCID mouse carrying a urokinase plasminogen activator (uPA) transgene under the control of the albumin promoter (Mercer et al., 2001). Expression of the uPA transgene in the mouse liver causes a gradual depletion of the mouse hepatocytes. Transplantation of normal human hepatocytes into these SCID-beige/Alb-uPA mice results in animals with chimeric human livers, which can then be infected with HCV. This model provides a unique opportunity to study virtually all aspects of the HCV life cycle, including viral entry, replication, and viral kinetics. The chimeric SCID/uPA mouse model also provides a unique opportunity to study HCV-infected animals, which have been transplanted with hepatocytes from different donors, facilitating the analysis of host-specific responses to HCV. However generation of such chimeric animals is laborious and requires special expertise to isolate and transplant human hepatocytes and maintain colony of fragile immunodeficient mice with an approximately 35% mortality in new-borns due to a defect in blood coagulation.

## 7.2. In Vitro model Systems.

### Cell culture Systems

Study of the viral life cycle requires a robust in vitro cell culture system. A number of reports have described the propagation of HCV particles in tissue culture, including one in which the propagated virus was subsequently shown to be infectious in chimpanzees (Shimizu et al., 1998). For reasons that are not evident, infection of primary hepatocytes and established cell lines with HCV have not only produced poor viral replication and low viral yields but have also suffered from poor reproducibility. There are several reports suggesting that HCV can also replicate in extra hepatic cells, in particular, in peripheral blood mononuclear cells (PBMC) (Cribier et al., 1995; Lerat et al., 1996). Recently, the in vivo HCV-infected B-cell lines directly established from chronically HCV-infected patients were described (Sung et al., 2003). At least one of these B-cell lines persistently produced infectious virions, which could establish secondary infection in primary human hepatocytes and lymphocytes in vitro. These findings, if confirmed in independent laboratories, would provide evidence that HCV infects B-cells during the course of natural infection.

Since primary hepatocytes are difficult to grow in cultures, numerous attempts to infect immortalized hepatocytes and hepatoma cell lines have been undertaken in the last years. For example, a nontumorigenic, immortalized human hepatocytes cell line, PH5CH was used to assess the infectivity of HCV positive sera (Ikeda et al., 1997; Ikeda et al., 1998). During the first twelve days of culture, the level of HCV positive-strand RNA increased and the viral RNA remained detectable for at least thirty days of cell cultivation. During cell incubation a strong selection for HCV variants with a particular sequence of HVR-1 of the E2 protein was observed suggesting that only certain variants can bind to or replicate in these cells. Thus, in vitro cell culture models, at least in some cases, can be used to demonstrate the infectivity of the virus but in general they are not suitable to study the viral life cycle due to very low levels of viral replication (detectable only by PCR), which is usually transient.

### **Transfection of cell line with viral cDNA.**

Since infection of cell cultures with HCV-containing sera did not provide reproducible results, several groups tried to develop the HCV cell culture system using transfection of cells with cloned HCV genome molecules (Yoo et al., 1995; Dash et al., 1997). Transfected cell lines, in which the viral RNA was expressed from chromosomally integrated viral cDNA under the control of constitutive or inducible promoters, were already described for several plus-strand RNA viruses (Boyer and Haenni, 1994). Application of this approach to HCV led to the establishment of a stable HCV replication in cultures of Huh7 or HepG2 cells (Yoo et al., 1995; Dash et al., 1997). For about sixty days post-transfection both positive and negative-strands of HCV RNA were detectable by RT-PCR in the transfected cells. Expression of viral core and non-structural proteins was recorded in the cytoplasm of transfected cells by immunostaining. Culture supernatants of the HepG2 transfected cells were infectious for Daudi lymphoma cells for three passages tested. It should be noted, however, that other laboratories did not confirm these experiments and it is not clear if the authors did observe the HCV replication or dealt with the transcription of HCV RNA from the integrated cDNA.

### **Subgenomic replicon**

Since several groups failed to demonstrate a replication of a full-length genome in transfected cell lines, an alternative strategy based on the construction of the bicistronic subgenomic selectable HCV RNAs replicon was suggested (Lohmann et al., 1999). A consensus HCV genotype 1b genome strain Con1 cloned from a liver of a patient with chronic hepatitis was the source for HCV sequence. To construct the selectable replicon, the structural genes of HCV were deleted and replaced by the gene encoding the neomycin phosphotransferase (*neo*) followed by the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV) (Fig.9). This element was required to allow translation of the HCV coding sequence. Two different versions of the replicon were generated, harbouring the NS2-5B or the NS3-5B coding region of the HCV genome. The replicon sequences were positioned downstream of a T7 RNA polymerase promoter and a unique restriction site was engineered at 3' end to allow synthesis of run off transcripts with authentic 5' and 3' termini. Upon transfection of cells and

Subsequent selection with neomycin (G418), non-transfected cells and cells in which the replicon RNA does not replicate will die. Only cells harbouring a high level of replicating HCV RNA will amplify both the HCV sequences and the *neo* gene and therefore will develop G418 resistance. The cells will grow under selection conditions and form colonies (Fig.10).

It was shown that a series of adaptive mutations arose during propagation of the subgenomic replicon-containing cells and that a large fraction of these mutations conferred greatly increased cloning efficiency of the replicon in Huh7 cells (Blight et al., 2000; Lohmann et al., 2001). Such adaptive mutations usually cluster in certain regions, such as the central region of non-structural protein 5A (NS5A), the C-terminal portion of the NS3 serine protease and the N-terminal portion of the NS3 RNA helicase domains as well as at two positions in NS4B. In Con1-based replicon for example, substitution of arginine for glycine at amino acid position 2884 of the HCV polyprotein was found to work as a highly adaptive mutation that increases colony formation efficiency of the replicon by several orders of magnitude (Lohmann et al., 2001). Additionally, combination of two amino acids substitutions located in NS3 (E1202G and T1280I) with one amino acid substitution located in NS4B (K1846T) of Con1-based replicon increases drastically replication level of the replicon, allowing monitoring of HCV RNA replication in a transient replication assay using a luciferase reporter gene. Furthermore, substitution of serine with isoleucine at amino acid position 2204 of the HCV polyprotein was found to work as a highly adaptive mutation, which was sufficient to increase drastically replication level of Con1-based replicon by several orders of magnitude (Blight 2000).

However, some mutations are clearly incompatible with each other, leading to defective replicon, when they were combined (Lohmann et al., 2001).

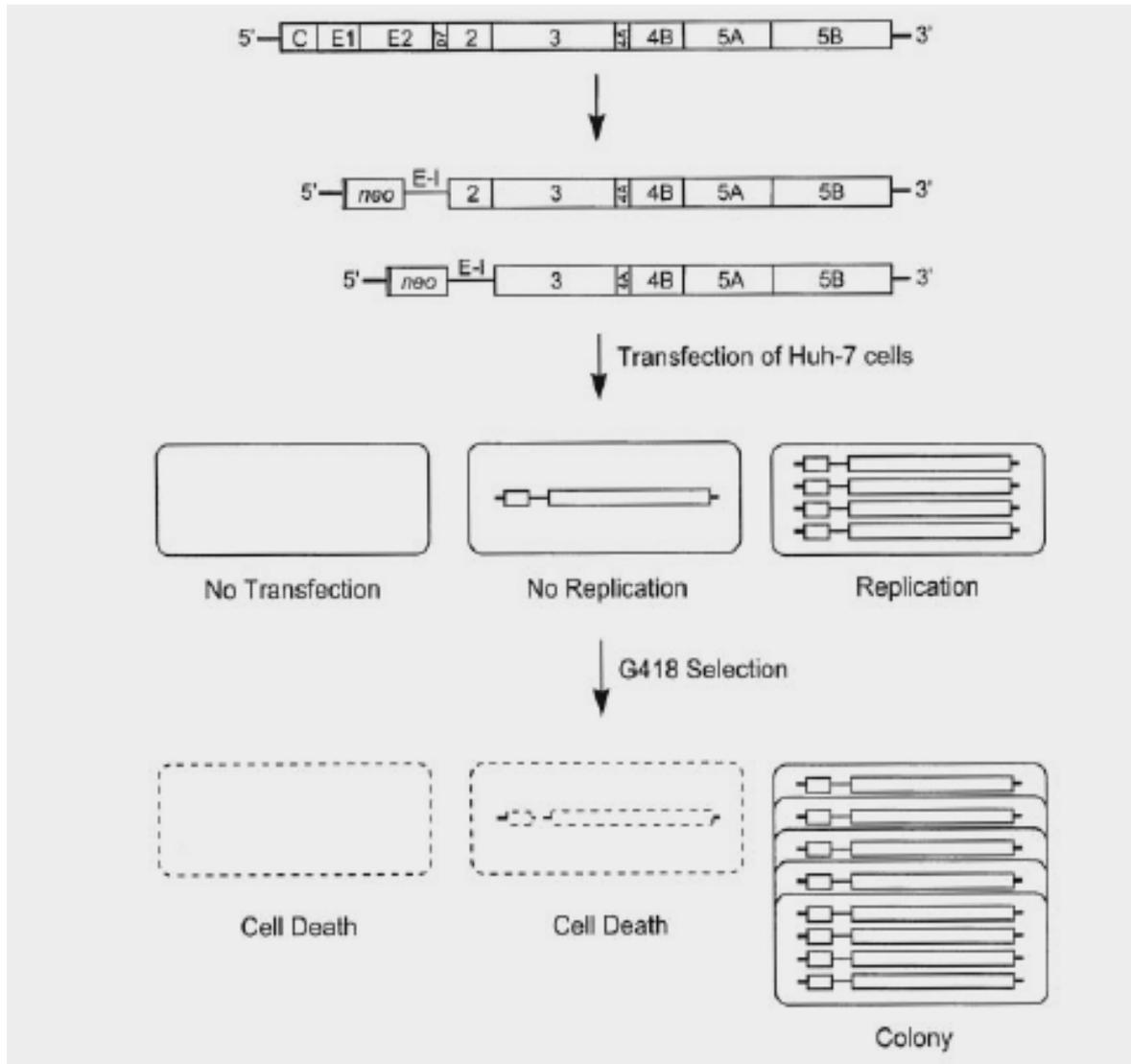
The efficiency of replicon RNA amplification was found to be determined by selection for particularly permissive cells within a given population of Huh7 cells (Lohmann et al., 2003; Blight et al., 2002), and even not all clones of Huh7 cells are able to support replication of HCV RNA. Treatment of a Huh7 cells bearing HCV replicon with interferon alpha or inhibitors of polymerase often results in cell lines that support higher levels of HCV RNA replication as compared with the original naïve Huh7 cells. Despite Huh7 cells, the spectrum of permissive host cells for HCV RNA replication has been expanded. Zhu and co workers for example reported the successful propagation of HCV replicon in Hela cells and the mouse hepatoma cell line Hepa1-6 (Zhu et al., 2003). Moreover, replications of HCV RNA in other

cell lines including HepG2 cells, IMY-N9 cells, Kidney 293 cells and Huh6 were also reported (Ali et al., 2003; Date et al., 2004; Kato et al., 2005; Windisch et al., 2005). One should mention, however, that these data need to be confirmed and that majority of laboratories still work with permissive clones of Huh7 cells. Although the mechanism governing the high permissiveness of cell lines supporting efficient replication of HCV RNA remains unclear, a single point mutation in the double-strand RNA sensor retionic acid-inducible gene I (RIG-I) has been postulated as a determinant of a high permissiveness for HCV RNA replication observed with an interferon alpha-cured Huh7-derived cell line designated Huh7.5 (Sumper et al., 2005).

Since the original reports of functional genotype 1b replicons (Lohmann et al., 1999; Grobler et al. 2003), replicons for genotype 1a (Blight et al., 2003; Liang et al., 2005) and for genotype 2a (Kato et al., 2003) as well as derivatives expressing quantifiable enzymes other than neomycin phosphotransferease such as luciferase and  $\beta$ -lactamase have been developed (Friebe et al., 2001; Murray et al., 2002). Additionally, subgenomic and genomic intergenotypic replicon chimera bearing sequences of 1a and 1b isolates (Gu et al., 2003; Lemm et al., 2005) or 1b and 3a isolates (Lanford et al., 2006) or 1b and 2a isolates (Piettschmann et al., 2005) were also reported. In table1, the functional subgenomic and genomic replicons of HCV reported to date are listed. The development of these replicons systems has facilitated genetic studies as well as drug screening and evaluation (Krieger et al., 2001; Murray et al., 2003). Moreover, the replicon system was used to investigate in vitro the effect of interferon- $\alpha$ ,  $\beta$ , and  $\gamma$  on the replication of HCV RNA (Frese et al., 2001; Guo et al., 2001; Lanford et al., 2002; Dash et al., 2005). These results suggest that the replicon system faithfully mimics at least some aspects of HCV replication in vitro. It is apparent that the absence of the structural proteins, and the ability to couple replication with virus particle assembly, is not required for efficient RNA replication at least in vitro.

**Table1.** HCV subgenomic and genomic replicons.

HCV strains	Genotype	Dicistronic subgenomic replicon	Dicistronic full-length replicon	Ability for a vigorous production of infectious progeny in cell culture	Permissive cell lines	Publications
Con1	1b	+	+	-	Huh7 cells	Lohmann et al., 1999; Pietschmann et al., 2002
H77	1a	+	+	+	Huh7 cells	Blight et al., 2003; Kyung et al., 2006
BK	1b	+	-	-	Huh7 cells	Grobler et al., 2003
O	1b	+	+	-	Huh7 cells	Ikeda et al., 2005
N	1b	+	+	-	Huh7 cells	Ikeda et al., 2002
M1LE	1b	+	-	-	Huh7 cells	Kishine et al., 2002
JFH-1	2a	+	+	+	Huh7 cells, Hela cells, 293 cells	Kato et al., 2003, 2005; Wakita et al., 2005



**Fig. 10. Schematic representation of the method used to establish HCV-replicon containing cell lines.** The structure of the HCV genome is given at the top. The subgenomic RNAs derived form, composed of the HCV 5'UTR plus a small fragment of the core-coding region (thin box), the *neo* gene, the encephalomyocarditis virus IRES (E-I), HCV NS2-5B or NS3-5B and the 3'UTR, are drawn below. Since core-coding sequence are requires for full IRES activity, the ~20 amino-terminal residues of the core protein are fused to the amino terminus of the neomycin phosphotransferase. Upon transfection of Huh7 cells, only those supporting replication of the HCV RNAs amplify the *neo* gene and develop resistance against the drug G418. Therefore, only these cells will form colonies, whereas untransfected cells and cells that do not support replication of these RNAs will be eliminated during selection. Adapted from Bartenschlager and Lohmann; 2000.

**Full-length, genomic HCV replicon.**

The replicon system has allowed genetic dissection of HCV RNA elements and proteins, provided material for biochemical and ultra structural characterization of the viral replication complex, and facilitated drug discovery efforts as well as the investigation of antiviral resistance. Despite these advances, the important questions about the production of the infectious viral particle, pathway of the virus entry, and the assembly of viral structural proteins and RNA into new virus particles still remain unanswered. Although dicistronic genome-length HCV RNAs harbouring adaptive mutations replicated efficiently in Huh7 cells and expressed the structural proteins, infectious particles were not released into the culture medium (Blight et al., 2002; Pietschmann et al., 2002, Ikeda et al., 2005). It was suggested that either the Huh7 cell line lacked some factors critical for particle formation and release or that the adaptive mutations required for efficient replication in tissue culture interfered with packaging, assembly or release of virus. The last hypothesis was supported by observations that mutations adaptive for Con1 genomic RNA in vitro (in Huh7 cells) significantly reduced the infectivity of this RNA in chimpanzees (Bukh et al., 2002).

Recently, a HCV genotype 2a subgenomic replicon, derived from a patient with fulminant hepatitis C, and designed as JFH-1 (Japanese fulminant hepatitis-1), was found to be able to replicate efficiently in Huh7 and in non-hepatic cell lines without requirement for adaptive mutations. Based on these observations, Wakita and colleagues transfected in vitro transcribed full-length JFH-1 RNA into Huh7 cells and found that this genome not only replicated efficiently but also produced virus that was infectious for naïve Huh7 cells (Wakita et al., 2005). Virus particles recovered from the culture medium had a density of about 1.15-1.17 g/ml and a spherical morphology with an average diameter of about 55 nm. Virions produced in this system were also found to be infectious when intravenously inoculated into a chimpanzee. Similarly, another group succeeded in establishing a highly efficient replication and virus production system by constructing chimeras comprising the core to E2 region from the genotype 2a clone J6 and the non-structural region from the JFH-1 clone (Lindenbach et al., 2005). Virus particles produced in tissue culture could be inhibited efficiently by interferon alpha as well as specific HCV protease and polymerase inhibitors, demonstrating the suitability of this system for antiviral testing.

Taking advantage of the JFH-1 genome and a highly permissive Huh7.5.1, Zhong and colleagues demonstrated that infectious particles were released with high efficiency following transfection with the full-length JFH-1 RNA (Zhong et al., 2005). Infectivity titers observed were in the range of  $10^4$  to  $10^5$  infectious units per millilitre, which was approximately 50-fold higher as compared to a previous study that also used the same JFH-1 clone but naïve Huh7 cells (Wakita et al., 2005). Virus spread efficiently throughout the culture and could be serially passaged without loss of infectivity. Thus, the JFH-1 replicon can support efficient production of infectious HCV in cell culture. It is not yet clear why this particular genome is capable of replicating without adaptive mutations, or how adaptive mutations preclude infectious particles production. More recently, production of infectious HCV in three-dimensional culture systems, but not in monolayer cultures, of Huh7 cells carrying the genome-length dicistronic viral RNA of genotype 1b (Con1) was reported (Murakami et al., 2006). Analysis of the culture fluid by a 10-60% (wt/vol) sucrose density gradient centrifugation have shown that HCV RNA and core protein were predominately detected in the 1.15-1.20 g/ml fractions with a maximal detection in the 1.18 g/ml fraction. Transmission electron microscopy of the 1.18 g/ml fraction revealed presence of particulate structures with diameters of 30-60 nm and a major particle size of 50 nm. Viral particles produced in these 3-D culture systems were shown to be infectious; their infectivity could be neutralized by monoclonal antibody directed against E2 protein. However the use of 3-D cell culture system is limited because the system is not accessible for all laboratories.

So far, a robust production of infectious virus progeny in cell culture and the spread of the virions are restricted to a particular Huh7 cell clone, namely Huh7.5.1 cell clone. To extend the studies on HCV replication and interaction with the cell host, additional cell lines, that can support replication of HCV RNA and robust production of infectious viral particles, are urgently needed.

## Conclusion

During the last 10 years a large bulk of information on biology of HCV and characteristics of its infection has been collected. Despite these new data many aspects of HCV replication and interaction of the virus with permissive cells remain unknown or unclear, and that significantly hampered our efforts to develop new prophylactic and therapeutic approaches to control HCV infection. One of the major drawbacks is the limitation of our knowledge of the mechanisms of HCV replication and of virus interaction with the infected cells. Even considering the breakthrough results obtained in recent years with HCV replicon system, essential details of these processes are still unknown, and require development and application of new experimental models and approaches. Thus, a very short range of available HCV replicon variants significantly limited our attempts to identify the crucial replication steps and to study the peculiarities of interaction of viral and cellular proteins in a replicative complex. Relatively small number of HCV replicons, including the chimeric ones, also hampered the studies of one of the most important aspects of HCV research, namely, of the mechanisms of HCV resistance to IFN- $\alpha$  in vivo. Despite a number of reports on sensitivity of several different cell lines to HCV, so far, only one line of human hepatocellular cells - Huh7 cells - are able to support a robust replication of both subgenomic and genomic HCV replicons. The reasons for that are not completely clear and search for new cell models for HCV replication remains an important avenue of HCV research. All these and many other intriguing aspects of HCV research may be studied using a relatively new approach, based on the development of chimeric HCV replicons, in which one or several genome fragments of one HCV isolate/strain are inserted into the backbone replicon sequence belonging to another HCV strain. Use of such chimeric replicons, however, is associated with an uncertainty, caused by the fact that in these chimeric replicons the sequence fragments are taken from unrelated HCV isolates or strains. Thus, for example, different biological potentials of a series of chimeric replicons, in which one virus gene is obtained from different unrelated HCV isolates, might be associated not with differences in activity or function of corresponding gene product *per se*, but with a distorted interaction of this protein with other proteins of the replicative complex, which are encoded by the “backbone” sequence. In order to avoid this restrictive factor, one would need to have a number of closely related HCV isolates in order to create a replicon, which subsequently could be used as a backbone for insertion of different genome fragments from these closely related virus isolates. Such HCV isolates are not easily

available. In 1978, several thousands women in Germany were infected with a single isolate of HCV genotype 1b in single-source outbreak caused by treatment with virus-contaminated anti-D immunoglobulin (Dittmann et al., 1991). The HCV isolate and infected patients were designated as HCV AD78 isolate and AD78 patients, respectively. Infection of different individuals with this virus strain led to evolution of virus genome sequences and, most probably, to appearance of isolates with different biological properties. Thus, for example, patients from the AD78 cohort responded differently to the therapy with IFN- $\alpha$ . HCV isolates from these IFN- $\alpha$ -responder and non-responder AD78 patients might be used in the context of HCV AD78-based replicon to study possible contribution of different structural and non-structural viral proteins to mechanisms of HCV resistance to IFN- $\alpha$ . This example, demonstrates that chimeric AD78-replicon might represent a unique system for studies of different aspects of HCV replication and evolution.

## **The goal and the aims of the study**

The major goal of the current study was to establish a new experimental tool, which should allow to study in more details several very important topics of HCV research. These topics include:

- Characteristics of HCV evolution.
- Significance of a variability of B- and T-cell viral epitopes for HCV replication.
- Dynamic and characteristics of antiviral humoral immune response.
- Mechanisms of HCV replication and virus interaction with permissive cells.

To reach this goal the following aims were formulated:

- Establishment of HCV AD78-based subgenomic replicon system and characterization of cell lines bearing the subgenomic replicon.
- Generation of HCV AD78-based full-length, genomic replicon system.
- Establishment of additional cell lines supporting replication of HCV RNA in vitro.
- Analysis of the influence of different mutations, including the CTL-escape mutations, on HCV replication.

As a result of this study, both subgenomic and genomic replicons based on the HCV AD78 sequence will be created. Generation of these replicons, as well as the availability of serial blood samples from a cohort of AD78-infected women, would allow to implement a number of new experimental approaches and receive the answers to several very important questions dealing with HCV evolution and role of humoral and cellular immune responses in virus elimination and resolution of HCV infection. Thus, it would become possible to create the AD78-specific cell targets for assessment of T-cell-mediated immune responses. The developed AD78-based replicons could be used to study the evolution of T-cell epitopes and significance of their variability for HCV replicative capacity. Finally, a full-length, genomic replicon may be used for establishment of AD78-specific system for virus-neutralization assessment and quantification. That should allow to analyze the evolution of B-cell epitopes and to identify possible B-cell escape variants. All these important topics are directly related to the major avenue of HCV research – development of prophylactic and therapeutic HCV vaccine.

## Materials

### 1. Equipment

#### Apparatus

Automatic incubator CO<sub>2</sub>

Heraeus Cytoperm 2

Bioimaging System (Gene genius)

Captair filetersystem

Centrifuge 3 LR

Centrifuge 5804

Centrifuge 5415 D

Elisa Reader A Sys Expert Plus

Gene Pulser II

Hybridization Oven

Horizontal Electrophoresis  
system (Mupid 21)

Inverted Microscope Carl Zeiss

Inverted Microscope TMS Nikon

Laminar Flow HSP 12

Light Cycler PCR system

#### Supplier

Kendro GmbH (Hanau)

Syngene (USA)

Captair GmbH (Düsseldorf)

Kendro GmbH (Hanau)

Eppendorf (Hamburg)

Eppendorf (Hamburg)

Asys Hiteck GmbH (Austria)

Bio Rad Laboratories GmbH (München)

Amersham Pharmacia Biotech GmbH (Freiburg)

Eurogentec (Belgium)

Zeiss (Göttingen)

Nikon (Düsseldorf)

Kendro GmbH (Hanau)

Roche Diagnostics GmbH (Mannheim)

Microplate Scintillation and Luminescence counter	Packard (USA)
Photometer Gene Quant	Amersham Biosciences Europe GmbH (Freiburg)
pH-Meter MP 220	Toledo(USA)
Thermocycler Gene Amp PCR system 2400	Roche Diagnostic system( Mannheim)
UV transluminator FLX-20M	MWG-Bio Tech(Ebersberg)
Vacuum Blotter	BioRad Laboratories GmbH(München)
Vertical Eltrophoresis system	Bio Rad Laboratories GmbH(München)

## **2. Chemicals**

<b>Products</b>	<b>Supplier</b>
Agar	Becton Dickinson GmbH (Heidelberg)
Agarose DNAses/RNAses free	SIGMA (Deisenhofen)
Agarose	Eurogentec (Belgium)
Ammonium Persulfate	Bio Rad Laboratories GmbH (München)
Ampicillin	ROTH GmbH (Karlsruhe)
Aqua-Roti-Phenol	ROTH GmbH (Karlsruhe)
BromophenolBlue	SIGMA Aldrich Chemie GmbH (Steinheim)
Crystal violet	SIGMA Aldrich Chemie GmbH (Steinheim)

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Chloroform	J.T. Baker (Hohenbrunn)
Dimethylsulfoxid	ROTH GmbH (Karlsruhe)
DMEM	PAA Laboratories (Celle)
DEPC	SIGMA (Deisenhofen)
0.5 M EDTA pH8.0	Ambion (UK)
Ethidiumbromid	ROTH GmbH (Karlsruhe)
Ethanol	ROTH GmbH (Karlsruhe)
Formaldehyde Loading dye	Ambion (UK)
Geneticin (G418)	PAA Laboratories (Celle)
Glycerin	SIGMA (Deisenhofen)
HEPES	PAA Laboratories (Celle)
Recombinant Interferon alpha	SIGMA (Deisenhofen)
Interferon Gamma	Roche (Mannheim)
Isopropanol	ROTH GmbH (Karlsruhe)
JT3*	Institute for Virology (Heidelberg)*
L-Glutamine	PAA Laboratories (Celle)
MTT	SIGMA (Deisenhofen)
Penicillin/streptomycin	PAA Laboratories GmbH (Celle)

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Sodium dodecyl sulfate (SDS)	Appli Chem (Darmstadt)
Sodium Hydroxide	ROTH GmbH (Karlsruhe)
Tris-Borate-EDTA (TBE)	Invitrogen GmbH (Karlsruhe)
3M Sodium Acetate pH5.5	Ambion ( UK)
Tris Base	Merck KGaA (Darmstadt)
Triton-X100	SIGMA Aldrich Chemie GmbH ( Steinheim)
TRIzol <sup>R</sup>	Invitrogen GmbH (Karlsruhe)
TMED	SIGMA Aldrich Chemie GmbH( Steinheim)
Trypsin/EDTA	Gibco BRL (Karlsruhe)
Trypan Blue	Gibco BRL (Karlsruhe)
Trypton	Becton Dickinson GmbH (Heidelberg)
Tween 20	SIGMA (Deisenhofen)
X-Gal	Bio Solve LTD (Netherland)

\*The inhibitor for HCV polymerase JT3 was kindly provided by the laboratory of Prof. Bartenschlager from the Institute of Virology of Heidelberg University.

### 3. Commercial Kits

<b>Designation</b>	<b>Supplier</b>
Expand Long System Template PCR System	Roche Diagnostics GmbH (Mannheim)
High Speed Midi Plasmid Kit	QIAGEN GmbH (Hilden)
Light Cycler RNA Amplification Kit (Hybridization probes)	Roche Diagnostics GmbH (Mannheim)
Luminescence Reporter gene assay (Luciferase)	Perkin Elmers. Applied Biosystem GmbH (Weiterstadt)
Nuc Away	Ambion (UK)
MAXiscript™ T3	Ambion (UK)
MEGAscript™ T7	Ambion (UK)
Mini elute reaction Cleanup Kit	QIAGEN GmbH (Hilden)
QIA Plasmid Mini Kit	QIAGEN GmbH (Hilden)
Quick change XL-Mutagenesis Kit	Stratagene GmbH (Heidelberg)
QIA amp Viral RNA Mini Kit	QIAGEN GmbH (Hilden)
QIA Quick Gel extraction Kit	QIAGEN GmbH (Hilden)
Thermoscript Reverse transcriptase	Invitrogen GmbH (Karlsruhe)
Topo TA cloning Kit	Invitrogen GmbH (Karlsruhe)

**4. Isotopes**

[ $\alpha$ -<sup>32</sup>P] –UTP 9.25 MBq, 250 uCi, 3000 CC/Mmol; Amersham Biosciences (UK).

**5. Plastic Products**

-Cell culture Flask; Greiner Bio-One GmbH (Frickenhausen).

-Falcon tube 50 ml and 15 ml Becton Dickinson (Heidelberg).

-2 ml, 1.5 ml and 0.2 ml tubes; Eppendorf AG (Hamburg).

- Electroporation cuvette (4 mm gap width); Bio-Rad (Germany)

**6. Membranes**

-Hybond N+ positively charged nylon; Amersham Biosciences Europe GmbH (Freiburg).

-Hybond-C nitrocellulose; Amersham Biosciences Europe GmbH (Freiburg).

## 7. Endonucleases and ligases

<b>Enzymes</b>	<b>Supplier</b>
AgeI	New England Biolabs GmbH (Frankfurt)
AatII	New England Biolabs GmbH (Frankfurt)
BglII	New England Biolabs GmbH (Frankfurt)
BsiWI	New England Biolabs GmbH (Frankfurt)
BsrGI	New England Biolabs GmbH (Frankfurt)
BspI68 (NruI)	Fermentas GmbH (Leon-Rot)
CiP	New England Biolabs GmbH (Frankfurt)
EcoRI	New England Biolabs GmbH (Frankfurt)
FseI	New England Biolabs GmbH (Frankfurt)
NheI	New England Biolabs GmbH (Frankfurt)
PmeI	New England Biolabs GmbH (Frankfurt)
PvuI	New England Biolabs GmbH (Frankfurt)
SalI	New England Biolabs GmbH (Frankfurt)
ScaI	New England Biolabs GmbH (Frankfurt)
SspI	New England Biolabs GmbH (Frankfurt)
T4 DNA Ligase	Fermentas GmbH (Leon-Rot)

## 8. Oligonucleotides

All the oligonucleotides used in this work have been synthesized by Biomers net GmbH (Ulm). In the following list are mentioned only primers used for the amplification of different HCV genome's regions.

Primer designation	Sequences	Targeted genome region
A3' -45	5' -GGA CCT TTC ACA GCT AGC CGT GAC TAG GG-3'	3'UTR
A3' -58	5' -TCA TGC GGC TCA CGG ACC TTT CAC AGC TAG-3'	3'UTR
HVC16As	5' -CCG ACG TTG AGT CGT ACT CCT CCA TGC CC-3'	NS3
HCV 1170s	5' -CGC CTT CTT GAC GAG TTC TTC TG-3'	IRES of EMCV
HCV 3337s	5' -GCC TRC CCG TCT CCG CCC G-3'	NS3
HCV 3425s	5' -CAT CAC GGC CTA CTC YCR AC-3'	NS3
HCV 5511As	5' -CTG CTT GAA TTG GTC GGC GAG-3'	NS3
Ky81sLc	5' -GGT GTA CTC ACC GGT TCC G-3'	5'UTR
P47Lc	5' -TGT GAG GAA CTA CTG TCT TCA CGC-3'	5'UTR
SV 49As	5' -GAA TGA TGG CCG GCC TTC CCG ACA AGA TGA TCC TG-3'	NS3
SV 79 S	5' -TGT GAG GAA CTA CTG TCT TCA CGC-3'	EMCV IRES
SV 82 S	5' -GAA TGT CGT GAA GGA AGC AG-3'	NS3
SV 365Bs	5' GAC TAC GTC CCT CCM GTG GTA C-3'	NS5A
SV 379As	5' -GTC CTC CGA TCG TTG TCA G-3'	3'UTR
SV 394S	5' -GTC GGC CGT CGA CAG CGG CAC RGC RAC-3'	NS5A
SV401As	5' - CAC ARG TTA CTT GAA AGC CTC TGC GGC CTG TCG AG-3'	NS3
SV406s	5' -CCG TGC ACC ATG GGC ACG AAT CC-3'	Core
HCV sensor	5' -GTGTCGTGCAGCCTCCAGG-Fluorescin	5'UTR
HCV -anchor	5' -LC-Red640-CCCCCCTCCCGGGAGAGCC-PH	5'UTR

## 9. Plasmids

### - Plasmids containing the HCV subgenomic replicon

pFKI389neo/NS3-3' and pFKI389luc/NS3-3' have been provided by Prof. R. Bartenschlager and Dr. V. Lohmann, Institute for Virology, University of Heidelberg. Both plasmids contain bicistronic replicon sequences composed of HCV IRES, neomycin phosphotransferase /luciferase gene, EMCV IRES and non-structural genes of HCV. Upstream of HCV IRES the promoter of T7 bacteriophage is located. It allows transcription of the bicistronic replicon into RNA. The IRES of HCV drives the translation of Neo/ Luciferase gene and the EMCV IRES drives the translation of HCV non-structural gene. The current subgenomic replicon is based on a patient-derived consensus isolates, designated Con1.

### - Plasmid containing the full-length genome of HCV AD78 strain

pHCV plasmid has been generated by Dr. K.Rispeter, Institute of Virology, University of Duisburg-Essen. It is composed of the full-length genome of the consensus sequence of HCV AD78 isolate cloned into a pCR2.1 topo vector.

### - Commercial plasmids

During this work, we have used two vectors for cloning of PCR products: pCR2.1Topo and pCR4.Topo vectors. Both plasmids were purchased from Invitrogen (Germany)

## 10. Buffers

-Northern Max 10x Denaturing Buffer; Ambion (UK): used for preparation of denaturing gel for RNA electrophoresis.

-Northern Max 10x Mops Running Buffer; Ambion (UK): used for electrophoresis of RNA.

-50 mM NaOH: 2 g NaOH in 1 l sterile H<sub>2</sub>O: used as a transfer buffer for Northern blot analysis.

-Ultra Hyb Hybridization Buffer; Ambion (UK): used for hybridization of labelled probe with target RNA.

-PBS cell culture grade; Gibco BRL (Karlsruhe)

-Cytomix pH (7.6): 120 mM KCl; 0.15 mM CaCl<sub>2</sub>; 10 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> ;( pH 7.6); 25 mM Hepes; 2 mM EGTA; 5 mM MgCl<sub>2</sub>; used for transfection of eukaryotic cells.

-Cell lysis buffer Cells-to-cDNA; Ambion (UK)

-Crystal violet solution: 1% Crystal violet; 20% EtOH in Water.

-IF buffer: 3% (w/v) BSA, 0.1 % (v/v) Triton-X 100 in PBS.

-Cell lysis buffer: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5 % (v/v) Triton-X100, 1 mM EDTA, and 1 mM PMSF; used for detection of HCV proteins in cells transfected with HCV replicon RNA

-Blocking buffer: 0.5% (v/v) Tween 20, 2% (w/v) non-fat milk in PBS.

-10 x Tris-SDS Glycine buffers: 10 g SDS, 30.3 g Tris, and 144.2 g Glycine in 1 l water.

-10 x proteins transfer buffer: 30.2 g Tris, 141.1 g Glycine in 1 l water.

-Wash buffer (T-PBS): 0.5 % (v/v) Tween 20 in PBS.

-20 x SSC buffer; Invitrogen (Germany).

## 11. Antibodies

All anti-HCV antibodies used in this work were kindly provided by Dr. V. Lohmann, Institute for Virology, University of Heidelberg. Following antibodies were used for detection of HCV proteins in transfected cells:

- Polyclonal antibodies anti-HCV NS3.

- Polyclonal antibodies anti-HCV NS4B.

- Polyclonal antibodies anti-HCV NS5A.

- Polyclonal antibodies anti-HCV Core

- Monoclonal antibodies anti-HCV NS5B

All the anti-mouse and anti-rabbit IgG were purchased from Dianova (Hamburg):

-HP conjugates IgG Anti-Mouse

-HP conjugates IgG Anti-Rabbit

## 12. Medium for culture of *E. coli* cells

### LB Agar

10 g Trypton  
5 g Yeast extract  
5 g NaCl  
15 g Agar

The medium was completed to 1000 ml with bidistilled water and sterilized by autoclaving. Ampicillin was added to the medium at a 100 µg/ml final concentration.

### LB Medium

10 g Trypton  
5 g Yeast extract  
5 g NaCl

The medium was completed to 1000 ml with bidistilled water and sterilized by autoclaving. Ampicillin was added to the medium at a 100 µg/ml final concentration.

## 13. Biological material

### HCV infected patients' sera.

Sera of patients infected with AD78 strain were kindly provided by Prof. M. Wiese, St.Georg Hospital, Leipzig. All these patients were infected with the same isolate of HCV ("so called AD78") after the treatment with contaminated anti-D immunoglobulin in 1978.

**Bacteria*****E.coli* Strains****Supplier**

Xl-10 Gold Ultracompetent cells

Stratagene GmbH (Heidelberg)

Top 10 Competent cells

Invitrogen GmbH (Karlsruhe)

**Eukaryocyte cell lines****Cells lines****Origine**

Huh7

Human hepatocarcinoma

Huh7 Trf1

Human hepatocarcinoma

## Methods.

### 1. Extraction of HCV RNA.

HCV RNA was extracted from patient's sera by using a QIAamp Viral RNA mini Kit. According to the manufacturer's instructions, 140  $\mu$ l of serum were mixed with the lysis buffer (200  $\mu$ l AVL buffer) and incubated for 10 min at room temperature. The lysate was mixed with 560  $\mu$ l of 100% EtOH, then transferred to the silica column and centrifuged for 1min at 8000 rpm. The column was successively washed with 500  $\mu$ l of AW1 and 500  $\mu$ l of AW2 buffers. The viral RNA was eluted in 50  $\mu$ l of elution's buffer (AVE) and directly used in the reverse transcription reaction or kept at - 80°C.

### 2. Extraction of cellular RNA with TRIzol Reagent.

Subconfluent cells were washed with D-PBS and lysed with 1ml of TRIzol for 5 min at room temperature. The lysate was mixed with 200  $\mu$ l of chloroform and incubated for 2 min at room temperature. The mixture was centrifuged for 15 min by 12000 rpm at + 4°C.

The supernatant (aqueous phase) was then transferred in a new Eppendorf cup, and 1 volume of isopropanol was added to 1 volume of the supernatant, mixed, and incubated for 10 min at room temperature to allow RNA precipitation. RNA was pelleted by centrifugation at + 4°C for 15 min by 12000 rpm. The pellet was washed with 75% EtOH and dried at room temperature. RNA pellet was resuspended in DEPC-treated water and quantified by spectrophotometry at 260 nm. RNA aliquots were conserved at - 80°C or used directly for reverse transcription or for Northern blot analysis.

### **3. Analysis of RNA by Northern blot.**

#### **3.1. RNA Agarose gel electrophoresis.**

All the materials used for gel preparation were treated with 0.2% SDS overnight and subsequently washed with DEPC-treated water. To prepare the gel, 1.2 g of DNAses- and RNAses-free agarose was dissolved in 90 ml of DEPC-treated water, and then 10 ml of denaturing buffer were added to the liquid gel when the temperature was lowered to about 55°C. To separate RNAs by electrophoresis, 1 volume of RNA was mixed with 3 volumes of formaldehyde loading buffer. The RNA was denatured at 65°C for 15 min then loaded on the gel. Separation of RNAs was performed in 1x MOPS running buffer for 3 h at 50 mV.

#### **3.2. Transfer of RNA from agarose gel to nylon membrane.**

Prior to transfer the RNAs from agarose gel to nylon membrane, the gel was incubated for 20 min in sterile water, then in the Transfer Buffer (50 mM NaOH). The membrane was also humidified with sterile water, and then incubated for 20 min in the Transfer Buffer. The transfer was performed under a pressure of 2.5 kPa using a Vacuum-Blotter system. To immobilize the RNA the membrane was backed at 80°C in a backoven for 1 h.

#### **3.3. Synthesis of radiolabelled probes.**

Probes targeting neomycin and  $\beta$ -Actin genes were synthesized from Hind III linearized pFKIneo and  $\beta$ -Actin plasmids, respectively. MAXIscript T3 kit for RNA in vitro synthesis was used for this purpose. Two  $\mu$ g of linearized plasmids were used in the reaction.

### 3.4. In vitro transcription reaction.

10x Transcription Buffer	2 $\mu$ l
CTP	2 $\mu$ l
GTP	2 $\mu$ l
ATP	2 $\mu$ l
[ $\alpha$ - <sup>32</sup> P UTP]	5 $\mu$ l
T3 RNA polymerase	2 $\mu$ l
DNA	x $\mu$ l
H <sub>2</sub> O	y $\mu$ l
	20 $\mu$ l

The reaction was carried out at 37°C for 1 h. DNA template was removed from the reaction by treatment with DnaseI, which was then inactivated with 1  $\mu$ l of 0.5 mM EDTA.

Radiolabelled probes were purified using a NucAway spin column according to the instructions of the manufacturer.

### 3.5. Prehybridization, hybridization and autoradiography.

The blot was prehybridized in the Pre-Hyb/Hybridization (UltraHyb) for 30 min at 68°C. The hybridization was carried out overnight at 68°C by adding the radiolabelled probes to the membrane in hybridization buffer. Prior to the hybridization, the membrane was cut approximately 1cm below the 28S rRNA band. The upper part of the membrane, containing the HCV replicon RNA, was hybridized with a <sup>32</sup>P-labeled negative-sense RNA riboprobe complementary to the HCV IRES and Neo gene. The lower part of the membrane, containing  $\beta$ -Actin mRNA, was hybridized with a <sup>32</sup>P-labeled  $\beta$ -Actin-specific antisense riboprobe.

The blot was washed twice for 15 min with Washing Buffer 1 (2x SSC, 0.1%SDS) at 60°C and subsequently washed twice for 15 min with Washing Buffer 2 (0.1x SSC, 0.1%SDS) at 60°C. The blot was enveloped in a plastic bag and exposed to X-ray film in an autoradiography cassette at ambient temperature.

#### 4. Amplification of RNA and DNA.

##### 4.1. Reverse transcriptase reaction.

Cellular RNA extracted from cells bearing the HCV subgenomic replicon or HCV RNA isolated from patient's sera were reverse transcribed into cDNA using Thermoscript or MMLV reverse transcriptase as following:

###### RT reaction Mix I

Primer (20 pmol/μl)	3 μl
<u>dNTPs(10 mM)</u>	<u>1 μl</u>
	4 μl

8 μl of RNA were added to 4 μl of Mix I and denatured for 5 min at 65°C and directly cooled down on ice.

###### RT reaction Mix II

5x RT Buffer	4 μl
0.1M DTT	2 μl
Rnases Inhibitor (20U)	1 μl
<u>RT</u>	<u>1 μl</u>
	8 μl

8 μl of Mix II were added to 8 μl of denatured RNA and incubate for 1 h at 37°C when M-MLV reverse transcriptase was used and at 50°C when Thermoscript reverse transcriptase was used.

#### 4.2. Polymerase chain reaction using Expand Long Template PCR System.

All the cDNA were amplified in a nested PCR. Two separate master mixes were prepared and mixed at a 50  $\mu$ l final reaction as following:

##### Master Mix I.

dNTPs(10mM)	10.0 $\mu$ l
Primer Sens (10pmol/ $\mu$ l)	1.5 $\mu$ l
Primer Anti-sens	1.5 $\mu$ l
<u>H<sub>2</sub>O</u>	<u>y <math>\mu</math>l</u>

Y depends on the amount of the input DNA. When the reaction corresponds to the first PCR, 7  $\mu$ l of H<sub>2</sub>O and the 5  $\mu$ l of the cDNA were used in the reaction. In the second PCR 11  $\mu$ l of H<sub>2</sub>O and 1  $\mu$ l of the first PCR were used.

##### Master Mix II

10 x Buffer 3	5.0 $\mu$ l
MgCl <sub>2</sub> (25mM)	1.5 $\mu$ l
Enzyme mix	0.5 $\mu$ l
<u>H<sub>2</sub>O</u>	<u>18.0 <math>\mu</math>l</u>
	25.0 $\mu$ l

Thermal cycling.

	Temperature	Time	Cycle number
Initial denaturation	94° C	2 min	1 x
Denaturing	94° C	30 sec	
Annealing	X° C	30 sec	35 x
Elongation	68° C	1 min/kb	
Final Elongation	68° C	7 min	1 x
Cooling	4° C	Unlimited	

### **4.3. Quantification of HCV RNA by real-time PCR**

The amount of HCV RNA in transfected cells was quantified by real-Time RT-PCR using a Light Cycler RNA Amplification Kit, and Light Cycler. Briefly, 20  $\mu$ l of lysis buffer were added per well of 96 well plate containing cells and incubate for 30 min at 80°C. Then 5  $\mu$ l of cells lysate were directly added to 15  $\mu$ l of the master mix and a one step RT-PCR reaction was performed as following: reverse transcription at 55 °C, 30 min; denaturing at 95 °C, 30 sec; PCR: 95 °C, 5 sec; 52 °C, 15 sec; 72 °C, 15 sec, 45 cycles; cooling at 40 °C, 30 sec.

To normalize total amount of RNA in each sample, the number of mRNA molecules for human glucose-6-phosphate dehydrogenase (h-G6PDH) was determined using the Light Cycler-h-G6PDH Housekeeping Gene Set according to the instructions of the manufacturer.

### **5. Purification of restricted DNA by gel extraction.**

Restricted plasmids or amplified DNA were separated by electrophoresis on agarose gel. The fragments of interest were cutted from gel and purified with a QIAgel mini Kit according to the instructions of the manufacturer.

### **6. Restriction of plasmid DNA with endonucleases.**

Plasmids were restricted either with one endonuclease or with combination of two enzymes. When the enzymes used for plasmid restrictions had different optimal temperatures or when they did not have compatible buffers, sequential restrictions were carried out using the optimal buffers and temperatures.

Restriction of plasmids with one endonuclease.

DNA (10 $\mu$ g)	x $\mu$ l
10 x Buffer	10 $\mu$ l
10 x BSA	10 $\mu$ l
Enzyme(50U)	y $\mu$ l
<u>H<sub>2</sub>O</u>	<u>z <math>\mu</math>l</u>
	100 $\mu$ l

The restriction was carried out for overnight at the optimal temperature depending on the endonuclease used.

Restriction of plasmids with two endonucleases.

DNA (10 $\mu$ g)	x $\mu$ l
10 x Buffer	10 $\mu$ l
10 x BSA	10 $\mu$ l
Enzyme 1(50U)	y $\mu$ l
Enzyme 2(50U)	z $\mu$ l
<u>H<sub>2</sub>O</u>	<u><math>\alpha</math> <math>\mu</math>l</u>
	100 $\mu$ l

The restriction was carried out overnight at the optimal temperature for both endonucleases.

**7. Ligation of DNA.**

Restricted plasmids were purified on a preparative agarose gel as previously described. Vector DNA and insert DNA were ligated using a T4 DNA ligase. A 1:5 molar ratio of vector to insert DNA was used in all cloning reactions. The conversion of molar ratios to mass was performed as following:

$$\frac{(\text{ng of vector}) \times (\text{kb size of insert})}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert.}$$

#### The ligation mixture:

Vector DNA	100 ng
Insert DNA	x ng
10X ligation buffer	2 $\mu$ l
T4 DNA ligase (Weiss units)	1 u
Nuclease-Free water to a final volume of	20 $\mu$ l

The mixture was incubated at 14°C for 16 h and 5  $\mu$ l of the mixture were used for transformation of *E.Coli* competent cells.

### **8. Transformation of X-L10 Gold ultra-competent *E.Coli* cells.**

Cells were thawed on ice and 2  $\mu$ l of  $\beta$ -Mercaptoethanol were added to 50  $\mu$ l of cell aliquot. Following 10 min of incubation on ice, 5  $\mu$ l of the ligation were added to the cells and the mixture was additionally incubated for 30 min on ice. The cells were heated at 42° C for 30 sec and directly cooled down on ice for 2 min. 400  $\mu$ l of Soc medium was added to the cells and cells were incubated at 37° C for 1 h on a shaker before plating on agar selective plates.

### **9. Preparation of Plasmid.**

One bacterial colony was picked up from the LB agar plate and cultivated overnight on a shaker at 37°C in LB medium. The culture was then centrifuged at 3000 rpm for 10 min and washed once with PBS prior to extraction of the plasmid. The extraction of plasmid was carried out with a High Speed Midi Plasmid preparation Kit according to the instruction of the manufacturer. DNA was quantified by spectrophotometry at 260 nm and the size of the plasmid was checked on agarose gel.

### 10. Purification of DNA with Phenol-Chloroform method.

Plasmids containing HCV replicon sequences were linearized by sequential restriction either with PvuI and ScaI in case of the HCV subgenomic replicon or with SspI and ScaI in case of the full length replicon. The Restricted Plasmids were then purified using a phenol-chloroform method as follows: The restriction mixture was adjusted with water to 500  $\mu$ l. An equal volume of aqua phenol was added to the reaction and mixed by vortexing. The mixture was centrifuged at 13000 rpm for 10 min at + 4°C. The supernatant (aqueous phase) was transferred into a new Eppendorf cup and 1 volume of a 1:1 phenol-Chloroform mixture was added to the supernatant and mixed by vortexing. The mixture was centrifuged for 10 min at 13000 rpm at +4°C. The supernatant was then treated with 1 volume of chloroform and centrifuged as described previously. The DNA was precipitated after addition of one volume of isopropanol in the presence of 50  $\mu$ l of 3 M sodium acetate at -20°C for overnight or for 20 min at -70 °C. The DNA was pelleted by centrifugation at 13000 rpm for 30 min at + 4°C. The DNA pellet was washed with 70% EtOH. The DNA was resuspended in DNases and RNase free water, and then quantified by spectrophotometry at 260 nm.

### 11. In vitro RNA transcription.

Restricted and purified plasmids were used for in vitro RNA transcription. Up to 2  $\mu$ g of plasmids were used in the in vitro transcription reaction as follows:

DNA	x $\mu$ l
ATP (10mM)	2 $\mu$ l
CTP (10mM)	2 $\mu$ l
GTP (10mM)	2 $\mu$ l
UTP (10mM)	2 $\mu$ l
10x Transcription Buffer	2 $\mu$ l
RNase Inhibitor (20U)	1 $\mu$ l
T7 RNA Polymerase	2 $\mu$ l
<u>H<sub>2</sub>O</u>	<u>y <math>\mu</math>l</u>
	20 $\mu$ l

The reaction was carried out at 37°C for 3 h, subsequently the template DNA was removed by treatment of the reaction with 1 µl of DNase I at 37°C for 15 min and stopped with 15 µl of 3 M NH<sub>4</sub>AC. The synthesized RNA was purified with TRIZol Reagent and quantified by spectrophotometry at 260 nm.

## **12. Introduction of mutations into HCV sequences.**

To generate single point mutations into the plasmid sequences a Quick-Change site-directed mutagenesis kit was used. The strategy of this technology is based on a PCR. Principle and instructions on this kit are described in the handbook provided by the supplier (Stratagene).

## **13. Culture of adherent cell lines**

Cell monolayers of the human hepatoma cell line Huh7 were routinely grown in Dulbecco's modified minimal essential medium supplemented with 2 mM L-glutamin, non-essential amino acids, 100 U penicillin, 100 µg of streptomycin and 10% fetal calf serum. In case of cells carrying HCV replicons, 250 or 500 µg of G418 (Geneticin) were added to the medium. Cells were passaged three times a week at a dilution of 1:2 to 1:3 depending on the confluence.

## **14. Transfection of cells by electroporation**

Huh7 cells were harvested with trypsin: EDTA (Gibco-BRL) and washed twice with PBS. Cells were resuspended in Cytomix at final concentration of  $1 \cdot 10^7$  cells/ml or  $1 \cdot 10^5$ . Cell suspension (500 µl) was mixed with 1.5 to 10 µg of replicon RNA transcribed in vitro or total cells RNA and transferred to an electroporation cuvette (4 mm gap width, Bio-Rad) and subjected to an electric pulse of 270 v and 975 µF using a Gene Pulser II apparatus.

After electroporation a cell suspension was immediately diluted with DMEM supplemented with 2 mM L-glutamine, nonessential amino acids, 10% fetal calf serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml, and seeded into the six wells plates or culture dishes. After 48 h, cells were cultivated in presence of G418 at 250 and 500 µg/ml final concentrations and medium was change twice weekly. Three or four weeks after transfection, G418-resistant colonies became visible and cells lines harbouring continuous replicon replication were established for further analysis.

### **15. Treatment of replicon-bearing cells with anti-HCV compounds.**

To assess the sensitivity of HCV replicons to antiviral compounds,  $2 \times 10^4$  replicon-bearing cells were seeded in triplicate cultures into a 96 wells plate. 24 h after cells were incubated with 0, 0.55, 1.67, 5, 15, 45, 135, 250 or 500 IU/ml of IFN-alpha or with 0, 1, 5, 10, 50, or 250 IU/ml of IFN-gamma or with 0, 0.04, 0.012, 0.37, 1.1, 3.3, 10 mg/ml of JT3 for 48 h or 72 h respectively. Cells were then washed with PBS and treated with cell lysis buffer (Ambion). Cell lysates were then directly used for HCV RNA and for GAPDH mRNA quantification by the reverse transcription real-time PCR.

### **16. Measure of luciferase activity in cell lysates.**

Cells were washed twice with Dulbecco's PBS  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ . Two hundred µl of Dulbecco's PBS were added to the cell monolayers, followed by 200 µl of Lucelite substrate and incubated on a shaker for 10 min in the dark at room temperature. Cell lysates were immediately transferred into a 96 wells plate and luciferase activity was measured with the luminometer. All cell lysates were measured in quadruplets and a mean value was calculated. Values obtained with cells harvested 4 h post-transfection were used to normalize the data for transfection efficiency.

## 17. Indirect immunofluorescence.

Cells were grown on chamber slides until 70 to 80% confluence and fixed after being washed three times with PBS in a mixture of acetone and methanol (1:1[vol/vol]) for 10 min at -20°C. Fixed cells were washed three times with PBS and incubated for 1h in IF buffer at +4°C. A primary antibody diluted in IF buffer was added at dilution 1: 500. The binding of primary antibodies was run for 1 h at +4°C. Cells were then washed three times with PBS and incubated with 1: 500 diluted anti-mouse or anti-rabbit FITC conjugates for 1 h at + 4°C.

## 18. SDS-PAGE and Western blot.

### 18.1. Preparation of polyacrylamid gels.

Reagents	Dividing gel (12%)	Concentration gel (5%)
Acrylamid stock (30% acrylamid, 8% bisacrylamid)	6 ml	0.8 ml
Bidistilted water	5.8 ml	3.6 ml
1.8 mM Tris-HCl pH 8.8	3 ml	-
1.25 mM Tris-HCl pH 6.8	-	0.5 ml
10%(w/v)SDS	150 µl	50 µl
TEMED	7.5 µl	5 µl
10% Ammonium persulfate (w/v)	100 µl	17 µl

### **18.2. Lysis of cells and electrophoresis of proteins.**

Cells were harvested from culture dish by treatment with Trypsin-EDTA and washed twice with PBS. Lysis of cells was performed in 1 ml of lysis buffer after 3 cycles of freezing and thawing. The lysate was then clarified by centrifugation at 10000 rpm for 10min at + 4°C. The supernatant was used for SDS-PAGE.

### **18.3. Blotting and detection of targeted proteins.**

After separation by electrophoresis, proteins were transferred from the gel to the nitrocellulose membrane and excess binding sites on the membrane were blocked overnight in the blocking buffer at room temperature with gentle shaking. Anti-HCV antibodies were diluted 1:1000 in the blocking buffer before incubation with the membrane for 1 h. Following three cycle of washing with T-PBS; the membrane was incubated for 1h with the conjugate (anti-mouse or anti-rabbit peroxydase-labelled IgG) diluted 1:10000 in the blocking buffer. Binding of the conjugate to anti-HCV on the membrane was detected by using ECL Western blotting detection reagents according to the instructions of the manufacturer.

## **19. Measure of cell viability by MTT assay.**

MTT assay was performed to estimate cell viability. Briefly, 10.000 cells in 190 µl media were seeded per well in a 96 well plate and incubated (37°C; 5% CO<sub>2</sub>) overnight. Different concentrations of compounds were prepared and added to the cells in 10 µl media. Cells were additionally incubated for 24 h. Media containing compounds were replaced with 180 µl of fresh culture media and 20 µl of MTT reagent (5 mg/ml) was added to the cells followed by a 3 h incubation time. Formazan (MTT metabolic product) was suspended in 200 µl of DMSO after removal of the culture media and the optical density, which correlates with cell density, was measure at 560 nm.

## Results.

### 1. Subgenomic replicon chimeras based on sequences of Con1 and AD78 strains.

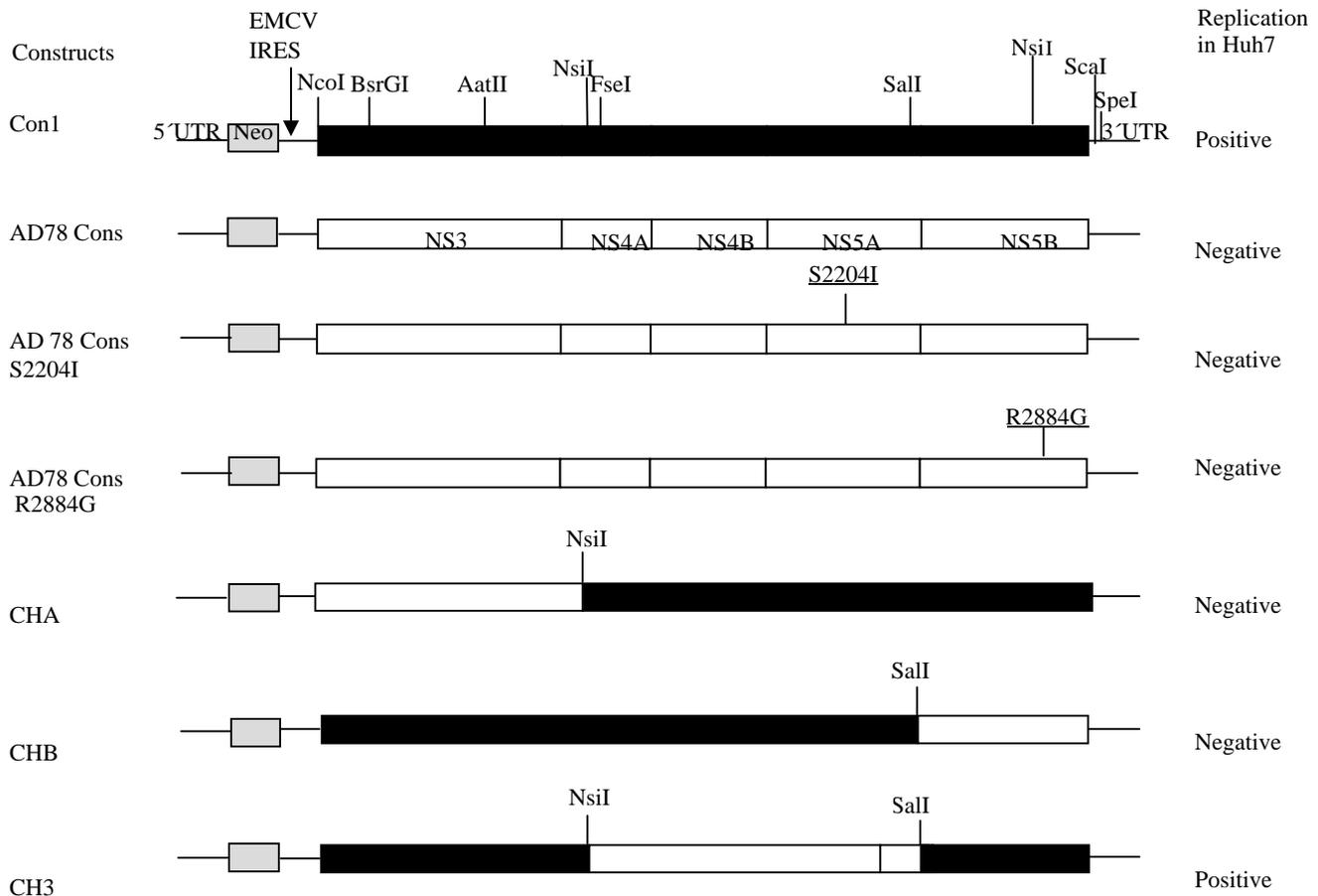
#### 1.1. Identification of defective genes within the consensus sequence of HCV AD78 strain.

The first attempt to create a subgenomic AD78 replicon was based on the use of the consensus sequence of this HCV strain obtained in our laboratory by Rispeter and coworkers (Rispeter et al., 1997). The fragment of this sequence, corresponding to the non-structural protein region from NS3 to NS5B, was inserted into the bicistronic vector, designed by Lohmann and co-workers (Lohmann et al., 1999) for generation of the subgenomic replicon based on the consensus sequence of the Con1 isolate of HCV (V.Lohmann and S.Viazov, unpublished results; Fig. 11, Con1). The obtained AD78 subgenomic replicon molecule (Fig. 11, AD78 Cons) contained a neomycin selectable marker downstream of the HCV 5'-UTR followed by the internal ribosome entry site (IRES) from the encephalomyocarditis virus (EMCV), the NS3-NS5B nonstructural coding region, and the HCV 3'-UTR (Fig. 11). The HCV 5'-UTR and 3'UTR of the established AD78 based replicon were derived from the Con1 sequence. The resulting AD78-based replicon was tested for replication ability in the "Lunet" clone of the cured Huh7 cells, which has been shown to be permissible for the replication of Con1 (Lohmann et al., 1999). Unfortunately, AD78 consensus replicon failed to replicate in cell culture, suggesting that AD78 consensus sequence contained some elements incompatible with the replication at least in the Huh7 cells.

There is an accumulating evidence that the HCV replicons, including Con1, BK, and H77, require cell culture adaptive mutations for an efficient replication. Different adaptive mutations were reported and some of them have to be combined for improving the fitness of HCV RNA replication in cell culture. Since the substitution of serine with isoleucine at the amino acid position 2204 in NS5A gene of HCV (S2204I) appeared to be the most effective Huh7 cell culture adaptive mutation, such a mutation was introduced into the AD78 consensus-containing replicon (V.Lohmann and S.Viazov, unpublished results).

Unfortunately, the amino acid exchange S2204I could not confer to AD78-based replicon the ability to replicate in Huh7 cells. In addition, a substitution of glycine for arginine at amino acid position 2884 was introduced into the NS5B gene of the defective AD78-based replicon. This mutation was shown to work as an efficient cell culture adaptive mutation in the Con1 replicon. Despite this modification, AD78 based replicon remained unable to replicate in cell culture.

To identify which genes of AD78 sequence are incompatible with the replication of AD78-based replicon in cell culture, a genetic approach was developed by using a prototype replicon Con1 as a backbone for generating Con1/AD78 chimeric replicons through a sequential replacement of non-structural genes of Con1 replicon with corresponding sequences of AD78 consensus sequence as depicted in Fig. 11. As a result, it was shown that substitution of the sequence fragment NsiI-SalI (partially covering NS4B to NS5A genes) of the Con1 replicon with the corresponding fragment of the consensus sequence of HCV AD78 led to a replication competent hybrid replicon termed CH3 (Fig. 11). On the contrary, substitutions of the fragment NcoI-NsiI (covering the whole NS3 gene and a small fragment of NS4A gene) as well as the fragment SalI-ScaI (covering the whole of NS5B gene) with the corresponding fragments from HCV-AD78 replicon led to replication defective hybrid replicons. These data clearly demonstrated that the inability of the AD78 replicon, constructed on the basis of the consensus sequence, to replicate in Huh7 cells was associated with the structure of its NS3 and NS5B genes.



**Fig. 11. Schematic representation of the subgenomic HCV replicons and their ability to replicate in Huh7 cells.** The 5'UTR and 3'UTR of HCV are depicted as solid lines, and the open reading frames are indicated as boxes. The sequences of HCV-AD78 and Con1 replicons are shown in white or in black, respectively. Neomycin selectable marker is designated as Neo and the internal ribosomal entry site of EMCV as EMCV IRES. The positions of the HCV non-structural proteins and restrictions sites of endonucleases used in the cloning strategies are indicated. The ability of hybrid constructs to replicate in Huh7 cells is indicated on the right site (V.Lohmann and S.Viazov, unpublished results).

## **1.2. Subfragment of AD78 NS3 that abrogates the replication of the chimeric Con1/AD78 replicon.**

The data reported in the previous section suggested that NS3 gene of AD78 replicon, based on the consensus sequence, most probably was defective, at least in the context of Huh7 cells. Considering these observations, we assumed that this gene might contain mutations that are deleterious for the replication of AD78 Cons replicon in cell culture. To analyse this hypothesis amino acids sequences corresponding to the NS3 gene of AD78 Cons replicon and Con1 replicon, belonging both to the genotype 1b of HCV, were compared as shown in Fig. 12. As a result, we could identify a difference of twenty-five amino acids residues between AD78 and Con1 NS3 genes. The intent being to identify which amino acid(s) is (are) deleterious, we tried to separate them into two groups. The first group of substitutions was located within the serine protease domain and the second one within the helicase domain of NS3 protein. For screening of both mutations' groups, two Con1/AD78 hybrid replicons bearing serine protease and helicase domains of AD78 consensus sequence in the context of Con1 replicon respectively were generated as follows. Due to the absence of unique restrictions sites in NS3 gene sequences of Con1 and AD78 strains, a number of intermediate plasmids were generated for facilitating the cloning procedures used for generating the Con1/AD78 hybrid replicons. First, an intermediate plasmid termed pFKI389NS3 has been created by cloning of a 3.5 kb fragment, which corresponds to the NS3-NS4A fragment of HCV Con1 amplified from pFKI389neo/NS3-3' plasmid, into a pCR4.Topo vector. Site-directed mutagenesis has been used to remove the restrictions site of BsrGI from the vector sequence and to introduce the restrictions site of FseI into the NS4A region of the cloned fragment. A second intermediate plasmid termed pAD78NS3 was generated by cloning into a pCR4.Topo vector of a 2.2 kb fragment corresponding to a NS3-NS4A fragment amplified from pAD78 plasmid. The restrictions site of BsrGI present in the sequence of the pCR4.Topo vector and in the NS3 sequence amplified from pAD78 have been removed by site-directed mutagenesis. Two additional intermediate plasmids were generated after subcloning into pFKI389NS3 plasmid of fragments BsrGI-AatII (containing the serine protease domain and 245 amino acids of the helicase domain from HCV-AD78) and AatII-FseI (containing the remaining 270 amino acids of the helicase domain of HCV-AD78) from pAD78NS3. Fig. 13 summarizes the cloning steps for generating pAD78NS3 and pFKI389NS3 intermediate plasmids.

Finally, two chimeric replicons constructs CH1a (containing BsrGI-AatII fragment of AD78 consensus sequence) and CH1b (containing AatII-FseI fragment of AD78 consensus sequence) were generated by subcloning of a BsrGI-EcoRI fragment from both previously created intermediate plasmids into the plasmid pFKI389neo/NS3-3' as shown in Fig. 14.

```

Con1      APITAYSQQTRGLLGCIITSLTGRDRNQVEGEVQVVSTATQSFLATCVNGVCWTVYHGAG
AD78 Cons .....K.....F....

Con1      SKTLAGPKGPITQMYTNVDQDLVGWQAPPGARSLTPCTCGSSDLYLVTRHADVIPVRRRG
AD78 Cons .....P.....S.....

Con1      DSRGSLSPRPVSYLKGSSGGPLLCPGSHAVGIFRAAVCTRGVAKAVDFVPVESMETTMR
AD78 Cons .....VA.....T.

Con1      SPVFTDNSSPPAVPQTFQVAHLHAPTGSKGSTKVPAAAYAAQGYKVLVLPNSVAATLGFGA
AD78 Cons .....S...

Con1      YMSKAHGIDPNIRTGVRTITTGAPITYSTYKFLADGGCSGGAYDIIICDECHSTDSTTI
AD78 Cons .....V..S...T.....S.

Con1      LGIGTVLDQAETAGARLVVLATATPPGSVTVPHPNIEEVALSSTGEIPFYGKAIPLETIK
AD78 Cons .....S.....N.....A.R

Con1      GGRHLIFCHSKKKCDELAAKLSGLGLNAVAYYRGLDVSVIPTSGDVIVVATDALMTGFTG
AD78 Cons ..G.....Q..S..V.....V.....

Con1      DFDSVIDCNTCVTQTVDFSLDPTFTIETTTVPQDAVRSRQRGRGTGRGRMGIYRFVTPGE
AD78 Cons .....KR.....

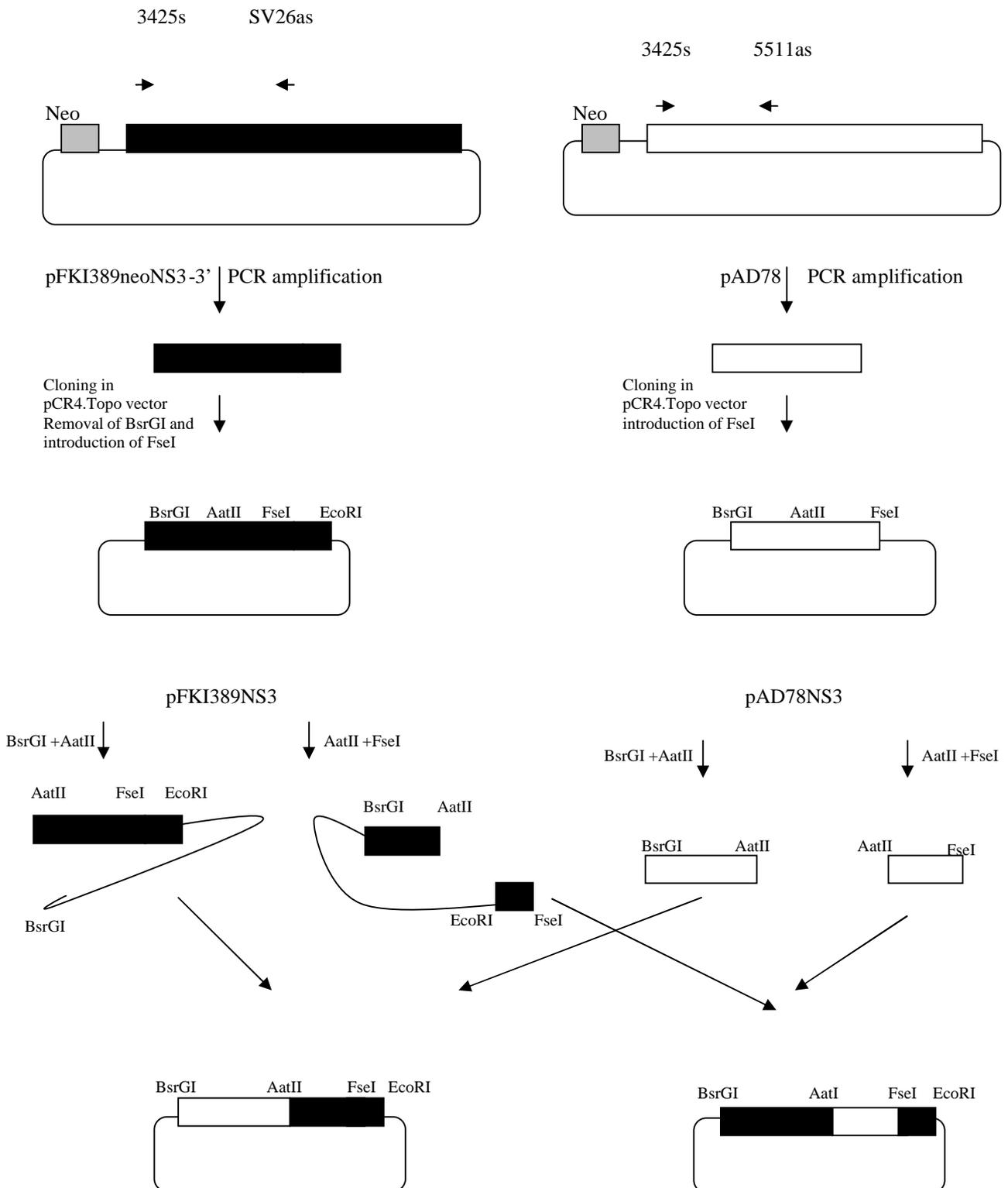
Con1      RPSGMFDSSVLCECYDAGCAWYELTPAETSVRLRAYLNTPLPVCQDHLEFWEVFTGLT
AD78 Cons .....

Con1      HIDAHFLSQTKQAGDNFPYLVAQATVCARAQAPPSWDQMWKCLIRLKP TLHGPTPLLY
AD78 Cons .....

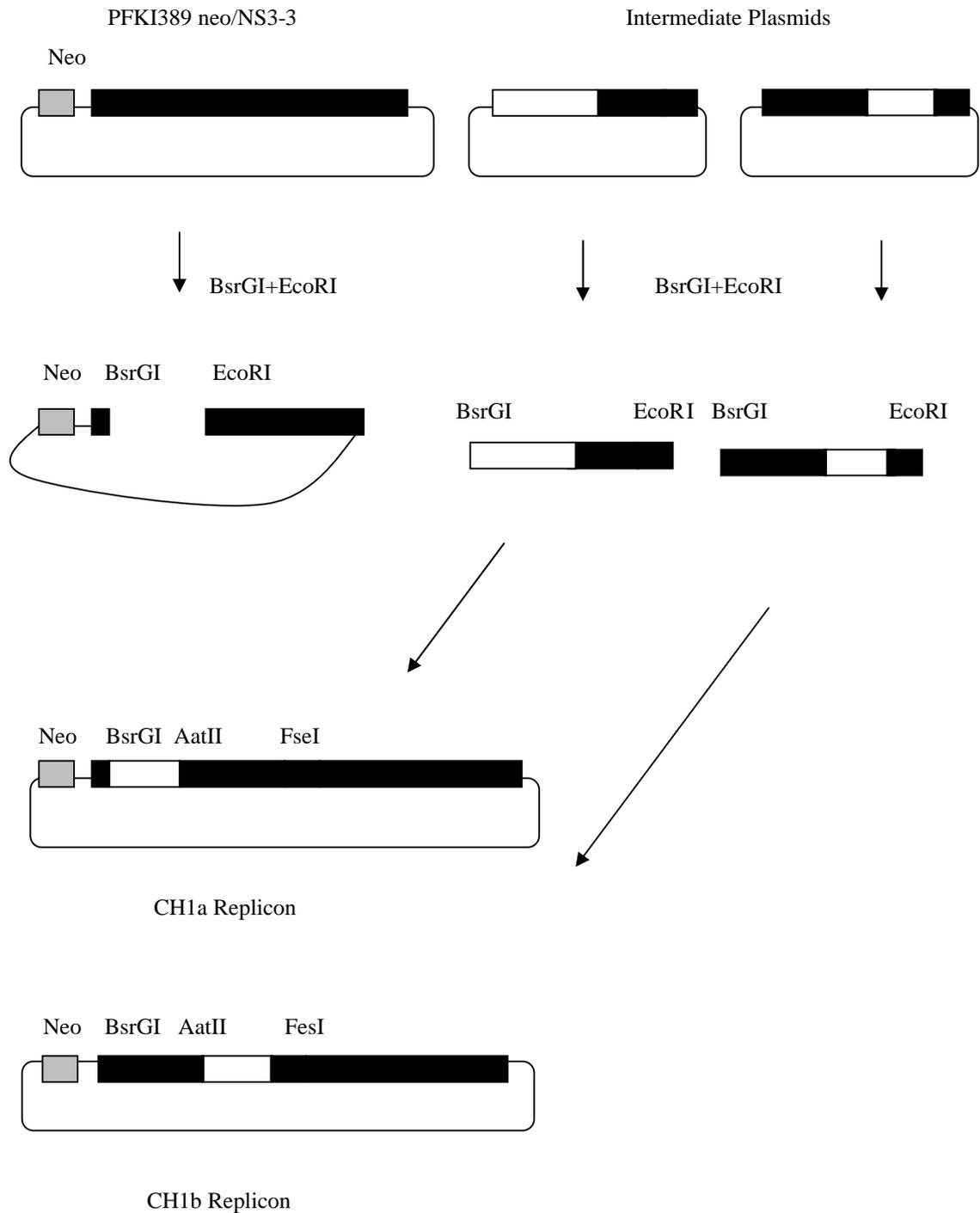
Con1      RLGAVQNEVTTHPITKYIMACMSADLEVVT
AD78 Cons .....L...V.....

```

**Fig. 12. Comparison of NS3 sequences of AD78 consensus with Con1 replicon.** On the top is represented the amino acid sequence of Con1 replicon and below of Con1 sequence is the amino acid sequence of NS3 gene of AD78.(.) indicates sequence identity.

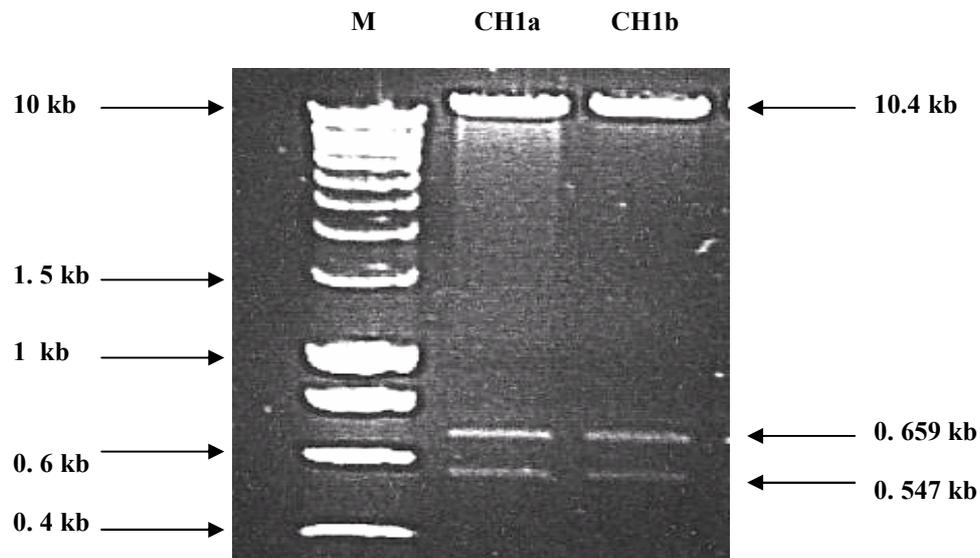


**Fig. 13. Creation of intermediate plasmids containing a hybrid NS3 gene.** NS3-NS4A fragment amplified from pFKI389neo/NS3-3' (represented in black) and pAD78NS3 (represented in white) were cloned into a pCR4.Topo vector. Following plasmid modifications by site directed mutagenesis the resulting intermediate plasmids were restricted either with BsrGI+AatII or with AatII+ FseI.

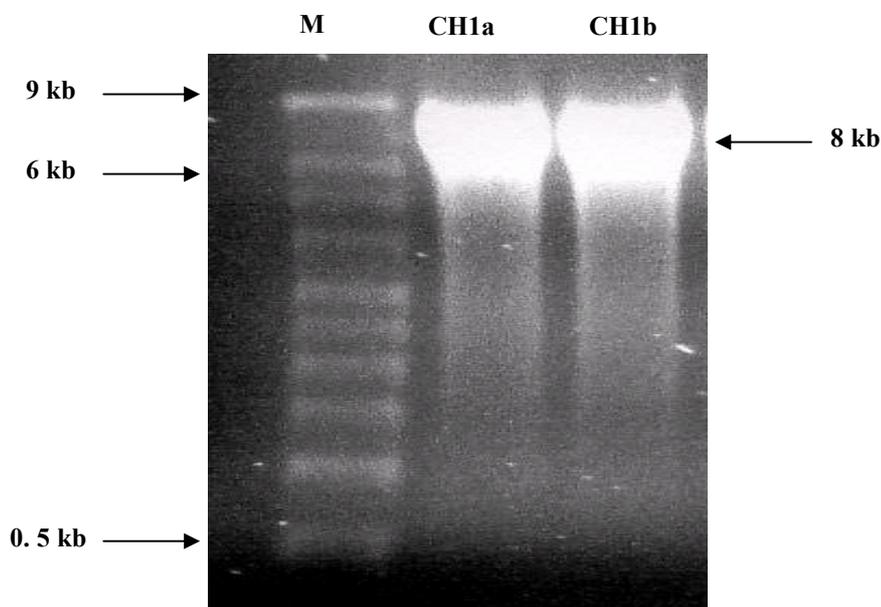


**Fig. 14. Cloning of the hybrid NS3 gene in the backbone Con1 replicon based.** The intermediate plasmids were restricted with BsrGI and EcoRI, the obtained fragments were cloned into the backbone replicon Con1 for getting CH1a (containing the BsrGI-AatII fragment of AD78 consensus sequence) and CH1b (containing the AatII-FseI fragment of AD78 consensus sequence) replicon. Sequence of Con1 and AD78 genomes are depicted in black or white, respectively.

For the assessment of an ability of CH1a and CH1b constructs to replicate in cell culture, plasmids were linearized and transcribed into RNA in vitro, with a subsequent transfection of Huh7 cells. The vector pFK that bears the HCV subgenomic replicon pFKI389neo/3-3' is a derivative of pBR322 vector. Since ScaI generates blunt ends and is a unique cutter in this plasmid, a recognition site of ScaI was engineered at the 3'UTR of HCV in pFKI389neo/NS3-3' for generating restricted plasmid with an exact 3'UTR of HCV. Because ScaI cuts badly the circular DNA, plasmids were first restricted with PvuI, which has a unique recognition site in the vector, and subsequently digested with ScaI. The restriction of CH1a and CH1b plasmids generated a big fragment of about 10.4 kb bearing an 8 kb HCV subgenomic replicon molecule and two small fragments of 0.112 kb and 0.547 kb (Fig. 15). The linearized plasmids were transcribed into RNA with the use of the promoter for bacteriophage T7 RNA polymerase cloned upstream of HCV 5'UTR. The correct size of HCV replicon RNA, which corresponds to an 8 kb fragment, was checked on agarose gel as shown in Fig. 16. The RNA was transfected into Huh7 cells by electroporation and cells were cultivated in presence of 250 µg/ml of G418. Under G418 selective pressure only cells, bearing HCV RNA that replicate efficiently, yield the Neo-resistant colonies and, cells bearing RNA that does not replicate die during the 3-4 weeks of G418 selection.



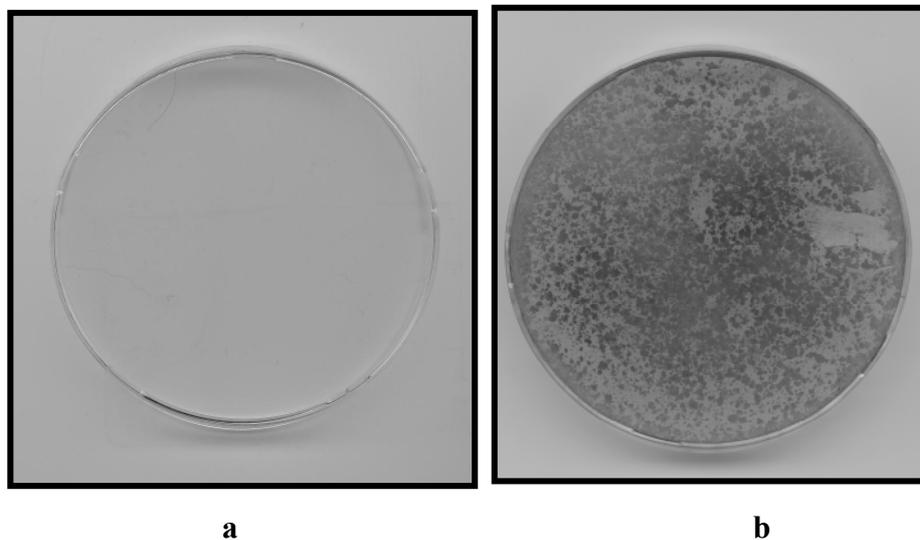
**Fig. 15. Gel electrophoresis of CH1a and CH1b replicon after restriction with PvuI and ScaI.** CH1a and CH1b plasmids were subsequently restricted with PvuI and ScaI. Fragments of 112 bp, 547 bp, and 10.4 kb size were generated. A 112 bp fragment is in general not visible on the gel. The 10.4 kb fragment bears an 8 kb HCV subgenomic replicon molecule. When the digestion with ScaI is incomplete an additional fragment of 659bp will appear on the gel.



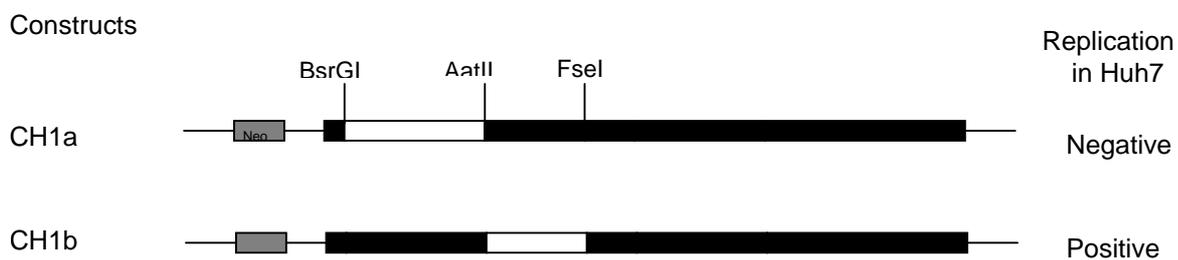
**Fig. 16. Gel electrophoresis of RNA synthesized from CH1a and CH1b restricted plasmids.** One  $\mu$ g of the restricted plasmid was used for synthesising the replicon RNA. Four  $\mu$ g of synthesized RNA were analysed by electrophoresis under denaturing conditions using formaldehyde as denaturant. M correspond to the RNA molecular weight marker. RNA synthesized from CH1a and CH1b plasmids are presented. HCV replicon RNA corresponds to a strong band of about 8kb.

Following 3 weeks of cultivation of transfected cells in the presence of G418, resistant colonies were obtained from cells transfected with RNA of CH1b (which contained the AatII-FseI fragment of AD78 consensus sequence) as shown in Fig. 17. In contrast, no colonies were obtained from cells transfected with RNA of CH1a (which contained the BsrGI-AatII fragment of AD78 consensus sequence). The efficiency of colony formation per microgram of transfected RNA observed was comparable to that, obtained with the parental Con1 replicon (data not shown).

A



B

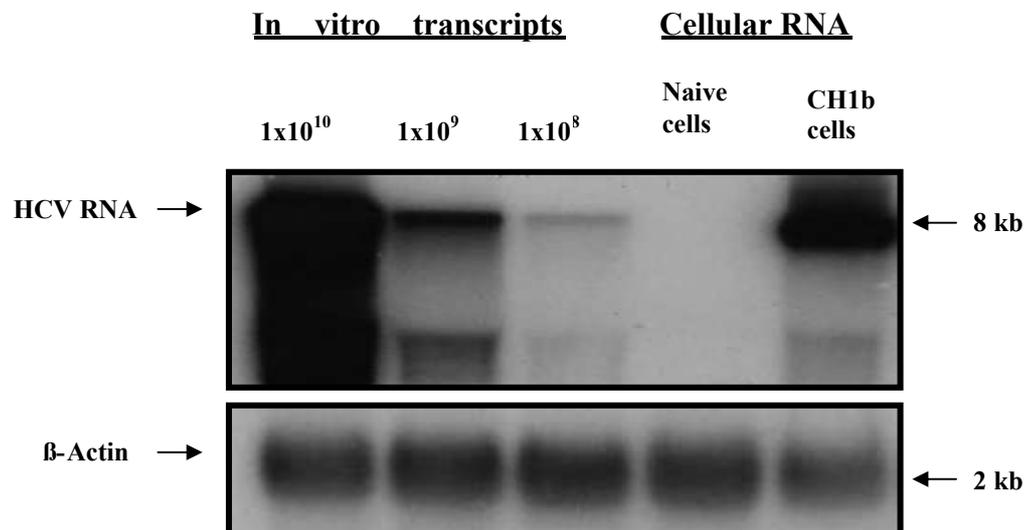


**Fig. 17. (A) Colony formation after transfection of Huh7 cells with CH1a and CH1b replicon RNA.** Huh7 cells were transfected with 2  $\mu\text{g}$  of in vitro synthesized RNA of CH1a and CH1b replicons. Transfected cells were cultivated for 3 weeks in presence of G418 (250  $\mu\text{g}/\text{ml}$ ), and resistant colonies were stained with crystal violet. (a) Huh7 cells transfected with CH1-a RNA; (b) Huh7 cells transfected with CH1-b RNA. **(B). Scheme of chimeric Con1/AD78 chimeric replicons CH1a and CH1b and their ability to replicate in Huh7 cells.** Sequence of Con1 and AD78 are shown in black and white, respectively.

For further analysis, the G418 resistant cell clones were mixed and a stable cell line bearing the CH1b replicon was established. To confirm the presence of HCV subgenomic replicon RNA in G418-resistant cell clones, the NS3 gene of HCV was amplified from the cellular RNA and submitted for sequencing. As shown in Fig. 19, the NS3 sequence amplified from the established cell line was identical to that of the CH1b construct, confirming that the G418 resistant cell clones contained the CH1b replicon RNA.

The presence of a replicating HCV RNA in these cells was also confirmed by Northern blot analysis as shown in Fig. 18. In comparison with the signal of the probe, hybridized with *in vitro* synthesized RNA used as a standard, the amount of HCV subgenomic RNA CH1b in G418-resistant cell clones was estimated to be more than  $10^9$  copies of HCV RNA per 5  $\mu$ g of cellular RNA.

Thus, the CH1b but not the CH1a replicon was able to replicate in Huh7 cells. This observation indicated that the failure of the AD78 Cons replicon to replicate in cell culture was associated with the defect in the fragment BsrGI-AatII of AD78 consensus sequence.



**Fig. 18. Detection of HCV subgenomic replicon RNA in selected cells.** Ten  $\mu$ g of total RNA from cloned cells bearing CH1b replicon was analysed by Northern blot. HCV RNAs and  $\beta$ -Actin mRNA were detected by hybridization to a riboprobe complementary to the neo gene or to  $\beta$ -Actin mRNA. Different amount of *in vitro* transcripts were used as standard. RNA isolated from naïve Huh7 cells served as negative control.

```

Con1      MAPITAYSQQTRGLLGCIITSLTGRDRNQVEGEVQVVSTATQSF LATCVNGVCWTVYHGA
CH1b-par  .....
CH1b-clo  .....

Con1      GSKTLAGPKGPITQMYTNVDQDLVGWQAPPGARSLTPCTCGSSDLYLVTRHADVIPVRRR
CH1b-par  .....
CH1b-clo  .....

Con1      GDSRGSLLSPRPVSYLKSSGGPLLCPSGHAVGIFRAAVCTRGVAKAVDFVPVESMGTTM
CH1b-par  .....
CH1b-clo  .....

Con1      RSPVFTDNSPPAVPQTFQVAHLHAPTGSKGSTKVPAAAYAAQGYKVLVNLNPSVAATLGFG
CH1b-Par  .....
CH1b-clo  .....

Con1      AYMSKAHGIDPNIRIGVRTITTTGAPITYSTYKFLADGGCSGGAYDIIICDECHSTDSTT
CH1b-par  .....
CH1b-clo  .....

Con1      ILGIGTVLDQAETAGARLVVLATATPPGSVTVPHPNIEEVALSSTGEIPFYGKAIP IETI
CH1b-par  .....
CH1b-clo  .....

                                AaTII
Con1      KGGRH LIFCHSKKKCDELA AKLSGLGLNAVAYRGLDVSVIPTSGDVIVVATDALMTGFT
CH1b-par  .....V.....
CH1b-clo  .....V.....

Con1      GDFDSVIDCNTCVTQTVDFSLDPTFTIETTTPQDAVSRSQRRGRTGRGRMGIYRFVTPG
CH1b-par  .....KR.....
CH1b-clo  .....KR.....

Con1      ERPSGMFDSSVLCECYDAGCAWYELTPAETSVRLRAYLNTPGLPVCQDHLEFWESVFTGL
CH1b-par  .....
CH1b-clo  .....

Con1      THIDAHFLSQTKQAGDNFPYL VAYQATVCARAQAPPSWDQMWKCLIRLKP TLHGPTPLL
CH1b-par  .....
CH1b-clo  .....

Con1      YRLGAVQNEVTTTHPITKYIMACMSADLEVVT
CH1b-par  .....L...V.....
CH1b-clo  .....L...V.....

```

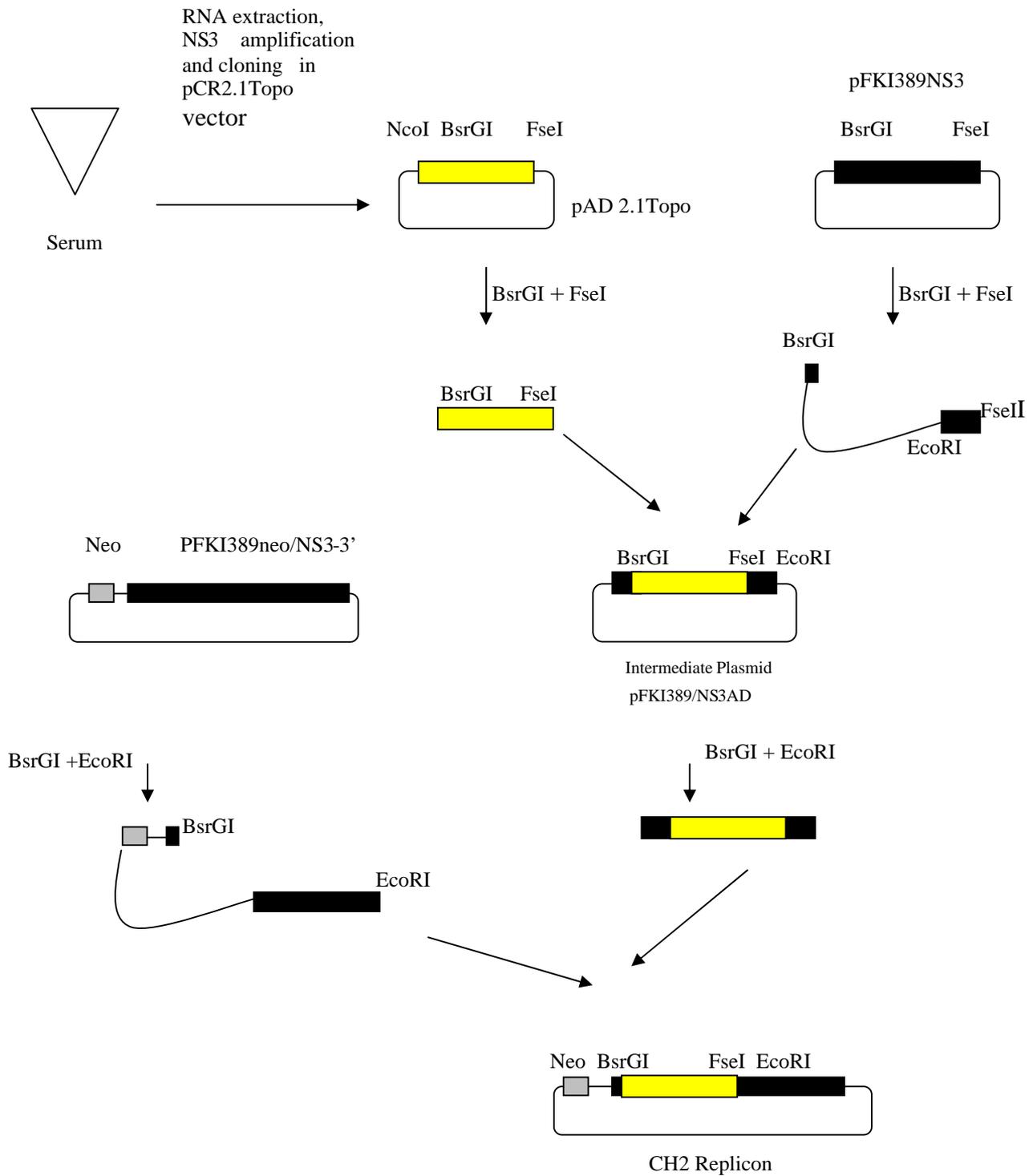
**Fig. 19. Alignment of the NS3 gene sequences from the parental CH1b replicon and the HCV RNA from the CH1b-transfected Huh7 cells.** Con1 indicates the NS3 sequence of Con1 replicon. CH1b-par and CH1b-clo represent the NS3 gene sequences of the parental CH1b and from the HCV RNA from the CH1b-transfected Huh7 cells, respectively. (.) Indicates sequence identity. AatII indicates the beginning of AD78 sequence in CH1b replicon

### **1.3. Chimeric replicon, bearing a NS3 sequence from an AD78-infected patient, is able to replicate.**

The results of the experiments reported in the previous section, clearly demonstrated that the fragment BsrGI-AatII, derived from the consensus sequence of HCV-AD78 strain, contains certain amino acid(s), which are deleterious for the replication of the chimeric replicon Con1/AD78 (CH1a). Given the relative high number (25) of amino acids difference observed between Con1 and AD78 consensus sequence, it would have been both time- and resource-consuming to identify potential lethal mutations by analysing each of the observed amino acid difference using the replicon system. The alternative way for identification of a functional NS3 gene of AD78 strain would have been the amplification of the NS3 gene sequence from individual patients infected with AD78 strain of HCV with a subsequent substitution of the corresponding gene of Con1 replicon with the amplified fragments, and testing of the obtained constructs in cell culture.

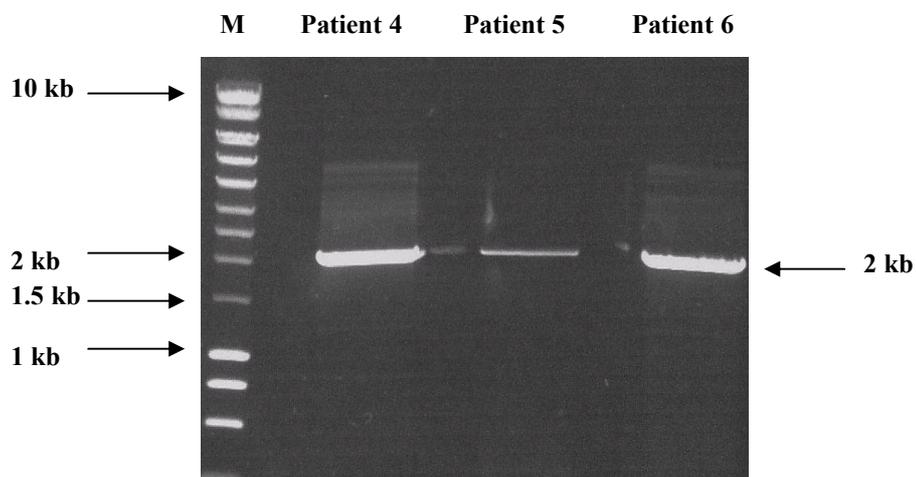
To obtain the NS3 gene of different AD78 isolates, the viral RNA has been extracted from serum of AD78-infected patients and amplified by RT-PCR. The amplified fragment of about 2 kb was purified by preparative agarose gel electrophoresis as shown in Fig. 20, and cloned into a pCR2.1 Topo vector. The obtained plasmids were used for sequencing of cloned NS3 genes. Because the recognition site of FseI was absent in the backbone replicon, the NS3 gene amplified from patients sera, which were cloned in pCR2.1Topo, were first subcloned in the intermediate vector pFKI389NS3 using BsrGI and FseI restrictions sites. Subsequently they were cloned in the backbone replicon Con1 using the BsrGI and EcoRI restrictions sites.

Overall, three hybrid Con1/AD78 replicons designated CH2-4, CH2-5, and CH2-6, which contained the NS3 gene sequence from AD78-infected patients P4, P5, and P6, respectively, were generated. The Fig. 20 summarizes the all cloning procedures used for preparation of a series of CH2 hybrid replicons.



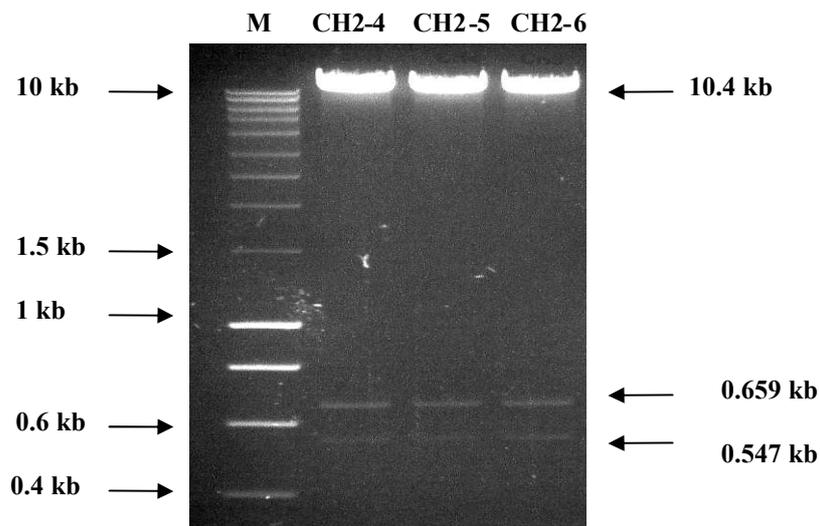
**Fig. 20. Cloning of NS3 gene amplified from HCV-AD78 infected patients into the backbone of replicon Con1.**

HCV NS3 gene was amplified from patients sera, cloned into pCR2.1Topo vector, and subcloned into the intermediate vector pFKI389NS3. The NS3 fragments were subsequently swapped with homologous ones from the backbone replicon Con1.

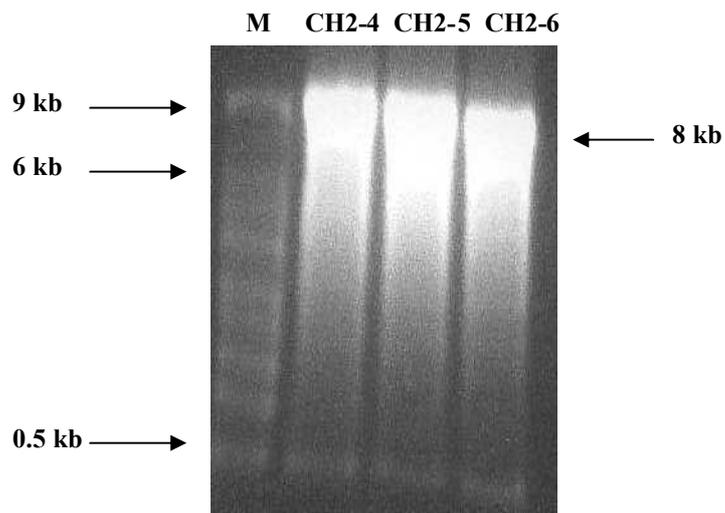


**Fig. 21. Electrophoretic analysis of NS3 gene amplified from three patients chronically infected with HCV AD78 isolate.** Viral RNA was extracted from patient sera and submitted to a RT-PCR as described in material and methods. The resulting NS3 fragment of about 2 kb was detected on agarose gel. M corresponds to the DNA molecular weight marker.

To test the ability of the obtained constructs to replicate in cell culture, the chimeric plasmids were restricted with PvuI and ScaI. The analysis of restricted plasmids by electrophoresis is shown in Fig. 22. The restricted plasmids were used for RNA transcription in vitro. The size of HCV RNA replicon was checked as shown in Fig. 23.



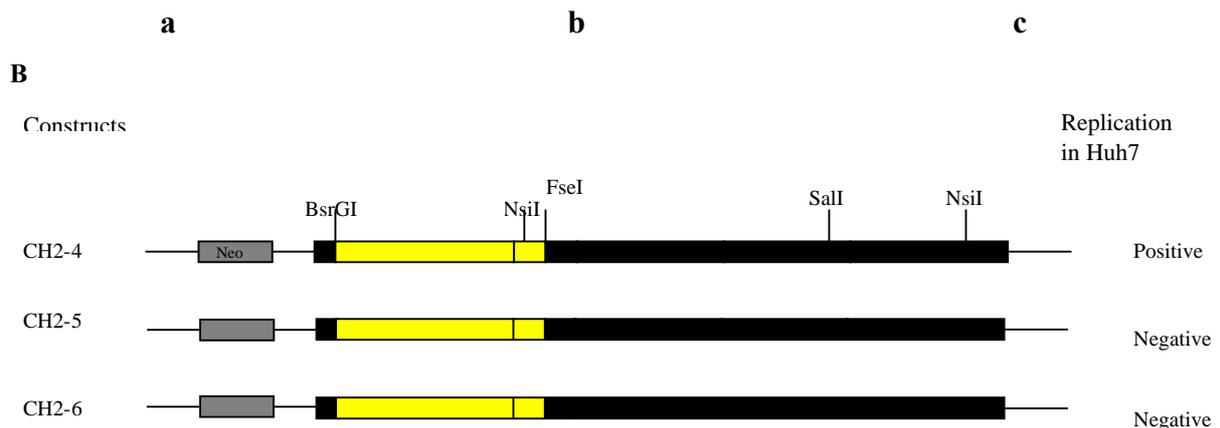
**Fig. 22. Electrophoretic analysis of CH2 plasmids restricted with PvuI and ScaI.** Plasmids bearing replicon constructs CH2-4, CH2-5 and CH2-6 were sequentially restricted with PvuI and ScaI. Fragments of 112 bp, 547 bp, and 10.4 kb size were generated. The big fragment (10.4 kb) bears an 8 kb HCV subgenomic replicon molecule. M correspond to the DNA molecular weight marker. Plasmids CH2-4, CH2-5 and CH2-6, are also presented on the gel.



**Fig. 23. Electrophoretic analysis of RNA synthesized from linearized CH2 plasmids.** One  $\mu\text{g}$  of the restricted plasmid was used for synthesising the replicon RNA. Four  $\mu\text{g}$  of synthesized RNA were analysed by electrophoresis under denaturing conditions using formaldehyde as denaturant. M correspond to the RNA molecular weight marker. RNA synthesized from CH2-4, CH2-5 and CH2-6 plasmids are presented. HCV replicon RNA corresponds to a strong band of about 8 kb.

Huh7 cells were transfected with RNA obtained from CH2-4, CH2-5 and CH2-6 plasmids. Transfected cells were cultivated in presence of G418 (250  $\mu\text{g/ml}$ ). Three week following the selection with G418, no colonies were formed within cells transfected with RNA from CH2-5 and CH2-6 replicons. However, G418-resistant colonies were obtained with cells transfected with CH2-4 RNA, as presented in Fig. 24. The calculated number of colonies formed per microgram of transfected RNA of CH2-4 was approximately eight. The efficiency of colony formation per microgram of transfected RNA was lower in comparison to that obtained with the CH1b replicon.

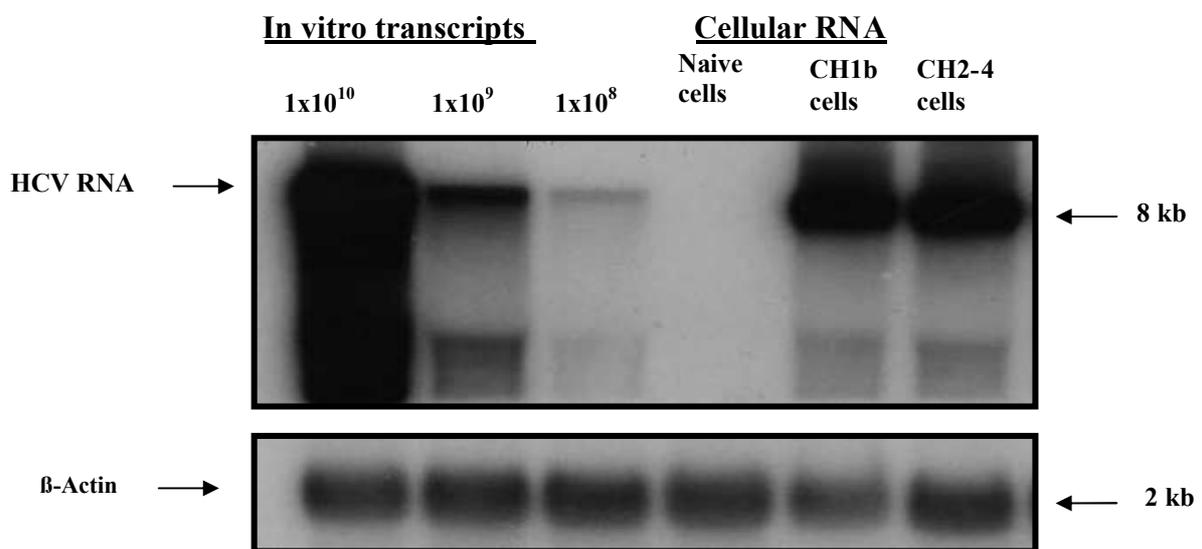
**A**



**Fig. 24. (A) Colony formation after transfection of Huh7 cells with CH2-4, CH2-5 and CH2-6 replicon RNA.** Huh7 cells were transfected with 5  $\mu\text{g}$  of in vitro synthesized RNA from CH2-4, CH2-5 or CH2-6 replicon. Transfected cells were cultivated for 3 weeks in presence of G418 (250  $\mu\text{g/ml}$ ), and resistant colonies were stained with crystal violet. (a) Huh7 cells transfected with CH2-4 RNA; (b) Huh7 cells transfected with CH2-5 RNA; (c) Huh7 cells transfected with CH2-6 RNA. **(B) Scheme of chimeric Con1/AD78 chimeric replicons CH2-4, CH2-5 and CH2-6 and their ability to replicate in Huh7 cells.** Sequences derived from patients infected with AD78 strain are shown in yellow. Sequence derived from Con1 is represented in black.

The G418-resistant cell clones obtained with CH2-4 replicon were expanded and cellular RNA was extracted for amplification of the NS3 gene of HCV. The presence of CH2-4 RNA in the G418 resistant cells was confirmed by comparison of NS3 sequence of the parental sequence with the sequence of the NS3 amplified from G418-resistant cells. The presence of a replicating HCV subgenomic RNA in resistant cell clones was also confirmed by Northern blot analysis (Fig. 25). The amount of replicon RNAs in G418-resistant cells was estimated to be comparable with that observed with CH1b replicon.

Thus, using the amplified NS3 sequence from one of the three AD78-infected patients we were able to identify a “functional” NS3 gene, namely, the gene that after insertion into the backbone of the Con1 replicon did not block its replication in Huh7 cells. These results open a way for subsequent experiments directed at development of a subgenomic replicon based on the sequence of HCVAD78 strain.



**Fig. 25. Detection of HCV RNA in transfected Huh7 cells.** Total RNA from cloned cells bearing CH1b (CH1b cells) and CH2-4 (CH2-4 cells) replicons was analysed by Northern blot. HCV RNAs and  $\beta$ -Actin mRNA were detected by hybridization to a riboprobe complementary to the neo gene or to  $\beta$ -Actin mRNA. Different amount of in vitro transcripts were used as standard. RNA isolated from naïve Huh7 cells served as negative control.

#### 1.4. Attempt to identify potential lethal mutations within the NS3 gene of Con1/AD78 replicons

Con1/AD78 hybrid replicons (CH2-5 and CH2-6) were cell culture replication defective. In order to identify whether the NS3 genes of these defective replicons contain potential lethal mutations, their amino acids sequences were compared with amino acids sequences of Con1, CH2-4 and AD78 consensus sequence (Fig. 26).

<i>BsrGI</i>	
Con1	SKTLAGPKGPITQMYTNVDQDLVGVWQAPPGARSLTPCTCGSSDLYLVTRHADVIPVRRRG
AD78 Cons	.....P.....S.....
AD78 -P4	.....P.S.....S.....
AD78 -P5	.....P.S.....IS.....
AD78 -P6	.....P.....S.....
	1177 <span style="float: right;">1205</span>
Con1	DSRGSLLSPRPVSYLKGSSGGPLLCPSGHAVGIFRAAVCTRGVAKAVDFVPVESMETMR
AD78 Cons	.....VA.....T.
AD78 -P4	.....V.....
AD78 -P5	.....VA.....T.
AD78 -P6	.....V.....A.
	1263
Con1	SPVFTDNSSPPAVPQTFQVAHLHAPTGSKGSTKVPAAAYAAQGYKVLVLPNSVAATLGFGA
AD78 Cons	.....S...
AD78 -P4	.....
AD78 -P5	.....S...
AD78 -P6	.....S.....
	1277
Con1	YMSKAHGIDPNIRITGVRTITTGAPITYSTYKFLADGGCSCGGAYDIIICDECHSTDSTTI
AD78 Cons	.....V..S...T.....S.
AD78 -P4	.....V.....S.....S.
AD78 -P5	.....V..S.....S.
AD78 -P6	.....V.....S.
	1386
Con1	LGIGTVLDQAETAGARLVVLATATPPGSVTVPHPNIEEVALSSTGEIPFYGKAIPETIK
AD78 Cons	.....S.....N.....A.R
AD78 -P4	.....NI.....
AD78 -P5	.....S.....N.....R
AD78 -P6	.....N.....A..
Con1	GGRHLIFCHSKKKCELAALKSGLGLNAVAYYRGLDVSVIPVATDALMTGFTG
AD78 Cons	..G.....Q..S..V.....V.....
AD78 -P4	E.....Q.....I.....N.V.....
AD78 -P5	.....V..Q.....I.....V.....
AD78 -P6	.....Q.....I.....S..V.....

```

Con1      DFDSVIDCNTCVTQTVDFSLDPTFTIETTTPQDAVSRSQRRGRGTGRGRMGIYRFVTPGE
AD78 Cons .....KR.....
AD78 -P4 .....R.....
AD78 -P5 N.....KR.....
AD78 -P6 .....Y.....R.....

Con1      RPSGMFDSSVLCECYDAGCAWYELTPAETSVRLRAYLNTPLPVCQDHLEFWESVFTGLT
AD78 Cons .....
AD78 -P4 .....
AD78 -P5 .....
AD78 -P6 .....

Con1      HIDAHFLSQTKQAGDNFPYLVAYQATVCARAQAPPSPWDMWKCLIRLKP TLHGPTPLLY
AD78 Cons .....
AD78 -P4 .....
AD78 -P5 .....
AD78 -P6 .....

Con1      RLGAVQNEVTTTHPI TKYIMACMSADLEVV
AD78 Cons .....L...V.....
AD78 -P4 .....L...V.....
AD78 -P5 .....L...V.....
AD78 -P6 .....AL.....

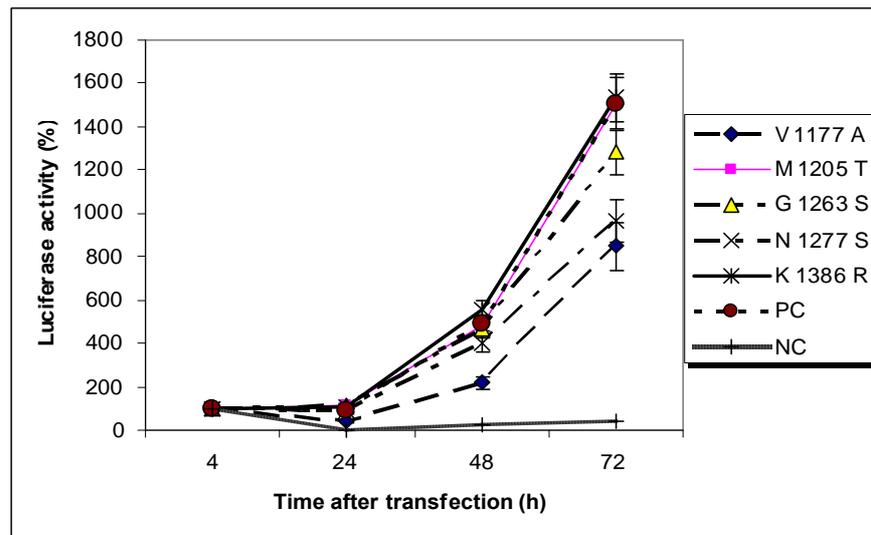
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**Fig. 26. Sequence alignment of NS3 gene amplified from patients infected with HCV AD78 isolate.** Con1NS3 sequence corresponds to the NS3 gene of Con1 replicon. AD 78 Cons represents the consensus sequence of HCV AD78 isolate. AD78-P4, AD78-P5 and AD78-P6 correspond to the NS3 gene amplified from patients infected with HCV AD78 isolate, cloned in CH2-4, CH2-5 and CH2-6. (.) Indicates match of the aligned sequences and the difference is represented by other amino acid letter.

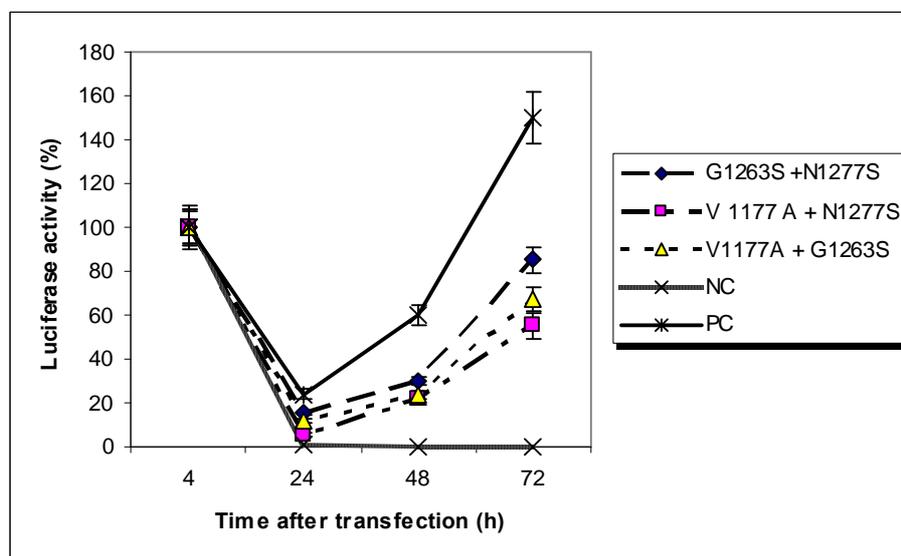
Data provided by sequence alignment revealed the presence at positions 1177, 1205, 1263, 1277, and 1386 of amino acid residues, which are absent from sequences of Con1 and CH2-4. This observation supposed that the amino acids residues found only in the NS3 genes of the defective replicons might represent lethal mutations. In order to test, whether these mutations are lethal, a prototype Con1 replicon, containing a fire luciferase gene as a reporter, was used as a tool. Selected mutations were introduced into the NS3 gene of Con1 by site-directed mutagenesis. Modified plasmids were linearized as previously described. RNA was transcribed from the linearized plasmids, and transfected into Huh7 cells. Replication of transfected RNA, which is proportional to the expressions level of the luciferase gene, was monitored by measuring luciferase activities in cells lysates.

As shown in Fig. 27, replication of Con1 was not affected by substitutions of methionine for theonine and of lysine for arginine, respectively, at the positions 1205 and 1386, whereas a slight reduction of Con1 replication was observed, when glycine was substituted for serine at position 1263. However, the substitution of valine for alanine and asparagine with serine at positions 1177 and 1277, correspondingly, showed a slightly more pronounced effect on the replication of Con1. Because all these mutations did not abolish the replication of Con1 when analyzed individually, we hypothesized that some of them might have a lethal effect only in combination with other substitutions (synergistic effect). Because substitutions V1177A, G1263S and N1277S somehow affected the ability of Con1 to replicate, we tested, whether combination of V1177A with G1263S, or with N1277s and combination of G1263 with N1277S would abolish the replication of Con1. None of these combinations, however, could abolish the replication of Con1. In fact, in these experiments we have observed the same effect as when these mutations were introduced individually into Con1 (Fig. 28).

Thus, using the mutational analysis we were not able to identify lethal mutation in NS3 genes of defective replicons. We have only observed that these mutations somehow affected the ability of Con1 to replicate without complete abolishment of HCV RNA replication. It remains unclear, why NS3 genes from defective sequences abolished the replication of Con1. The results of the experiments suggest that amino acids residues observed at positions 1177, 1205, 1277 1263, and 1386 of defective NS3 proteins from different AD 78 isolates are not directly involved in the inhibition of replication. Alternative explanation might be that different combination of some of these mutations play a negative role in blocking the HCV RNA replication. Finally, we can not also exclude a possibility that despite presence of all of these substitutions the expressed NS3 proteins are functionally active but not completely compatible with products of other genes of Con1 co-expressed by hybrid replicons in Huh7 cells.



**Fig. 27. Influence of AD78 mutations on the replication fitness of Con1 replicon.** Mutations observed in NS3 gene sequences amplified from AD78 isolates were introduced into Con1 replicon. Five  $\mu\text{g}$  of each mutated Con1 replicon RNA were transfected into naïve Huh7 cells. The replication of HCV RNA was monitored by measuring the level of luciferase activity in cell lysates. Luciferase activity were measured at different time point and normalized for difference in transfection efficacy using the activity at 4 h after transfection. PC corresponds to the original RNA from Con1 and NC is the replication-defective  $\Delta\text{GDD}$  mutant of Con1. Amino acid substitutions introduced into Con1 NS3 genes are indicated. Results are presented as mean  $\pm$ SD of the three independent experiments.



**Fig. 28. Analysis of synergic effect of AD 78 mutations on Con1 based replicon fitness.** Mutations observed in NS3 gene sequences amplified from AD78 isolates were inserted in indicated combinations into Con1 replicon. Five  $\mu\text{g}$  of each mutated Con1 replicon RNA were transfected into naïve Huh7 cells. The replication of HCV RNA was monitored by measuring the level of luciferase activity in cell lysates. Luciferase activity were measured at different time point and normalized for difference in transfection efficacy using the activity at 4 h after transfection. PC corresponds to the original RNA from Con1. NC is the replication-defective  $\Delta\text{GDD}$  mutant of Con1. Mutations introduced in Con1 replicon are indicated. Results are presented as mean  $\pm$ SD of the three independent experiments.

### 1.5. Influence of amino acids change within the CTL NS3-1395 epitope on HCV RNA replication.

Recently, two groups have presented evidence for the emergence of escape variants of the HLA-B8 restricted CTL epitope NS3-1395 HSKKKCDEL located within the non-structural protein 3 (NS3) of HCV. According to one report, mutations were selected in two patients with acute infection who mount an immunodominant CTL response against this epitope (Timm et al., 2004). In the other study accumulation of viral variants in subjects expressing the restricting HLA-B8 allele could be demonstrated in a cohort infected by a single source of HCV genotype 1b termed HCV AD78 (Ray et al., 2005). Moreover, both studies provide evidence for reversion of the observed escape mutation back to the consensus sequence in the absence of immune selection pressure. One of these escape variants was characterized by an exchange of lysine for arginine at position 1397 (K1397R) of the epitope, another one had arginine instead of lysine at position 1398 (K1398R) (Table2).

**Table 2.** Polymorphism in the position1397 and 1398 of the HLA B\*08 restricted HCV NS3-1395 epitope. Position 1395 to 1403 relative to the H77 polyprotein (Gen Bank/EMBL/DDBJ accession no.AF009606).

HLA allele	Sequence*	Publication
<b>B*08-</b>	<b>H S K K K C D E L</b>	<b>Timm et al., 2005</b>
<b>B*08+</b>	. . . <b>R</b> . . . . .	<b>Timm et al., 2005</b>
<b>B*08-</b>	. . . . .	<b>Ray et al., 2005</b>
<b>B*08+</b>	. . <b>K/R</b> . . . . .	<b>Ray et al., 2005</b>

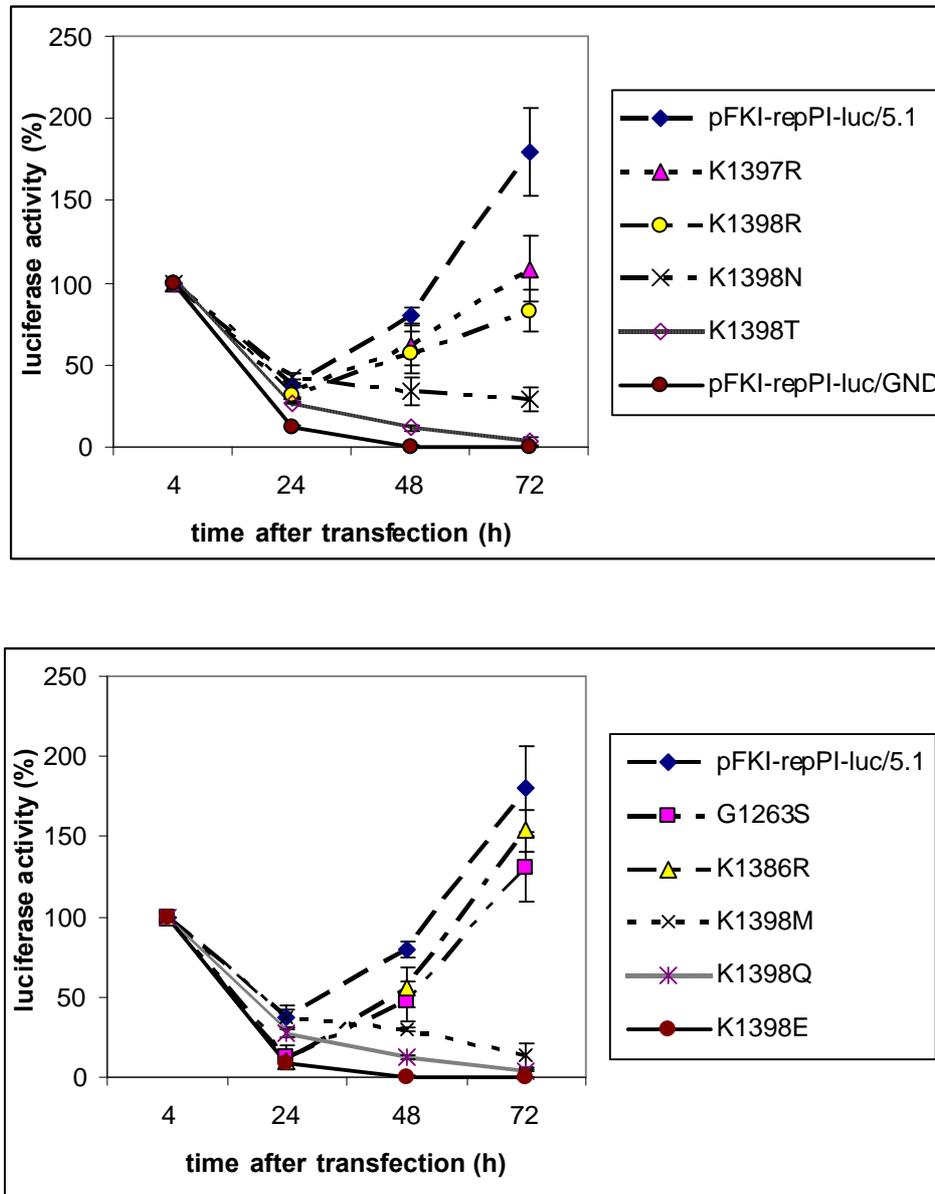
We used the site-directed mutagenesis to introduce these two mutations into the sequence of bicistronic subgenomic HCV construct pFK-rep PI-luc/5.1 that encode the luciferase reporter gene and HCV genes for non-structural proteins of Con1 isolate (Friebe et al., 2001). The original HCV NS3 protein encoded by this construct contained the prototype HSKKKCDEL sequence of the CTL epitope. In addition, site-directed mutagenesis was used to introduce five amino acids substitutions at position 1398 of the epitope. As a result; we obtained constructs that contained at position 1398 of the CTL epitope NS3-1395 all possible amino acid exchanges that theoretically might have occurred at this particular position as consequence of a single nucleotide mutation of the corresponding codon. These amino acids included K, present in the prototype sequence, R, observed in the described escape variant, and N, M, T, Q, and E, which were absent in any of HCV 1b sequences submitted to the Gen Bank (Table 3). In parallel, site-directed mutagenesis was used to introduce two mutations G1263S and K1386R in NS3 region of HCV pFK-rep PI-luc/5.1 replicon molecule. These two mutations were located outside of known CTL epitopes and were present in NS3 sequences of number of HCV type 1b isolates from the Gen Bank.

The original plasmid and the modified plasmids were linearized and subject to RNA transcription *in vitro*. The transcribed RNAs were used for transfection of Huh7 cells. Transfected cells were incubated for 72 h and the luciferase activity, which is a direct correlate of the efficiency of HCV RNA replication in cells, was measured at 4, 24, 48 and 72 h posttransfection. Activities at 4 h were used for correction of differences in transfection efficacy. Replication-defective RNA with an amino acid substitution in the active site of NS5B polymerase (pFK-repPi-luc/GND) was used as a negative control.

Altogether, nine mutated variants of HCV were prepared and assessed for their replicative potentials. The generalized results of the three independent experiments are presented in Fig. 29.

**Table3.** HCV NS3-1395 CTL epitope variants used in this work.

Replicon variant	Sequence									
pFKI-rep PI-luc/5.1 (Prototype sequence)	CAT	TCC	AAG	AAG	AAA	TGT	GAT	GAG	CTC	nt*
	H	S	K	K	K	C	D	E	L	aa*
K1397R	.	.	AGG	.	.	.	.	.	.	nt
	.	.	R	.	.	.	.	.	.	aa
K1398R	.	.	.	AGG	.	.	.	.	.	nt
	.	.	.	R	.	.	.	.	.	aa
K1398N	.	.	.	AAC	.	.	.	.	.	nt
	.	.	.	N	.	.	.	.	.	aa
K1398M	.	.	.	ATG	.	.	.	.	.	nt
	.	.	.	M	.	.	.	.	.	aa
K1398T	.	.	.	ACG	.	.	.	.	.	nt
	.	.	.	T	.	.	.	.	.	aa
K1398Q	.	.	.	CAG	.	.	.	.	.	nt
	.	.	.	Q	.	.	.	.	.	aa
K1398E	.	.	.	GAG	.	.	.	.	.	nt
	.	.	.	E	.	.	.	.	.	aa



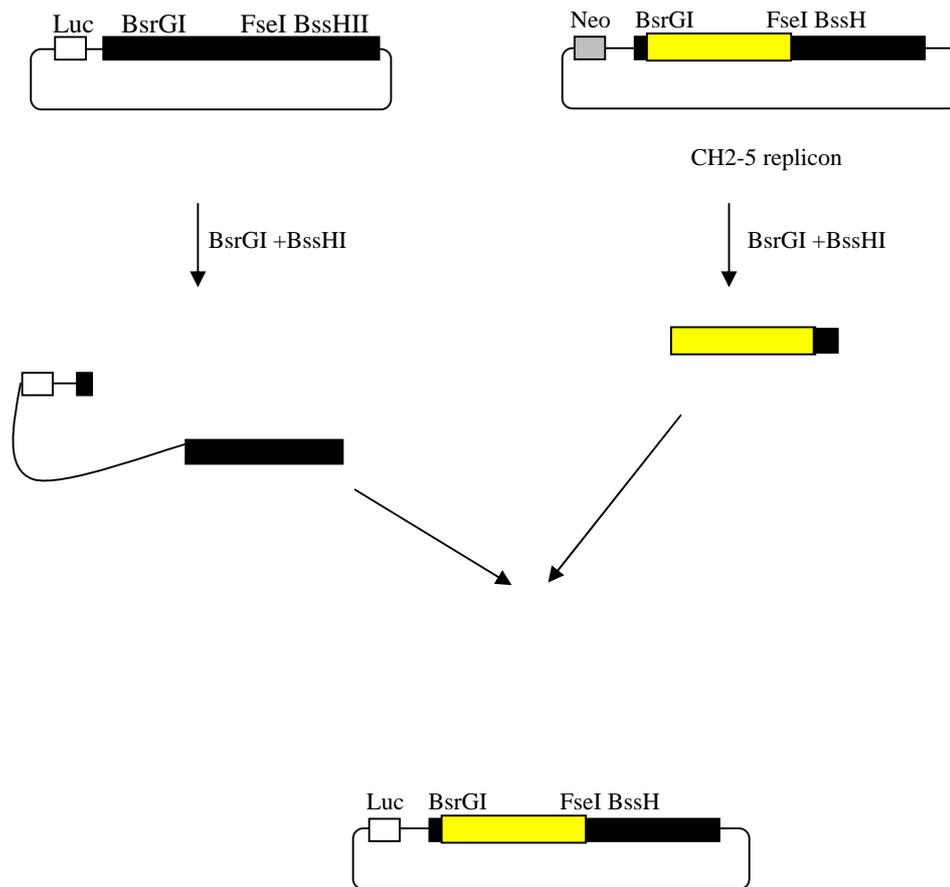
**Fig. 29. Influence of the amino acid substitution in the NS3-1395 CTL epitope on transient HCV RNA replication in Huh7 cells.** Replicons carrying mutations were transfected into Huh7 cells that were harvested after 4, 24, 48 and 72 h post transfection. Luciferase activity measured in the 4 h cell lysates was set as 100% for every experiment. Results are presented as mean  $\pm$ SD of the three independent experiments.

The introduction of substitution K to R at position 1397 of the NS3-1395 epitope (K1397R) of the HCV replicon led to the evident reduction in the levels of luciferase activity. In a second set of experiments the replicative ability of replicons, in which lysine at position 1398 of NS3-1395 epitope was substituted with different amino acids, was determined. The modified replicon construct K1398R, with arginine instead of lysine at this position, repeatedly demonstrated lower levels of replication. Thus, both escape mutations K to R at positions 1397 and 1398), which emerged during infection of HLA-B8 positive patients, led to a diminished efficacy of HCV RNA replication *in vitro*. Luciferase activities found with other five variants of replicon mutated at position 1398 (K1398N, K1398M, K1398T, K1398Q, and K1398E) were much lower and identical or very close to activities determined with defective replicon carrying an inactive NS5B polymerase (pKFI-rep PI-luc/GND) or, in other words, with all these mutations an impairment of HCV replication was observed. In contrast, two point mutations G1263S and K1386R, which presumably reflect the natural polymorphism of HCV NS3 sequence, did not lead to a significant reduction of the efficacy of HCV RNA replication.

### 1.6. *Trans-complementation of defective NS3 genes can not be achieved in vitro.*

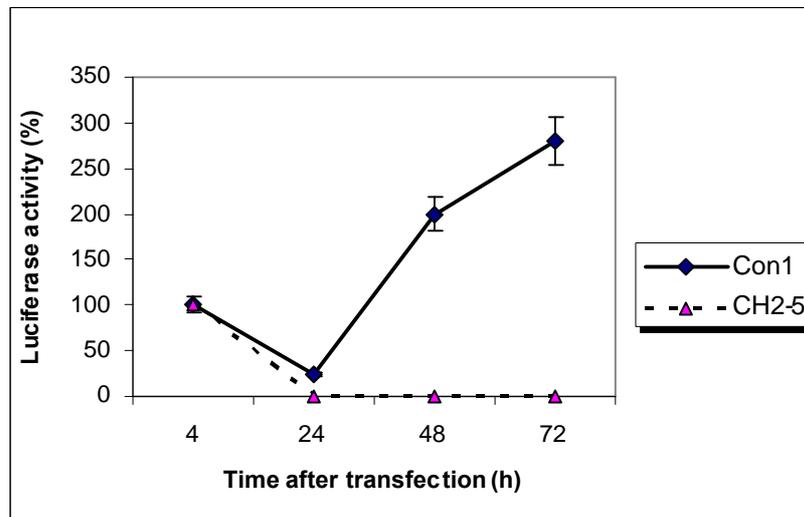
The data, presented in the previous sections, demonstrated that chimeric Con1/AD78 replicons CH2-5 and CH2-6 were not able to replicate in cell culture. Because all Con1/AD78 constructs are identical, with the only difference that each of them contains a NS3 gene derived from different HCV AD78 isolates, we hypothesized that CH2-4 replicon would be able to rescue the replication of CH2-5 or CH2-6, when these defective RNAs are transfected into Huh7 cell clone harbouring a replicating CH2-4 replicon. For these experiments it was essential to develop CH2-5 or CH2-6 variants, containing the fire luciferase gene as reporter instead of Neo-resistance gene, and to monitor the replications level of the defective replicon by a quantification of the expressions level of luciferase in the cells. To obtain defective replicon with luciferase as reporter gene, a prototype Con1 based replicon was used as a backbone, into which the NS3 gene of defective replicon CH2-5 was subcloned, using BsrGI and BssHI restrictions sites (Fig. 30). Obtained plasmid was subsequently restricted with PvuI and ScaI. The linearized plasmid was used for RNA transcription *in vitro*. The prepared RNA was used for transfection of Huh7 cells (Fig. 31 and 32). CH2-5 RNA was not able to replicate in naïve Huh7 cells in contrast to a positive control - Con1 replicon, which demonstrated, a high level of replication correlating with the increasing activity of luciferase measured in cells lysates (Fig. 31). These data demonstrated again that CH2-5 is defective for replication in cell culture.

To assess a possible rescue of the defective replicon, Huh7 cell clone bearing CH2-4 replicon was supertransfected with RNA derived from a defective replicon CH2-5, containing the luciferase gene. In parallel, these cells were also transfected by replication-competent Con1 replicon RNA. The transfection experiments were repeated four times and produced highly reproducible results. The results of three independent experiments are presented on Fig. 31 and 32. The RNA of Con1 was able to replicate in the retransfected CH2-4 cells but to the much lower levels than in the naïve Huh7 cells (Fig. 31). In contrast, the CH2-4 cell clone did not support the replication of CH2-5 RNA (Fig. 32). These results suggested that the replication of CH2-5 replicon bearing a defective NS3 gene could not be rescued by the replication complex of a stably replicating replicon CH2-4.

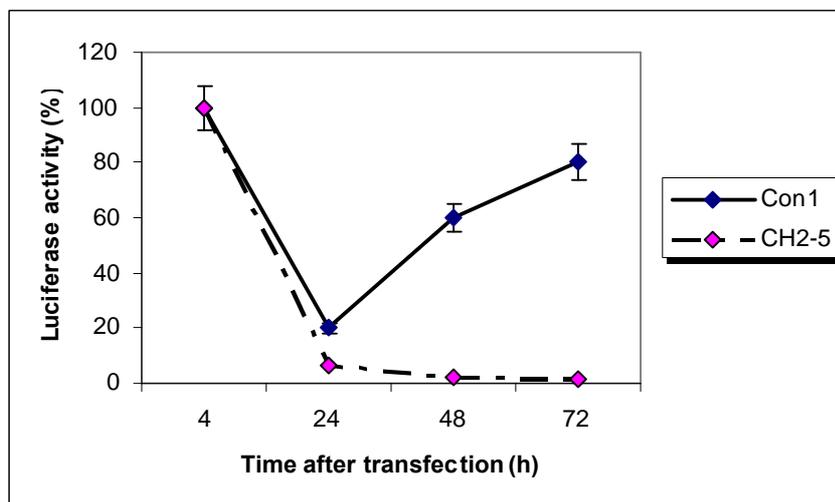


**Fig. 30. Creation of CH2-5 replicon bearing a luciferase gene.**

Sequence derived from patient 4 and Con1 replicons are depicted in yellow and in black, respectively. White box correspond to sequence of luciferase reporter gene.



**Fig. 31. Monitoring of the replication of Con1 and CH2-5 in naïve Huh7 cells.** Cells were transfected with 5  $\mu$ g of in vitro synthesized RNA. Replication of HCV RNA was monitored by measuring the level of luciferase activity in cell lysates. Luciferase activity were measured in cell lysates at different time point and expressed as percent of RLU. Results are presented as mean  $\pm$ SD of the three independent experiments.

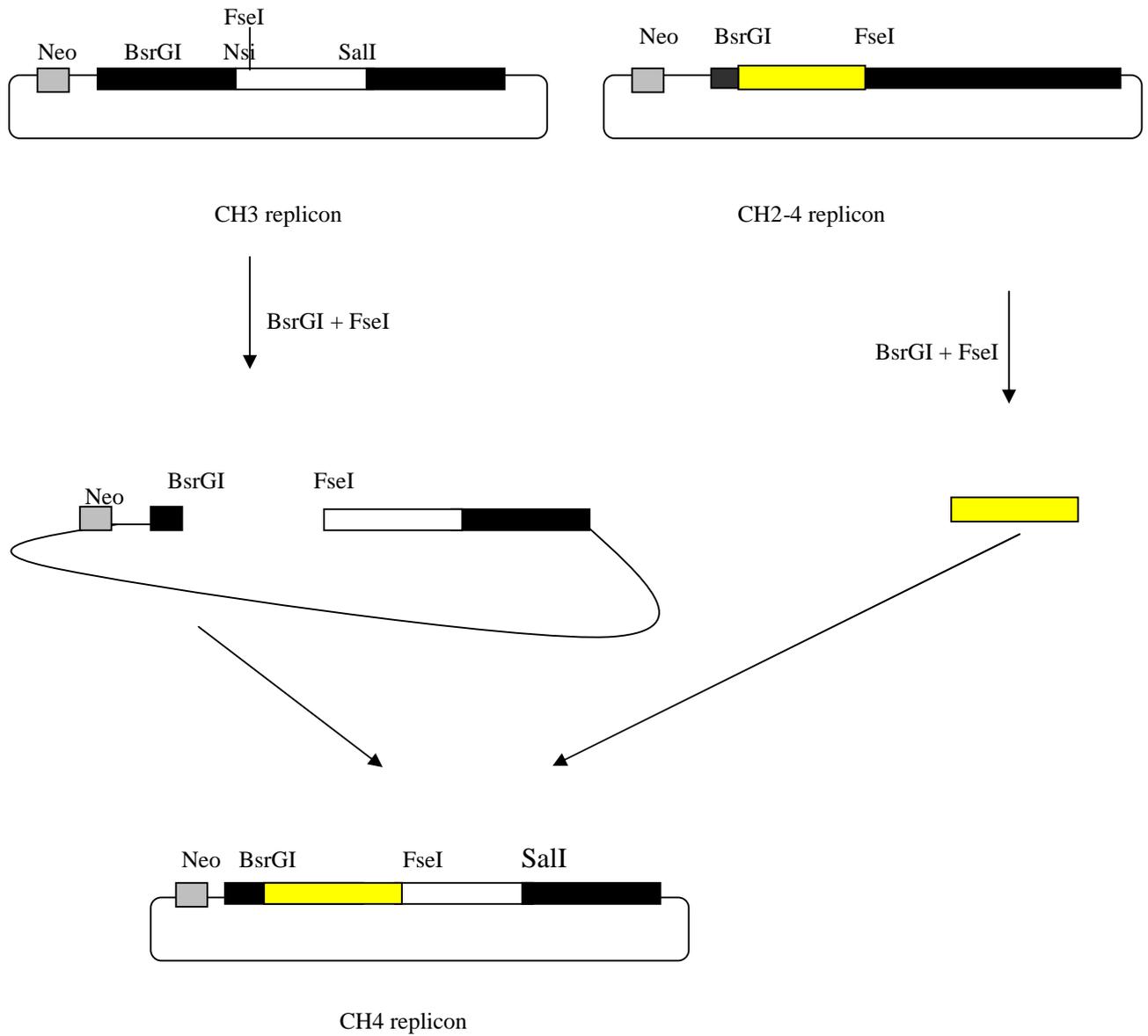


**Fig. 32. Replication of Con1 and CH2-5 in cell clones bearing CH2-4 replicon.** Cell clone CH2-4 was transfected with 5  $\mu$ g of in vitro synthesized RNA from Con1 and CH2-5 constructs. Replication of HCV RNA was monitored by measuring the level of luciferase activity in cell lysates. Luciferase activity were measured in cell lysates at different time point and expressed as percent of RLU. Results are presented as mean  $\pm$ SD of the three independent experiments.

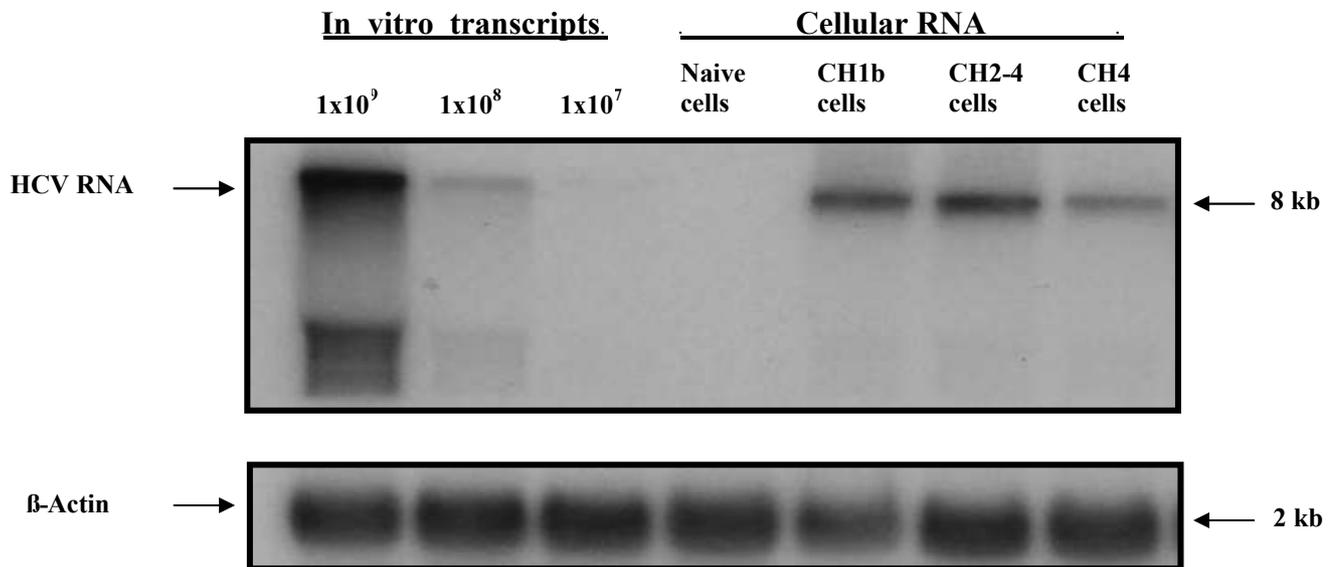
### **1.7. Generation of a Con1/AD78 chimeric replicon bearing the NS3 to NS5A AD78 sequence.**

One of the aims of our study was to develop a subgenomic replicon based on HCV AD78 sequence. In the experiments described above we have demonstrated a viability of the chimeric Con1/AD78 replicon CH3 bearing the sequence fragment NsiI-SalI (partially covering NS4B to NS5A genes) of the consensus sequence of HCV AD78 in the context of Con1 replicon (Fig. 11). In section 1.3 we reported on the development of the chimeric Con1/AD78 replicon CH2-4, which contained the NS3 sequence of AD78 isolate in the backbone of the Con1 sequence. This replicon was also able to replicate in the Huh7 cells (Fig. 24, 25). In the next stage of our study we decided to generate a new version of the chimeric Con1/AD78 replicon by transferring of the AD78-specific sequence (corresponding to a fragment of NS3) from CH2-4 replicon into the CH3 replicon. The details of the cloning procedure with a generation of a new chimeric Con1/AD78 replicon CH4 are presented on Fig. 33.

CH4 plasmid was linearized with PvuI and ScaI as previously described. HCV replicon RNA was synthesized and analyzed by agarose gel electrophoresis. Huh7 cells were transfected by electroporation and cultivated in presence of G418 (250 µg/ml). As expected, a number of G418 resistant colonies were observed after three weeks. These colonies were pooled and expanded in order to obtain a Huh7 cell clone persistently expressing a CH4 replicon. The presence of CH4 replicon RNA in the established cell clone was confirmed by sequencing of NS3 and NS5A genes amplified from cellular RNA. Additionally, Northern blot analysis (Fig. 34) confirmed the presence of replicating HCV replicon RNA in CH4 cell line.

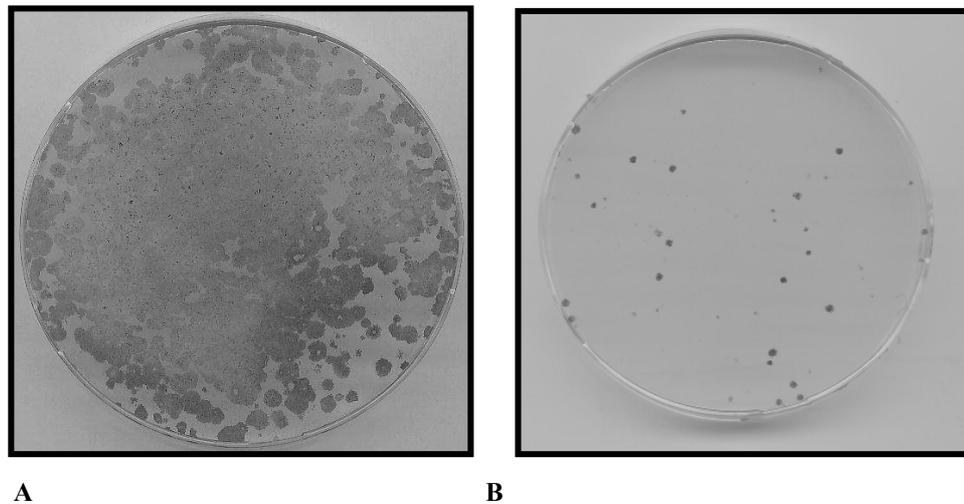


**Fig. 33. Construction of the chimeric replicon CH4.** CH3 represents the chimeric replicon containing a NS5A gene from AD78 consensus sequence. CH4 was obtained by swapping the NS3 gene of CH3 with the homologous gene derived from AD78 patient 4. Sequences derived from patient 4 and Con1 replicon are depicted in yellow and black, respectively. White box represents sequence of AD78 consensus sequence. Neomycin selectable marker is designated as Neo.



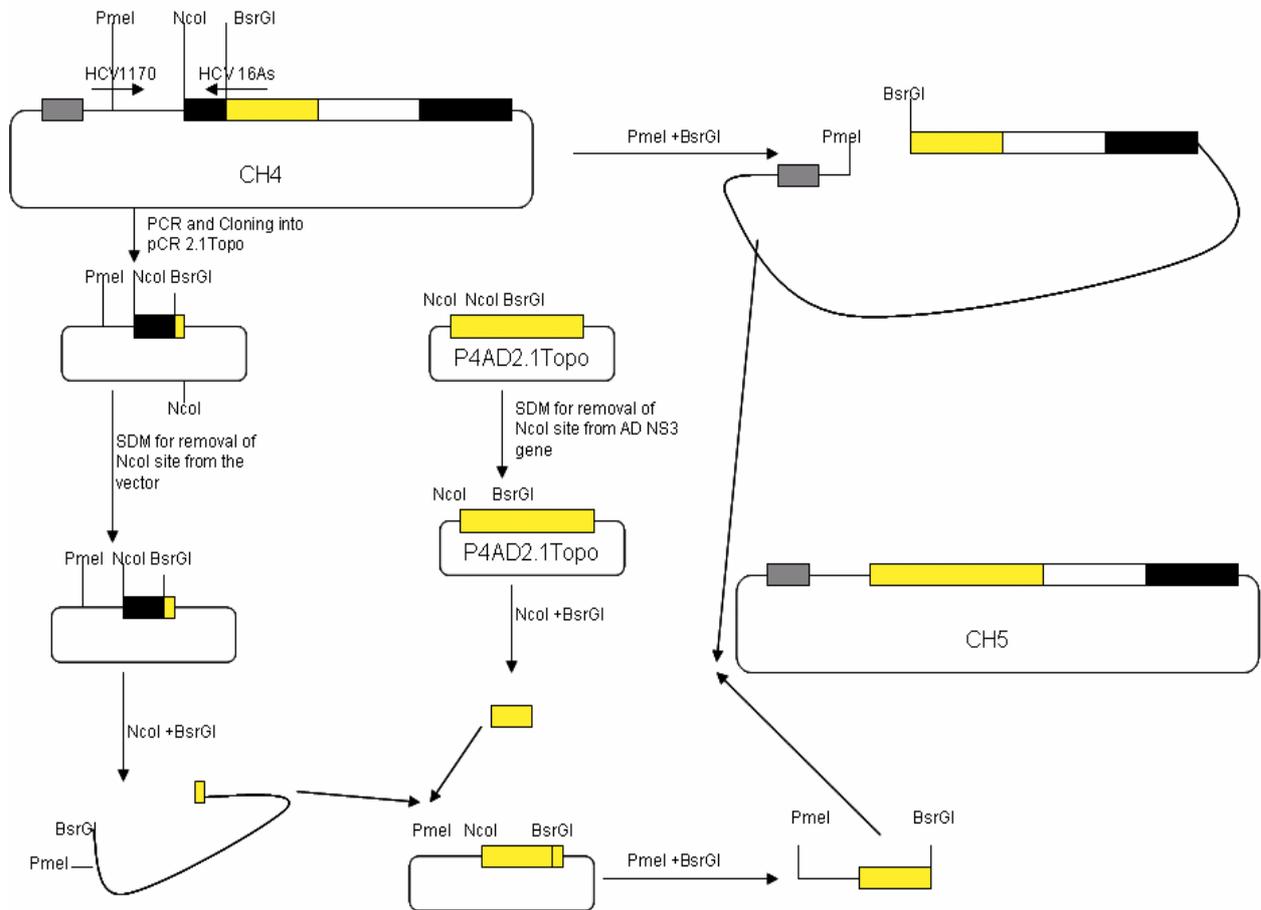
**Fig. 34. Northern blot analysis of cellular RNA from Huh7 cells bearing the chimeric replicons.** Total RNA from Huh7 cell clones bearing CH1b, CH2-4, and CH4 replicons was analysed by Northern blot. HCV RNAs and  $\beta$ -Actin mRNA were detected by hybridization to a riboprobe complementary to the Neo gene or to  $\beta$ -Actin mRNA. Different amount of in vitro transcripts were used as standard. RNA isolated from naïve Huh7 cells served as negative control.

The efficiency of colony formation after transfection of Huh7 cells with the transcripts of CH4 plasmid was higher than that observed with the RNA transcribed from the CH2-4 replicon construct (Fig. 35). Additionally, we have observed that transfection of cells with the total RNA extracted from the established CH4 cell line yielded more G418-resistant colonies than transfection with the RNA, transcribed in vitro from the linearized CH4 plasmid. The last observation most probably reflects the appearance of adaptive mutation(s) during the selection of G418-resistant CH4 cells. If this is the case, then this adaptive mutation(s) occurred within the Con1 sequence because the sequencing of the AD78-specific fragment amplified from the CH4 cells did not reveal any amino acid substitution in comparison with the original AD78-specific sequence present in the original CH4 construct.



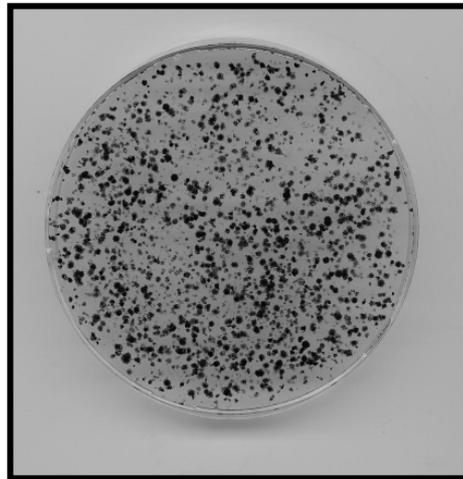
**Fig. 35. Colony formation efficiency of CH4 replicon versus CH2-4 replicon.** Huh7 cells were transfected with 5  $\mu$ g of CH4 or CH2-4 transcribed RNA. Transfected cells were cultivated 3 weeks in presence of G418 (250  $\mu$ g/ml), and resistant colonies were stained with crystal violet. (A) Huh7 cells transfected with CH4 RNA. (B) Huh7 cells transfected with CH2-4 RNA.

Within the NS3 gene of CH4 as well as of CH2-4 replicons the first 77 amino acids were derived from the Con1 replicon. We attempted to generate a new variant of chimeric Con1/AD78 replicon with a complete AD78-specific NS3 gene sequence. Considering the fact that the NS3 gene fragment, amplified from AD78-infected patient 4, contained more than one recognition site for NcoI, it was difficult to clone the complete sequence of this gene in one step. For getting a new replicon variant with a complete NS3 sequence from patient 4, a series of cloning was performed (Fig. 36). First, an intermediate plasmid was created by cloning of the Pme-BsrGI fragment amplified from CH4 into the pCR2.1Topo vector. The recognition site of NcoI was subsequently removed from the vector sequence by site-directed mutagenesis. The NS3 gene amplified from patient 4 was also cloned into pCR2.1Topo vector. From this vector an NcoI recognition site in the NS3 sequence was removed by site-directed mutagenesis. An additional intermediate vector was created by swapping an NcoI-BsrGI fragment from the first intermediate vector with the homologous fragment from patient 4. Subsequently the PmeI-BsrGI fragment from CH4 was swapped with the corresponding fragment from the last intermediate plasmid that contains BsrGI-NcoI fragment from patient 4. The obtained replicon was designated as CH5 (Fig. 36).



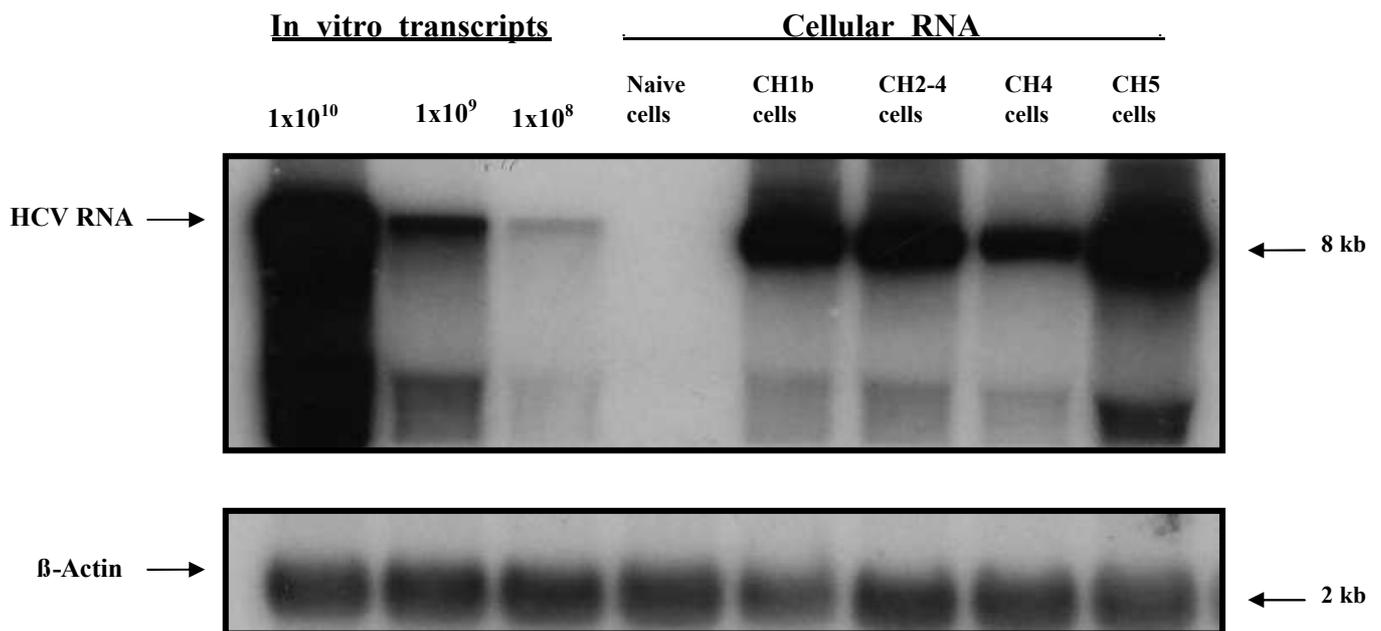
**Fig. 36. Generation of the chimeric Con1/AD78 replicon CH5.** Sequences derived from patient 4, AD78 consensus sequence, and Con1 replicons are depicted in yellow, white and black, respectively. Sequence of EMCV IRES is represented as solid line.

The prepared CH5 plasmid was linearized with PvuI and ScaI and subjected to RNA transcription in vitro. The correct size of the HCV subgenomic replicon was confirmed by analysis of the synthesized RNA on agarose gel. To assess an ability of CH5 to replicate in vitro, Huh7 cells were transfected with CH5 RNAs and transfected cells were cultivated in presence of G418 (250 µg/ml). G418-resistant cell clones were observed 3 weeks following the selection (Fig. 37). Because the expansion of individual cells clones turned to be difficult, cell clones were mixed to obtain a cell line CH5 that continuously expressed the HCV RNA.



**Fig. 37 . Colonies formation of Huh7 transfected with CH5 RNA.** Two µg of in vitro transcribed RNA from CH5 construct were transfected into Huh7 cells. Cells were cultivated in the presence of G418 (250 µg/ml). Three weeks following the selection, G418 -resistants cell clons were obtained and stained with crystal violet.

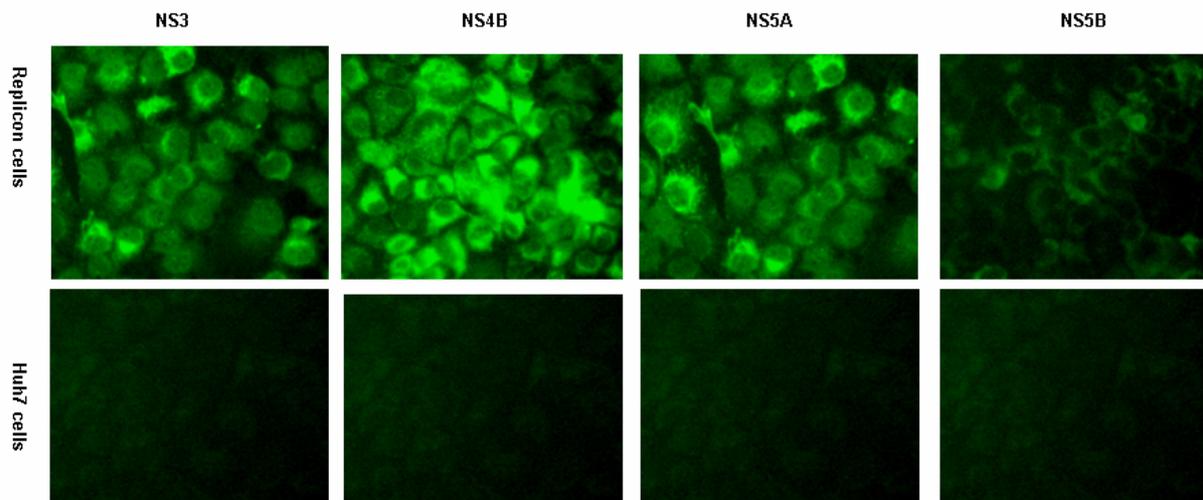
To confirm the presence of subgenomic HCV RNA in G418-resistant cells, total RNA was isolated from CH5 cell line and analyzed by Northern blot. As shown in Fig. 38, an 8 kb band, which was equivalent in size to the synthetic HCV subgenomic RNA used for RNA transfection was detected in the total RNA sample extracted from CH5 cells.



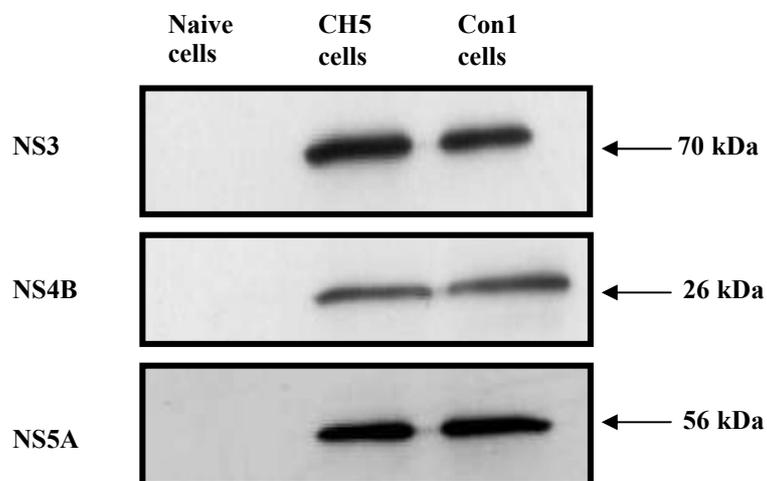
**Fig. 38. Northern blot analysis of cellular RNA of cells transfected with CH5 RNA.** Total RNA from Huh7 cells bearing CH1b, CH2-4, CH4 and CH5 replicons was analysed by Northern blot. HCV RNAs and  $\beta$ -Actin mRNA were detected by hybridization to a riboprobe complementary to the Neo gene or to  $\beta$ -Actin mRNA. Different amount of in vitro transcripts were used as standard. RNA isolated from naïve Huh7 cells served as negative control.

Production of HCV non-structural proteins (NS3, NS4B, NS5A, and NS5B) in CH5 cell clone was demonstrated by indirect immunofluorescence and by Western blot (Fig. 39). No signal was observed in untransfected Huh7 cells. Taking together, these results allowed us to conclude that CH5 RNA replicates efficiently in Huh7 cells.

A



B

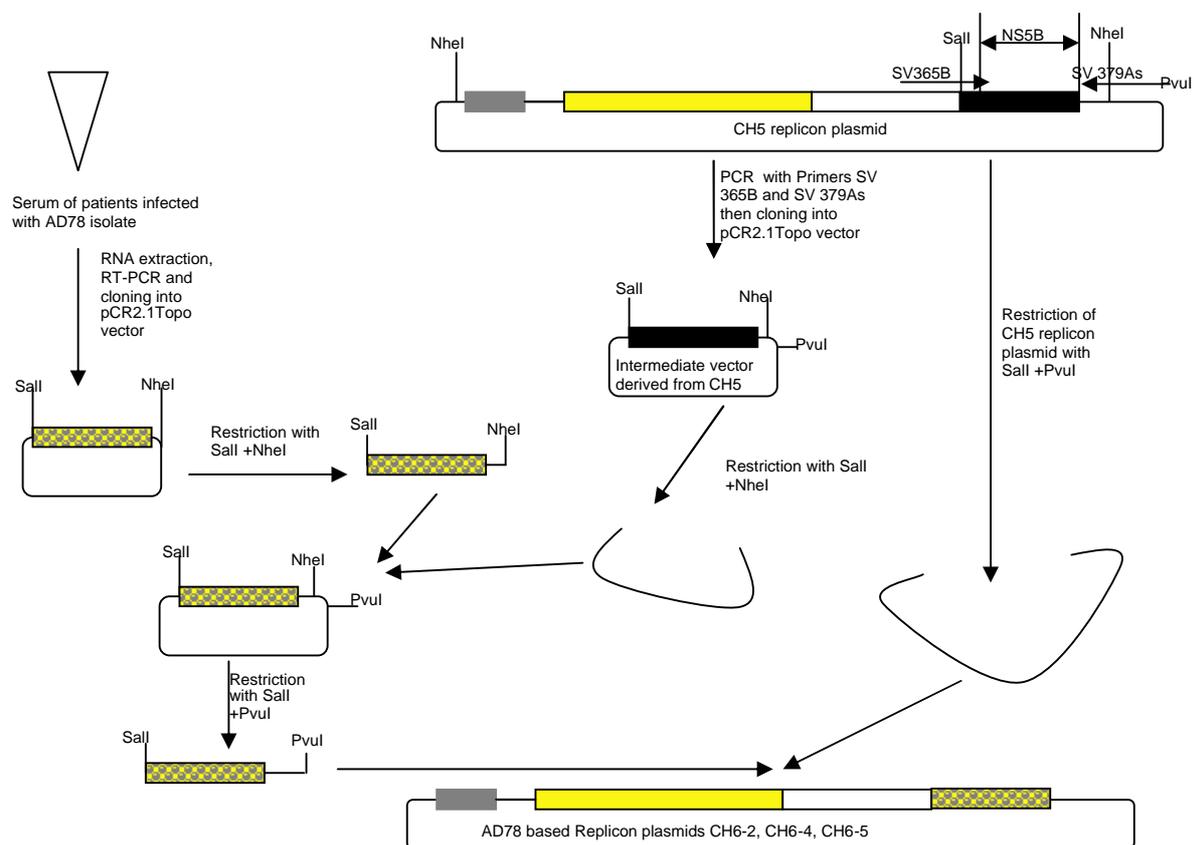


**Fig. 39. Detection of HCV proteins in CH5 cell clone.**

(A) Indirect immunofluorescence detection of HCV Antigens. CH5 cells lines and untransfected Huh7 cells were grown on coverslips. After fixation with Methanol-Acetone mixture (1:1[vol/vol]), cells were incubated with antibodies anti-NS3, anti-NS4B, NS5A and anti-NS5B respectively. (B) Western blot detection of HCV protein in transfected cells. Equal amount of supernatant from cell lysates of untransfected Huh7 cells (Naïve cells), Huh7 CH5 replicon cells (CH5 cells), and Huh7 Con1 replicon cells (Con1 cells) were separated by SDS-PAGE and analyzed by using antibodies against NS3, NS4B and NS5A proteins of HCV.

### 1.8. Identification of a functional NS5B sequence of AD78 strain.

We have demonstrated that the chimeric replicon CH5, which bears the NS3 to NS5A sequence derived from the AD78 strain in the back-bone of Con1 replicon, replicates efficiently in Huh7 cells. Thereby, to generate a complete AD78-based replicon we needed to install a functional NS5B sequence of AD78 strain into the CH5 replicon. Considering the fact that the consensus sequence of AD78 NS5B turned out to be “non-functional” (Fig. 11), an alternative approach would have been the use of NS5B sequence from patients infected with HCV AD78.



**Fig. 40. Cloning procedure used for generation of AD78 based replicon variants.** The sequence covering up the fragment from the carboxyterminal domain of NS5A to the polyU/UC tract of HCV AD78 was amplified from infected patient sera and cloned into intermediate vector, which contained the corresponding fragment derived from CH5 replicon. Cloning of AD78 sequences into the intermediate vector was performed by using SalI and NheI enzymes. AD78-based replicons CH6-2, CH6-4 and CH6-5 were obtained after substitution of the sequence covering up the fragment from the carboxyterminal domain of NS5A to the polyU/UC tract of CH5 replicon with the homologous sequence of the intermediate vector containing sequences amplified from patients' sera by using SalI and PvuI enzymes.

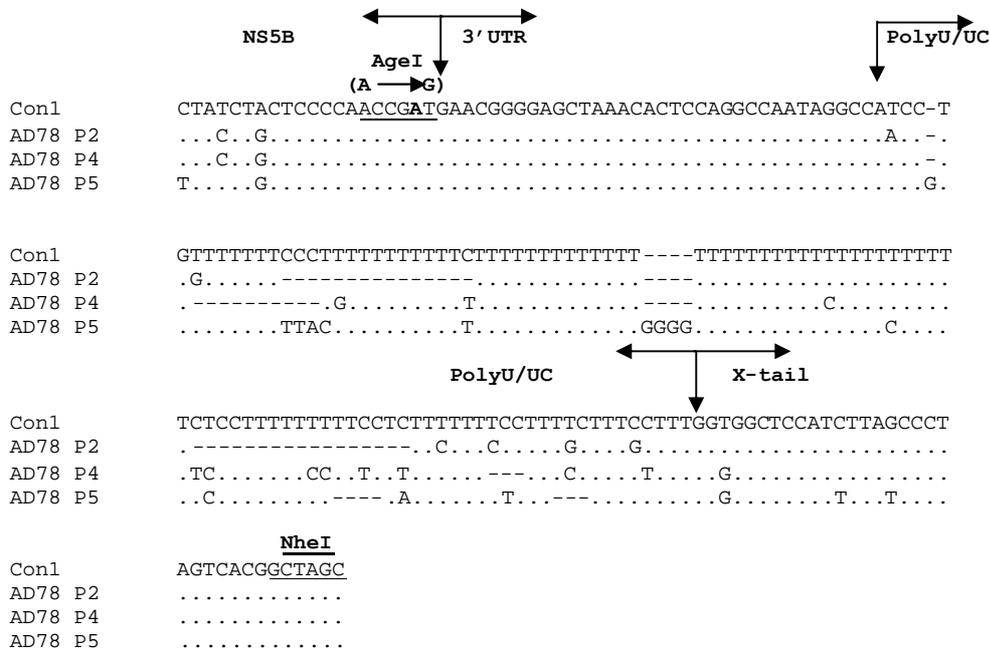
To accomplish this task, sequences, corresponding to the fragment from the carboxyterminal domain of NS5A to the poly-U/UC tract, were amplified from three AD78-infected patients and subjected to a cloning procedure depicted at Fig. 40. As a result, three variants of AD78-based replicon were generated. These replicon variants were designated CH6-2, CH6-4, and CH6-5.

To assess the replication potentials of these AD78-based replicons, three prepared plasmids were restricted with PvuI and ScaI as previously described. HCV replicon RNAs were transcribed and transfected into Huh7 cells. Transfected cells were cultivated for 5 weeks in presence of G418 (250 µg/ml). Despite several attempts, no G418-resistant colonies appeared after this manipulation. These results suggested that the amplified NS5B sequences from patients' sera were "defective" and somehow prevent the HCV RNA replication.

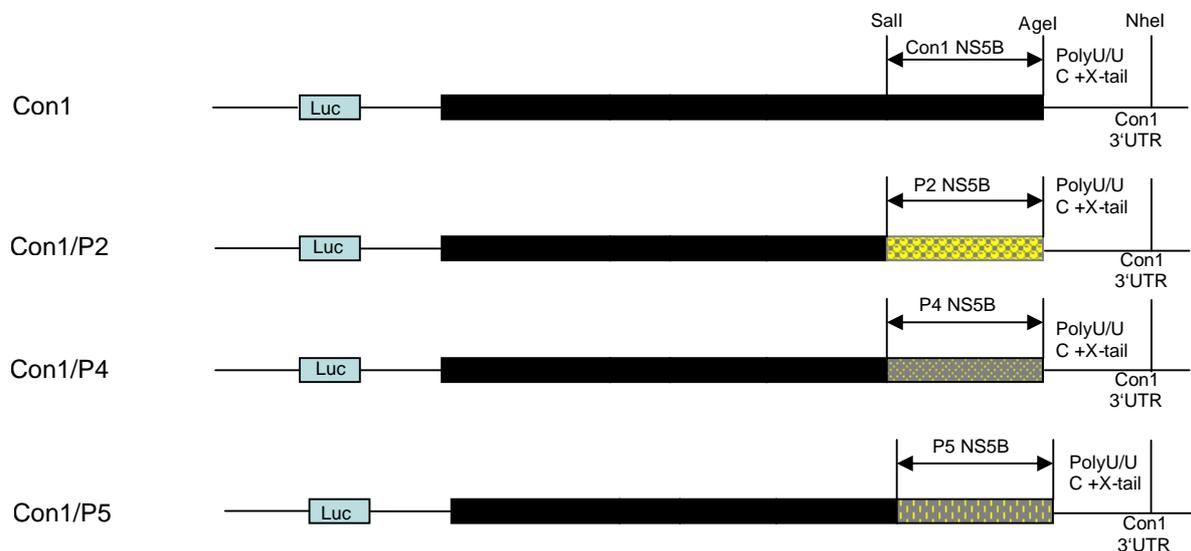
In order to identify a possible structure element of NS5B sequence, responsible for such "replication-inhibitory effect", three AD78 sequences were compared with the corresponding "functional" sequence of Con1 replicon (Fig. 41 A). First, we could observe that these three sequences display different length of their polyU/UC tract. Since data reported in previous studies (Friebe et al., 2002; Gates et al., 2003) suggested that the length of the polyU/UC sequence influence replication of HCV in Huh7 cell culture, we hypothesized that the difference in the polyU/UC length observed among all three sequences amplified from AD78 patients would be the reason why these sequences are defective. To test this hypothesis we have substituted the NS5B encoding sequence of the Con1-based replicon, expressing luciferase as a reporter gene, with the corresponding fragments from three AD78-derived sequences (Fig. 41B). As a result, three variants of chimeric Con1/AD78 replicon (Con1/P2, Con1/P4, and Con1/P5) that differed only in their NS5B sequences were generated; the polyU/UC fragment in all these constructs was identical and derived from Con1(Fig. 41 B). These three plasmids were linearized, transcribed into RNAs and the RNAs were transfected into Huh7 cells as previously described. The replication of these RNAs was monitored by measuring the luciferase activity in cell lysates at different time points after transfection as previously described. The Con1 replicon and the replication-defective  $\Delta$ GDD mutant of Con1 replicon were used as a positive and a negative controls, respectively. The results of these experiments were negative, even 72 h after transfection all three Con1/AD78 replicon chimeras failed to replicate in cell culture.

These data suggested that the failure of all three AD78-based replicons CH6-2, CH6-4 and CH6-5 to replicate was not due to the difference observed in the length of their polyU/UC tract and, most probably, were associated with differences in the sequence of their NS5B genes.

A



B



**Fig. 41. Generation of Con1/AD78 chimeras bearing NS5B genes amplified from different patients' sera.** (A) Comparison of the 3'UTR sequence of Con1 with sequences of AD78 isolates amplified from patients P2, P4 and P5. Substitution of A for G (A to G) shown on the alignment was performed to create a recognition site of AgeI at the end of NS5B sequence of Con1 and all the three AD78 sequences in order to facilitate cloning of NS5B amplified from different patients by using Sall and AgeI enzymes for cloning. (B) Representation of different chimeras obtained after substitution of the NS5B gene of Con1 based replicon with the homologous sequence amplified from patients infected with AD78 isolate. The 3'UTR of Con1 based replicon was conserved in all these three hybrid constructs.

Comparison of the sequences of three defective AD78-based replicons with that of the consensus AD78 sequence and with those from several functional HCV replicons was performed (Fig. 42). It has shown that all AD78-related sequences (within the Sall-AgeI fragment used for the swapping) contained some unique amino acid exchanges at several positions (Fig. 42). Additional investigations of HCV sequences published in the HCV database (Los Alamos HCV Sequence Database) also revealed the presence of uncommon amino acids residues within the Sall-AgeI fragment of all AD78-related sequences. In order to generate a functional NS5B sequence of the AD78 isolate, an attempt was made to use the site-directed mutagenesis to change a number of amino acid residues in the AD78 sequence corresponding to the Sall-AgeI fragment. A sequence P5, derived from AD78-infected patient 5, and which differed within the Sall-AgeI fragment from the sequence of Con1 replicon only by eight amino acids, was chosen as a target. Among these eight substitutions, three were found to be common for all our AD78 sequences, including the consensus sequence of AD78 strain. Five substitutions were present only in the sequence amplified from patient P5. The detailed information on all these substitutions is presented in Table 4. We decided to perform a sequential site-directed mutagenesis of these eight amino acid residues in the chimeric plasmid Con1/AD78-P5, which contained the Sall-AgeI fragment of P5 in the back-bone of Con1 replicon. As a result of the mutagenesis, all these eight amino acids were substituted with their counterparts present in the Con1 sequence. Table 5 illustrates the scheme of the sequential site-directed mutagenesis of the plasmid Con1/AD78-P5. Overall, eight variants of Con1/AD78-P5 replicon bearing the mutated Sall-AgeI fragment of P5 were generated. Mutant variants Con1/P5 M1, M2, M3 M4 M5, M6, M7 and M8 were tested for replication in Huh7 cells. Con1 and defective mutant Con1/GDD replicon RNAs were used as positive and negative controls, respectively. As a result, a minimal luciferase activity comparable to the levels detected in the lysates of cells transfected with RNA of the defective mutant Con1/GDD was detected 72 h after transfection in the lysates of cells transfected with RNA from mutant variants Con1/P5 M1 to Con1/P5 M5 (Fig.43). In contrast, in the lysates of cells transfected with RNA of mutants Con1/P5 M6, Con1/P5 M7 and Con1/P5 M8, which have been obtained by a sequential mutation of the Con1/P5 M5, luciferase activity were detected with different levels 72 h after transfection (Fig. 43).

	SalI	413
Con1	ESTVSSALAE <del>L</del> ATKTFGSS <del>E</del> SSAVDSGTATASPDQPSDDGDAGSDVESYSSMPPLEGE <del>P</del> G	
HCV-BK	..S.....L...A.....K.....	
HCV-O	.....G.....P..G.....A.....	
HCV-M1LE	.....G...A.....P...A.....K.....	
HCV-N	D....V.....L..A.....P...T..N.GKD..A..C.....	
AD78 -Cons	.....G.....P.....G..T.....	
AD78 -P2	.....G.....P.....S.....	
AD78 -P4	.....G.....P.....G..T...G.....	
AD78 -P5	.....G.....P.....S..... <b>H</b> .....	
	← NS5A      NS5B →	
	430	
Con1	DPDLS <del>D</del> GSWSTVSE <del>E</del> ASE <del>D</del> VVCCS <del>S</del> MSY <del>T</del> WTGALITPCAAE <del>E</del> T <del>K</del> L <del>P</del> INALS <del>N</del> SL <del>R</del> HH <del>N</del> LV	
HCV-BK	.....S.....M.....	
HCV-O	.....S.....M.....	
HCV-M1LE	.....S.....P.....M.....	
HCV-N	.....G.S.....S.....M.....	
AD78 Cons	...N.....S.....M.....	
AD78 -P2	..G.....S.....M.....	
AD78 -P4	.....G..A.....SS.....R.M.....	
AD78 -P5	..... <b>E</b> .....S.....M.....	
	50	
Con1	YATTSRSASL <del>R</del> Q <del>K</del> KVTFDR <del>L</del> QVLD <del>D</del> HYR <del>D</del> V <del>L</del> KEMKAKAST <del>V</del> KAKLLS <del>V</del> E <del>E</del> ACKLTPPHSA	
HCV-BK	.....G.....	
HCV-O	.....Q.....	
HCV-M1LE	.....G.....I.....	
HCV-N	.....G.....I...R.....	
AD78 Cons	.....Q.....R.....	
AD78 -P2	.....Q.....V...R.....	
AD78 -P4	.....Q.....M.....R.....	
AD78 -P5	.....Q... <b>R</b> .....R.....	
	120	
Con1	RSKFGYGAKDVRNLSKAVNH <del>I</del> RSVW <del>K</del> DLLEDTETPIDTTIMAKNEVFCVQPEKGG <del>R</del> KPA	
HCV-BK	K.....H.....V.....	
HCV-O	.....H.....V.....	
HCV-M1LE	K..Y.....R.....E.....S.....	
HCV-N	K.....R.I.....E.....V.....V.....	
AD78 Cons	K.....I... <b>N</b> ...E.....	
AD78 -P2	K.....I... <b>N</b> ...E...A.....G.....	
AD78 -P4	K.....I... <b>N</b> ...E.....	
AD78 -P5	K.....I... <b>N</b> ...E.....	
	209	
Con1	RLIVFPDLGVRVCEK <del>M</del> ALYDVVSTLPQAVMGSSYGFQYSPGQRVEFLVNAW <b>K</b> AKKCPMGF	
HCV-BK	.....V.....T..S..N...	
HCV-O	.....S..T...	
HCV-M1LE	.....K.....T..S...	
HCV-N	.....K...S..N...	
AD78 Cons	.....V.....H.....Y..K... <b>SS</b> ..N...	
AD78 -P2	.....K... <b>SS</b> ..N...	
AD78 -P4	.....K... <b>SS</b> ..N...	
AD78 -P5	.....K... <b>SS</b> ..N...	
	CTL epitope	
	248	
Con1	AYDTRCFDSTVTENDIRVEES <del>I</del> YQCCLAP <b>E</b> ARQAIRSLTERLYIGGPLTNSKGQNC <del>G</del> YR	
HCV-BK	S.....K.....	
HCV-O	S.....KL..K.....V.....	
HCV-M1LE	S.....K.....S.....	
HCV-N	S.....I.....	
AD78 Cons	.....I.....	
AD78 -P2	.....I.....	
AD78 -P4	.....I.....	
AD78 -P5	..... <b>D</b> .....	
	525	566
Con1	AATCGKYL <del>F</del> NWAVR <del>T</del> KL <del>K</del> LTPIPAASQLDLSSWFVAGYSGGDIYHSL <b>S</b> RARPRWFMWCLL	
HCV-BK	.....K.....R...G.....L...	
HCV-O	.....R...G.....	
HCV-M1LE	.....K.....R...G.....	
HCV-N	.....K.....G.....L...	
AD78 Cons	.....R...G..... <b>P</b> ...WF...	
AD78 -P2	.....A..R..G.....H... <b>H</b> .....	
AD78 -P4	.....R...G..... <b>P</b> .....	
AD78 -P5	..... <b>P</b> .....R...G..... <b>P</b> .....	

**Fig. 42. Amino acids sequences comparison of NS5B genes from functional replicons of genotype 1b with sequences of AD78 isolate. Bold letter correspond to amino acids residues only found in the sequence amplified from patient 5 or common to all AD78 sequence.**

**Table 4.** Difference in amino acid composition within the SalI-AgeI fragment between Con1 and AD78 P5 sequences.

HCV Proteins	Amino acid position	Amino acid residue in Con1	Amino acid residue in AD78 P5
NS5A	413	Y	H
	430	D	E
NS5B	50	K	R
	120	R	N*
	209	K	S*
	248	E	D
	525	L	P
	566	R	P*

(\*) Amino acid residues present in the sequences of all AD78 isolates.

**Table 5.** Scheme of the site-directed mutagenesis of the chimeric Con1/AD78-P5 replicon.

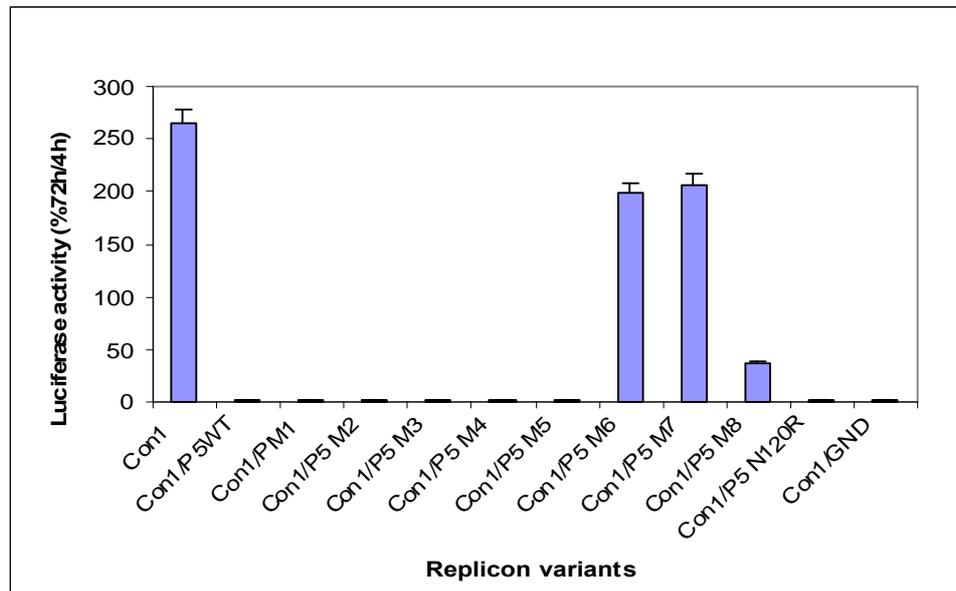
Con1/AD78-P5 mutant variants	Amino acids substitutions
Con1/P5 wt	Without mutation
Con1/P5 M1	R50K
Con1/P5 M2	R50K+ E248D
Con1/P5 M3	R50K + E248D + P525L
Con1/P5 M4	R50K + E248D + P525L + H413Y
Con1/P5 M5	R50K + E248D + P525L + H413Y + D430E
Con1/P5 M6	Con1/P5 M5 + N120R
Con1/P5 M7	Con1/P5 M6 + S209K
Con1/P5 M8	Con1/P5 M7 + P566R

The detected luciferase activity from Con1/P5 M8 transfected cells was about 7 folds lower than that detected in the lysates of cells transfected with RNA of Con1 replicon. Con1/P5 M6 and Con1/P5 M7 transfected cells, however, displayed a luciferase activity slightly lower (1.6 and 1.2 times, respectively) than that detected in the lysates of cells transfected with RNA of Con1 replicon. These observations suggested that mutants Con1/P5 M6, Con1/P5 M7 and Con1/P5 M8 were able to replicate in Huh7 cells and that the mutated NS5B gene of AD78 isolate (or more precisely, the SalI-AgeI fragment of P5) can be used for the generation of a subgenomic replicon based on the complete sequence of AD78 isolate.

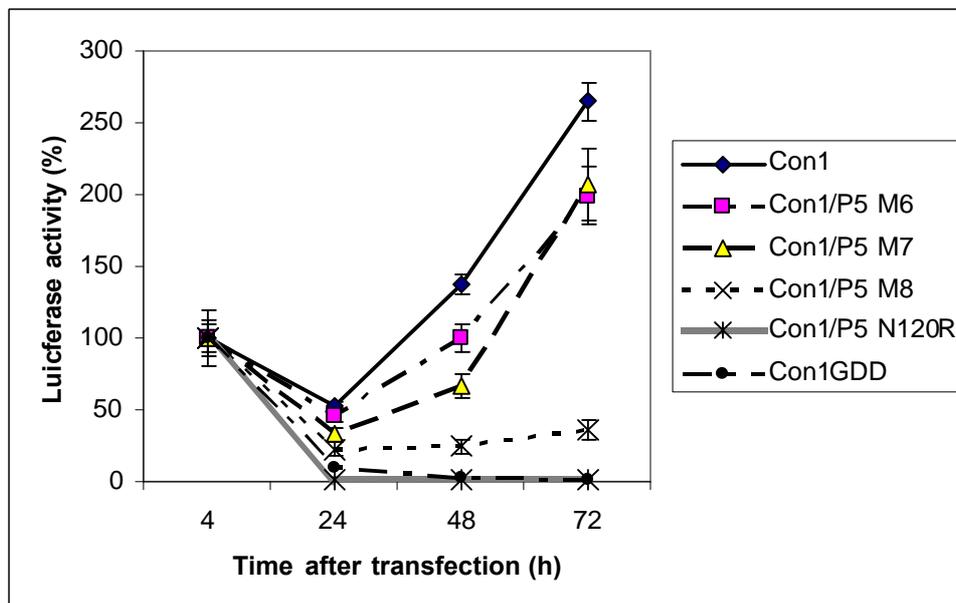
Since the substitution of asparagine for arginine at position 120 (N120R) within the NS5B sequence of Con1/P5 M5 rescues the replication of this construct, the question was to know if individually this mutation could have the same effect on Con1/AD78-P5 wild type. Despite several attempts, introduction of N120R mutation into the Con1/AD78-P5 wild type construct did not lead to a rescue of Con1/AD78-P5 replication (Fig. 43). This observation suggests that the five amino acids residues previously mutated in Con1/AD78-P5 might have direct impact on its replication.

Because Con1/P5 M6 and Con1/P5 M7 RNAs replicate efficiently, we assumed that substitution of the SalI-AgeI fragment in CH5 construct with the corresponding fragment from Con1/P5 M6 would lead to a viable replicon based on AD78 sequence. Such a construct designated AD78M6 replicon was prepared and tested for a replicative ability in Huh7 cells. As expected, the AD78 RNA was able to replicate under G418 selection and formed colonies (Fig. 44). G418-resistant cells were expanded and replication of HCV RNA in the established cell line was confirmed by Northern blot and Western blot analysis (Fig. 45).

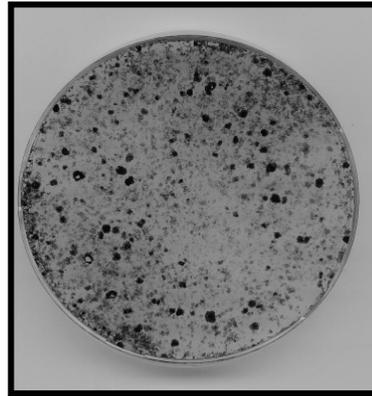
A



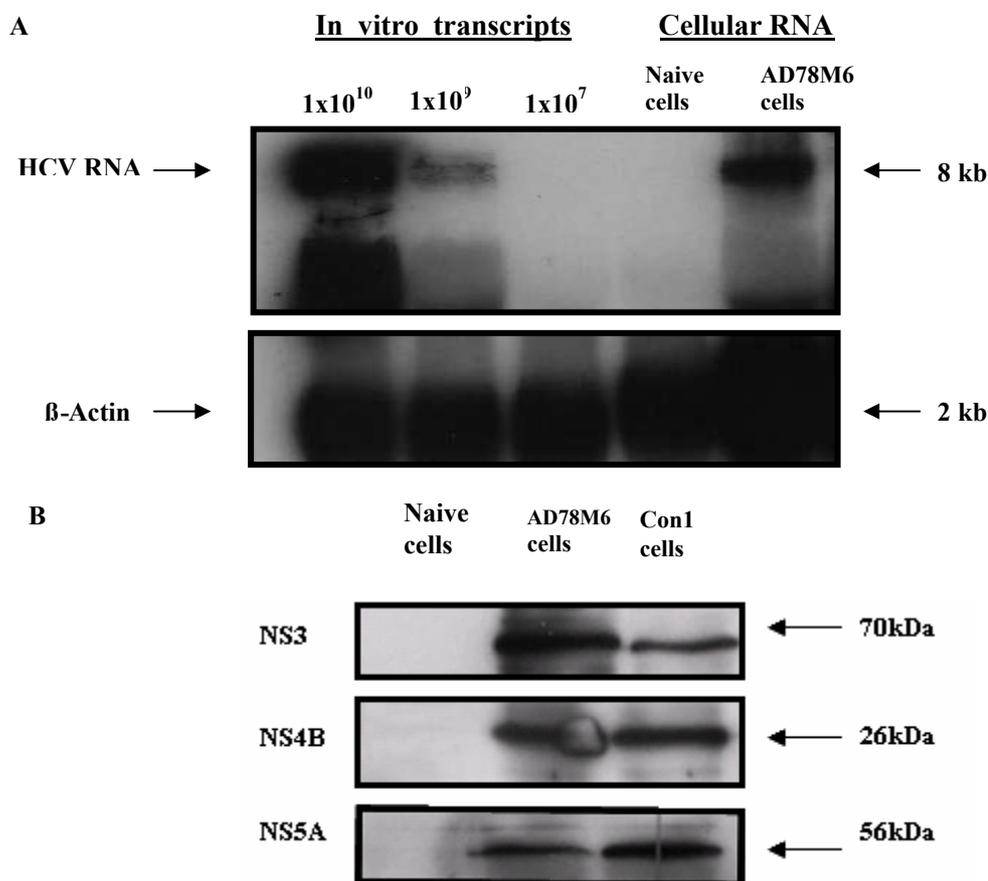
B



**Fig. 43. Replication of the hybrid replicon Con1/AD78-P5 after sequential substitutions of the uncommon amino acids residues found in NS5B of the amplified sequence.** Huh7 cells were transfected with RNA from different variants. Luciferase activity in the lysates of transfected cells was measured at different time point and normalized for difference in transfection efficacy using the activity at 4 h after transfection. Con1 and Con1/GND defective mutant RNAs were used as positive and negative controls respectively. (A) Replication ability of all Con1/AD78-P5 mutants at 72 h after transfection. (B) Replication ability of functional Con1/AD78-P5 mutants measured at different time points after transfection. Results are presented as mean  $\pm$ SD of the three independent experiments.



**Fig. 44. Colony formation after transfection of Huh7 cells with AD78-based replicon RNA.** One  $\mu\text{g}$  of in vitro transcribed RNA from AD78 construct were transfected into Huh7 cells. Cells were cultivated in presence of G418 (250  $\mu\text{g}/\text{m}$ ). Three weeks following the selection, G418 -resistants cell clons were obtained and stained with crystal violet.



**Fig. 45. (A) Northern blot analysis of cellular RNA from Huh7 cells bearing the AD78 replicon.** Total RNA from Huh7 cell clones bearing AD78 M6 replicon was analysed by Northern blot. HCV RNAs and  $\beta$ -Actin mRNA were detected by hybridization to a riboprobe complementary to the Neo gene or to  $\beta$ -Actin mRNA. Different amount of in vitro transcripts were used as standard. RNA isolated from naïve Huh7 cells served as negative control. **(B) Western blot detection of HCV protein in transfected cells with AD78M6 replicon.** Equal amount of supernatant from cell lysates of untransfected Huh7 cells (Naïve cells), Huh7 AD78 M6 replicon cells (AD78M6 cells), and Huh7 Con1 replicon cells (Con1 cells) were separated by SDS-PAGE and analyzed by using antibodies against NS3, NS4B and NS5A proteins of HCV.

## **2. Characteristics of Huh7 cell clones bearing the intragenotypic chimeric Con1/AD78 replicon CH5.**

### **2.1. Mutations in the CH5 replicon.**

Appearance of adaptive mutations in the coding sequence of HCV subgenomic replicons during G418 selection was reported (Blight et al.; 2000, Lohmann et al.; 2001). One might have expected to see the emergence of adaptive mutations during the selection or/and propagation of CH5 replicon. To identify possible amino acid exchanges in this replicon, which might have occurred during the G418 selection, total cell RNA was prepared from CH5 cell clone 1 month after transfection, the sequence corresponding to non-structural genes (NS3 to NS5B) of CH5 replicon was amplified using expand long PCR system, and the resulting fragments were subjected to direct sequencing in order to determine the predominant sequence of the replicating HCV RNA. Comparison of the parental CH5 sequence with that amplified from CH5 cell clone revealed only one transversion from cytosine to guanine at position 8652 (the number corresponds to the nucleotide number of the HCV genotype 1b genome). This transversion resulted in the substitution of arginine for glycine at amino acid position 2884 (R2884G) in the predominant sequence of NS5B of CH5 (Table 6).

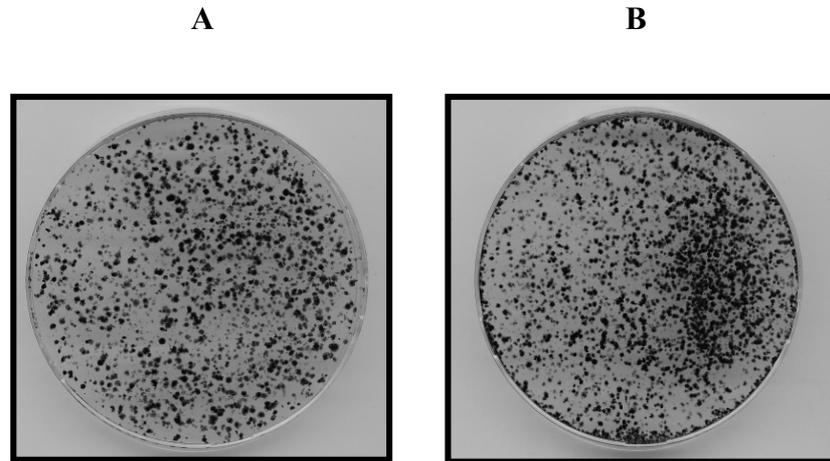
Since mutations accumulate in HCV genomes during virus replication, we were interested to know, whether a long-term cultivation of CH5 cell clones would result in appearance and accumulation of additional mutations in the non-structural genes of CH5 replicon. To address this issue, CH5 cell clone was cultivated during one year. The fragment, corresponding to non-structural genes (from NS3 to NS5B) and 3' UTR of CH5 RNA were amplified, and subjected to direct sequencing. The sequencing data demonstrated a conservation of the R2884G mutation in the NS5B protein of CH5 replicon, which has already been observed after one month of replication. Besides this mutation, an additional substitution of adenine for guanine was observed at nucleotide position 4827. This mutation resulted in the substitution of lysine for glutamic acid at amino acid position 1609 in the NS3 gene of CH5. Thus, the average mutation rate observed within the polyprotein of CH5 replicon was equal to  $3.9 \times 10^{-4}$  substitutions per amino acid position per year.

**Table 6.** Amino acid mutations occurred in the CH5 genome during selection and cultivation of G418-resistant clones.

Amino acids position	CH5 parental	CH5 clone 1 month cultivation	CH5 clone 1 year cultivation	Genes/Function
1609	K	K	E	NS3/Helicase
2884	R	G	G	NS5B/Polymerase

## 2.2. Evidence for adaptive mutations in CH5 replicon.

Since HCV subgenomic RNA usually require cell culture adaptive mutations for efficient replication in vitro (Blight et al.; 2000, Krieger et al.; 2001, Lohmann et al.; 2001), it was important to find out whether the mutations, which appeared in the NS3 and NS5B proteins of CH5 replicon, were adaptive mutations. In the first series of experiments, the efficacy of colony formation of total RNA from CH5 cells was compared with that of RNA, obtained by transcription in vitro from the parental CH5 construct. The Huh7 cells were transfected with 10 $\mu$ g of cellular RNA (containing 1x10<sup>8</sup> copies of HCV subgenomic RNA) extracted from CH5 cell clone bearing both K1609E and R2884G substitutions or with 1 $\mu$ g (corresponding to 2x10<sup>12</sup> copies of HCV subgenomic RNA) of RNA transcripts. As shown in Fig. 46, transfection of Huh7 cells with both RNA preparations resulted in appearance of comparable number of G418-resistant colonies. Taking into account the significant difference in the copy number of CH5-specific RNA between these two preparations (1x10<sup>8</sup> copies vs. 2x10<sup>12</sup> copies) one may conclude that efficacy of colony formation of the mutated CH5 RNA is evidently higher than that of the parental or “wild type” CH5 RNA.

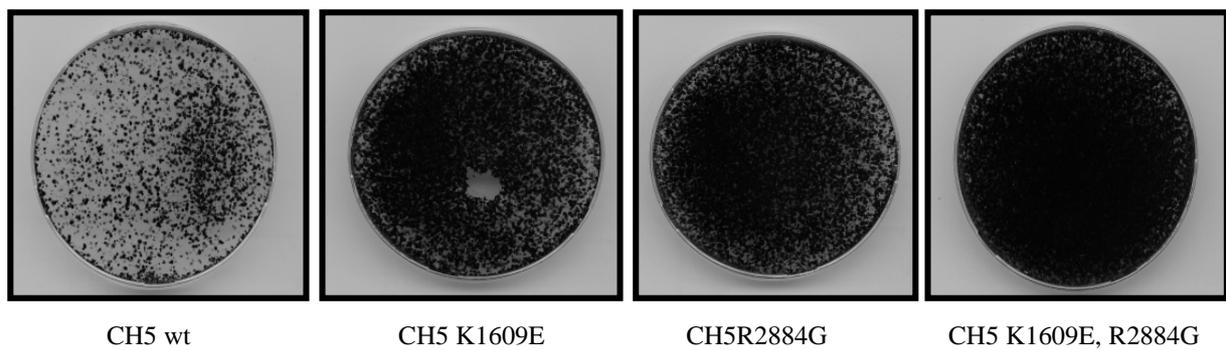


**Fig. 46. G418-resistant cell colonies present 3 weeks after transfection with CH5wt and cellular RNA extracted from CH5 cell clone.**  $1 \times 10^5$  Huh7-cells were independently transfected with  $1 \mu\text{g}$  of in vitro transcripts of CH5 wt or with  $10 \mu\text{g}$  of cellular RNA extracted from CH5 cell clone. Transfected cell were cultivated in a 10-cm-diameter culture dish and submitted to  $250 \mu\text{g/ml}$  of G418. Resistant cells clones were visible 3 weeks post-transfection and stained with crystal violet. (A) Presents cell lines transfected with  $1 \mu\text{g}$  of in vitro transcripts of CH5wt and (B) Presents cell lines transfected with  $10 \mu\text{g}$  of cellular RNA extracted from CH5 cell clone.

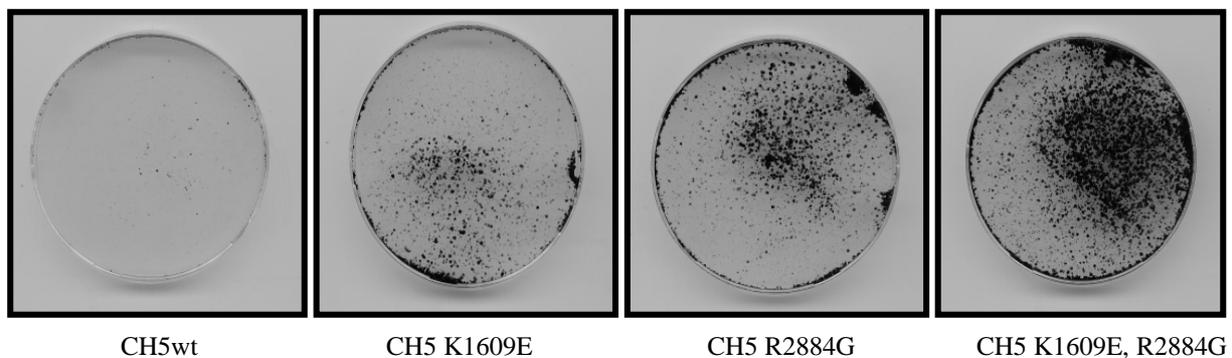
In the second series of experiments, the influence of each of the revealed mutation on HCV RNA replication was investigated. To this end, a site-directed mutagenesis was used to introduce these two mutations individually into the parental CH5 plasmid. As a result, three variants of the mutated CH5 plasmid were obtained: CH5 K1609E, CH5 R2884G, and CH5 K1609E, R2884G. The last plasmid carried both mutations. Naïve Huh7-cells were transfected with  $1 \mu\text{g}$  of in vitro transcripts of CH5 wild type, CH5 K1609E, CH5 R2884G and CH5 K1609E, R2884G. Cells were cultivated with  $250 \mu\text{g/ml}$  or  $500 \mu\text{g/ml}$  of G418 and after 3 weeks of selection the efficiency of colony formation (ECF) was determined. As presented in Fig. 47, transfection with CH5 wild type (wt) RNA resulted in a fewer number of colonies than transfection with CH5 K1609E or CH5 R2884G RNAs. The last two RNAs produced a comparable number of resistant colonies. The highest number of G418-resistant colonies was observed after transfection of cell with CH5 RNA, which carried both mutations.

Thus, the introduction of K1609E or R2884G mutations into the CH5 construct led to a significant increase in the efficacy of colony formation, indicating that both mutations function as cell culture adaptive ones. Combination of both these mutations increased the efficacy of colony formation of CH5 RNA even more significantly, suggesting that both mutations are compatible and demonstrate a synergistic effect.

**A**



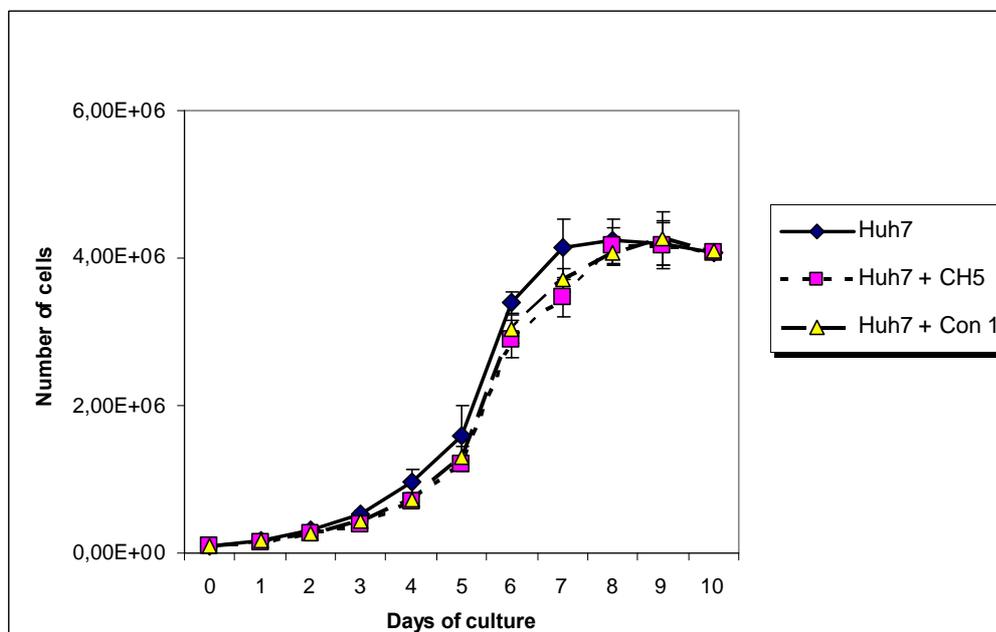
**B**



**Fig. 47. Identification of K1609E and R2884G as adaptive mutations for CH5 replicon.** K1609E or R2884G mutations were introduced into CH5 replicon for getting CH5 K1609E and CH5 R2884G replicons respectively. Both mutations were then combined into CH5 replicon for obtaining CH5 K1609E, R2884G. In independent experiments, Huh7 cells were transfected with 1 $\mu$ g of in vitro transcripts from CH5wt, CH5 K1609E, CH5 R2884G or CH5K1609E, R2884G. Cells were submitted to G418 pressure selection for 3 weeks and visible G418-resistant cell clones were stained with crystal violet (A) Selection of resistant cell clones with 250  $\mu$ g/ml of G418. (B) Selection of resistant cell clones with 500  $\mu$ g/ml of G418.

### 2.3. Replication of CH5 replicon does not affect the growth of Huh7 cells.

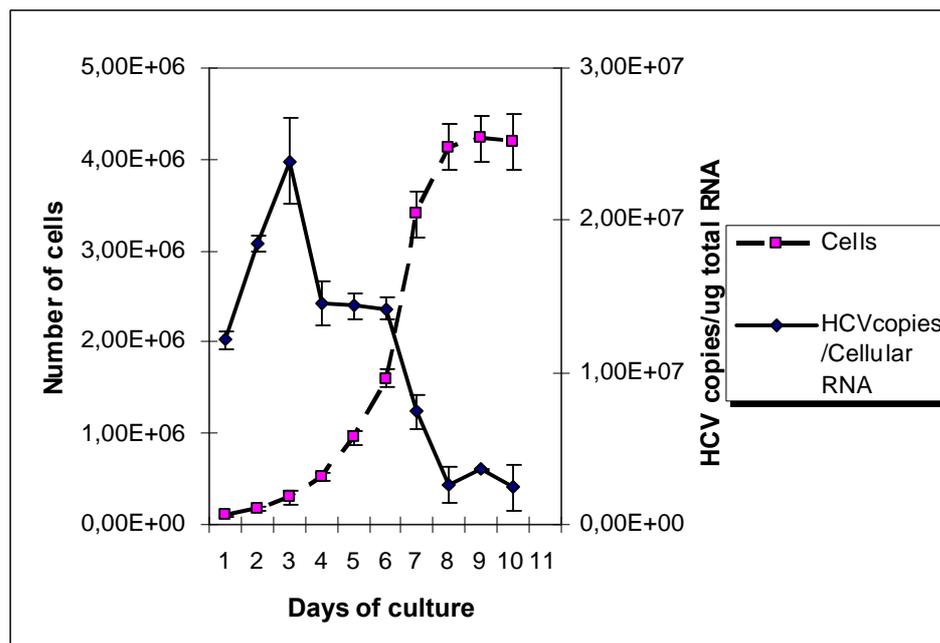
In order to analyze whether the replication of HCV subgenomic replicon RNA caused any modification on Huh7 cell growth, naïve cells and two replicon-bearing cell lines (CH5 and Con1) were analyzed. Cells were cultivated for 10 days and growth rates were determined by daily counting. As shown in Fig. 48, growth of naïve Huh7 cells was comparable to those of replicon-bearing cell lines. The mean doubling time of naïve Huh7 cells, Con1 replicon cells and CH5 replicon cells were  $29.1 \pm 4$  h,  $32 \pm 3$  h and  $34 \pm 2.5$  h, respectively. Naïve Huh7 cells reached saturation at day 7, whereas Con1 and CH5 bearing cell clones reached saturation at day 8. Saturation densities determined when cells reached confluence were  $4.2 \pm 0.6 \times 10^5$  cells/cm<sup>2</sup> for naïve Huh7 cells,  $4 \pm 0.3 \times 10^5$  cells/cm<sup>2</sup> for Con1 replicon bearing cells, and  $3.9 \pm 0.5 \times 10^5$  cells/cm<sup>2</sup> for CH5 replicon bearing cells. Thus, no significant difference was observed between the growth rates of naïve and replicon-bearing Huh7 cells, allowing concluding that the subgenomic HCV RNA did not influence the growth of Huh7 cells.



**Fig. 48. Growth curves of naïve and replicon bearing Huh7 cells.**  $10^5$  cells were seeded in triplicate into six-well plates. Cells were harvested daily for counting during 10 days of cultivation. Cell number corresponds to the mean of 3 wells counting. Standard deviation bars are shown.

#### 2.4. Influence of cell growth on replication of CH5 replicon.

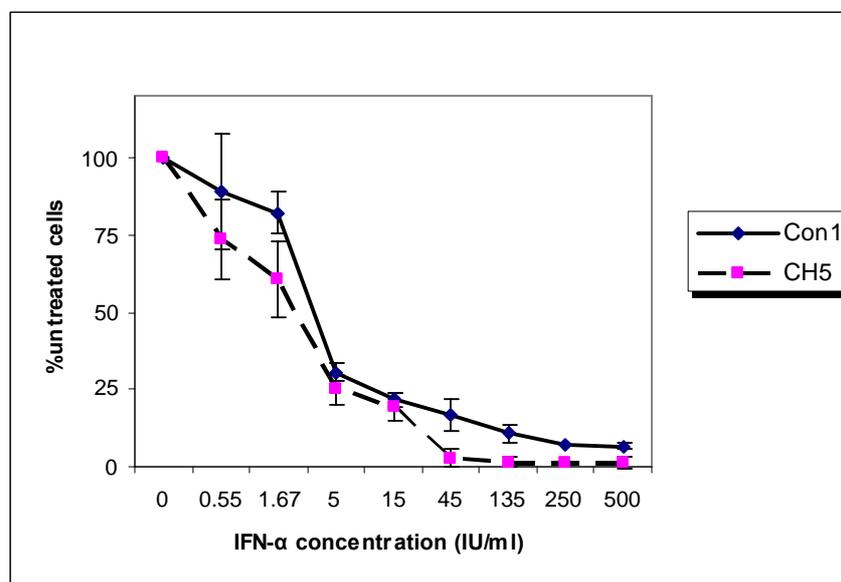
In the special series of experiments we investigated if the cell growth (cell density) has any influence on the replication kinetics of CH5 replicon. To address this issue, the CH5 replicon bearing cell lines were cultivated for 10 days and total RNA was daily extracted for quantification of the amount of HCV RNA. As shown in Fig. 49, the levels of HCV RNA per microgram of cellular RNA increased during the first three days of cultivation but dropped gradually during the next days. While cells in the early logarithmic phase of growth carried the highest amounts of HCV RNA, the level of HCV RNA decreased drastically in the late logarithmic and the stationary phase of cell growth. This observation suggested that HCV RNA replication was dependent on availability of putative cell host factors, which may vary in abundance or activity during the cell growth.



**Fig. 49. Quantification of HCV RNA and the number of cells at the time of harvest.**  $1 \times 10^5$  cells were seeded onto six well plate in triplicate and cultivated for ten days. Cells were daily harvested for counting and for RNA extraction. The amounts of HCV RNA were quantified by using real-time PCR. Mean data and standard deviation bars are shown.

## 2.5. Sensitivity of CH5 replicon to interferon-alpha.

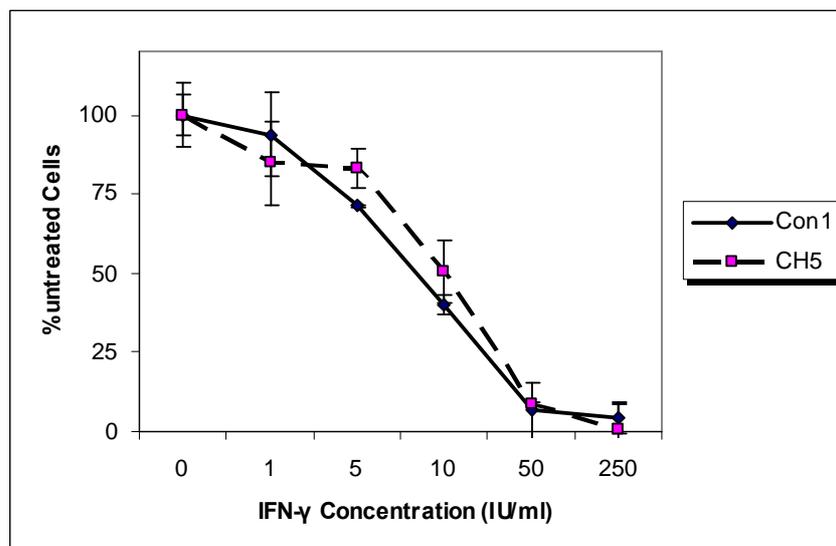
Interferons are well characterized components of the innate host defense against viral infections. Type 1 interferons (interferon-alpha and interferon-beta) are produced by virus infected cells and constitute the primary defense mechanism against viral replication. Interferon-alpha (IFN- $\alpha$ ) is also used for treatment of HCV infected patients and HCV replicon system might be used for the analysis of the mechanisms of interferon-resistance. We have performed a series of experiment to see if IFN- $\alpha$  inhibits the replication of CH5 replicon in vitro. To address this issue, CH5 replicon bearing cells were cultivated in presence of different concentrations of IFN- $\alpha$  for 48 h, the amounts of HCV RNA were quantified by real-time PCR, and the IC<sub>50</sub> was determined. Since Con1 replicon was shown to be sensitive to IFN- $\alpha$ , Con1 replicon bearing cells were used as control. As shown in Fig. 50, IFN- $\alpha$  inhibits replication of both CH5 and Con1 replicons in a dose-dependant manner. The 50% inhibitory concentrations ranged between 3 and 5 IU/ml of IFN- $\alpha$ .



**Fig. 50. Sensitivity of Con1 and CH5 replicon to IFN- $\alpha$ .** Replicon-bearing cells were seeded onto 96 well plates. IFN- $\alpha$  at different concentrations was added to the cells 24 h after seeding. The amounts of HCV RNA were quantified by real-time PCR 48 h after treatment with IFN- $\alpha$ . Experiments were performed in triplicates. Mean data and standard deviation bars are shown.

## 2-6. Sensitivity of CH5 replicon to interferon-gamma.

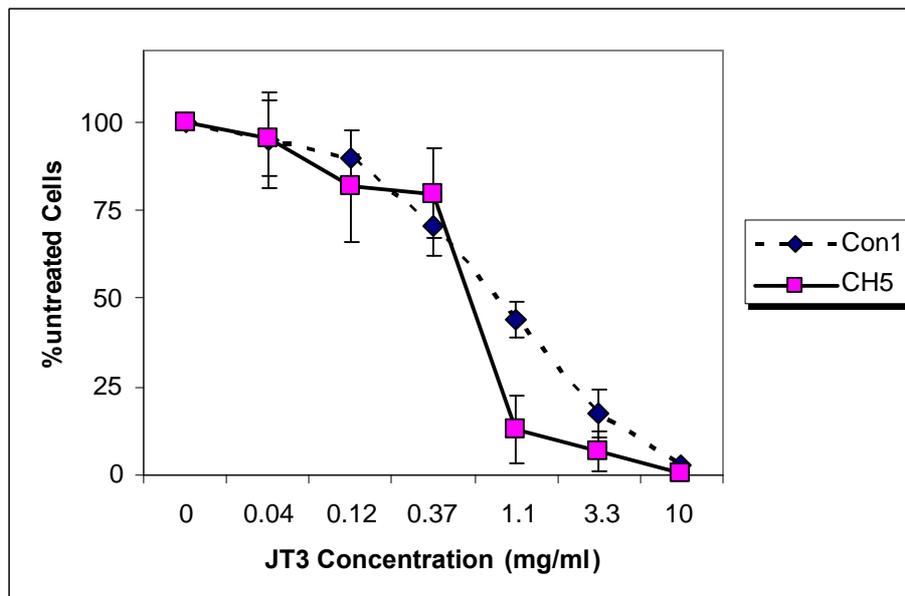
Interferon gamma (IFN- $\gamma$ ) is involved in the natural defenses of the organism against viral infection. IFN- $\gamma$  is synthesized only by certain cells of the immune system including natural killer cells, CD4 Th1 cells, and CD8 cytotoxic suppressor cells. We ask whether this cytokine inhibits the replication of CH5-replicon in vitro. To analyze the question replicon bearing cells were treated with different concentrations of IFN- $\gamma$  and the amount of HCV RNA in treated cells were quantified by real-time PCR. Since Con1-replicon was shown to be sensitive to IFN- $\gamma$ , Con1 replicon-bearing cells were used as a control. As shown in Fig. 51 IFN- $\gamma$  inhibits the replication of CH5 replicon in a dose dependant manner. The IC<sub>50</sub> of IFN- $\gamma$  was found ranged between 8 and 10 IU/ml of.



**Fig. 51. Sensitivity of Con1 and CH5 replicon to IFN- $\gamma$ .** Replicon-bearing cells were seeded into 96 well plates. IFN- $\gamma$  at different concentrations was added to the cells 24 h post seeding. The amounts of HCV RNA were quantified by real-time PCR 48 h after treatment with IFN- $\gamma$ . Experiments were performed in triplicates. Mean data and standard deviation bars are shown.

### 2-7. Sensitivity of CH5 replicon to a polymerase inhibitor JT3

JT3 is an inhibitor of HCV polymerase and was demonstrated to inhibit *in vitro* replication of at least several HCV replicons (R. Bartenschlager, personal communication). We were interested in checking whether JT3 exhibits an inhibitory effect on replication of CH5 replicon. To this end, the replicon-bearing cells were treated with different concentrations of JT3 and the amounts of HCV RNA in treated cells were quantified by real-time PCR. We used Con1-bearing cells as control in this experiment. As a result, we could observe that JT3 inhibits CH5 replicon in a dose-dependent manner (Fig. 52). The  $IC_{50}$  values varied between 0.37 and 1.1 mg/ml of JT3.



**Fig. 52. Inhibition of CH5 replication by JT3.** Replicon bearing cell lines (Con1 and CH5) were treated with different concentration of JT3. HCV RNA amounts in treated cells were determined 72 h after treatment with JT3 by real-time PCR. Experiments were performed in triplicates. Mean data and standard deviation bars are shown.

### 3. Attempts to establish a new cell line permissive for replication of HCV RNA.

As has already been discussed in the introduction, a search for new cell systems, which may support the HCV RNA replication, remains an important task. We have attempted to transfect a number of cell lines with Con1 and CH5 replicon constructs and to establish HCV RNA containing G418-resistant cell clones. Table 7 illustrates the results of these experiments.

**Table 7.** Replication of HCV subgenomic replicon RNA in different cell lines.

Cell lines	Origin	Replication	
		Con1-ET replicon	CH5 replicon *
Huh7 “Lunet“	Human hepatoma	Positive	Positive
Huh7 Trf-1	Human hepatoma “CK2- $\alpha$ “ deficient	Negative	Positive
HepG2	Human hepatocellular carcinoma	Negative	Negative
KB	Human carcinoma	Negative	Non tested
FL	Human amnion	Negative	Non tested
Vero	African green monkey	Negative	Non tested
L	Mouse fibroblasts	Negative	Non tested
SVV	EBV immortalized human B-cells	Negative	Non tested
SVS	EBV immortalized human B-cells	Negative	Non tested
Jac1	Mouse B-cells	Negative	Non tested
Caco	Human carcinoma	Negative	Non tested
RD	Human rhabdosarcoma	Negative	Non tested
L41	Human myeloleukemia	Negative	Negative

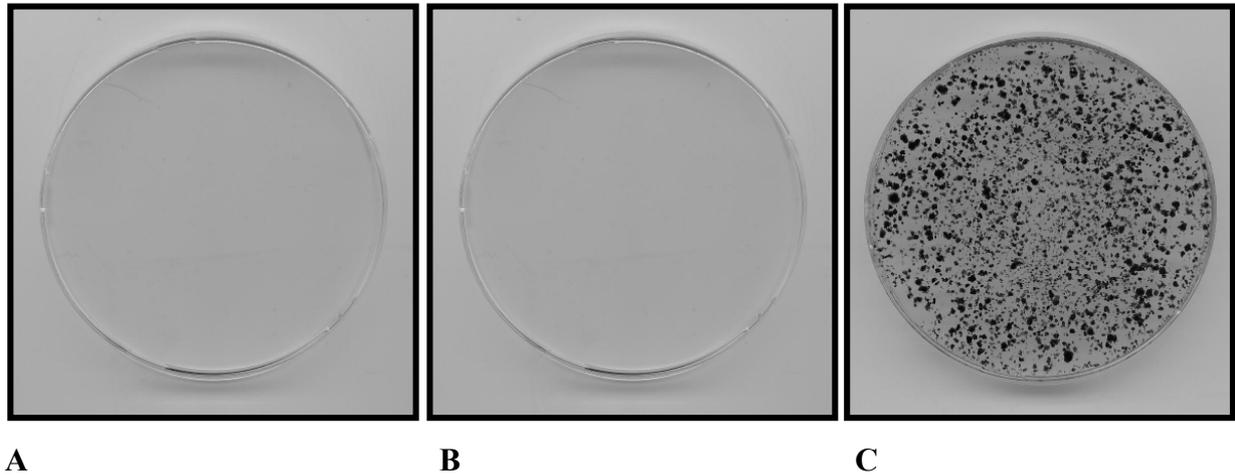
\* CH5 replicon variant used in this study contained both K1609E and R2884G adaptive mutations.

Despite numerous attempts, a successful transfection with the establishment of G418-resistant cell clones was achieved only with the Trf1 variant of the Huh7 cells, which is characterised by a defect in cell surface protein trafficking (Stockert et al., 1995). Recent data suggest that Trf-1 cell line is more resistant to TNF- $\alpha$ , to virus-induced cell death, and to other apoptotic stimuli than a naïve Huh7 cells (Hilgard et al., 2004). In the current study we have transfected Trf-1 cells with the RNA transcribed in vitro from the CH5 wild type, CH5 K1609E, CH5R2884G and CH5K1609E, R2884G variant replicons. Transfected cells were submitted to selection with 250  $\mu$ g/ml of G418. Four weeks following transfection, G418-resistant cell colonies were obtained only with cells transfected with RNA from CH5 K1609E, R2884G variant replicon (Fig. 53). Despite several attempts, we have never been able to obtain G418-resistant clones with cells transfected with CH5-wt RNA or with CH5 RNA bearing single mutations K1909E or R2884G. These observations suggest that single adaptive mutations K1609E and R2884G are not sufficient to enhance replication of HCV RNA in Trf-1 cells. However, combination of both mutations efficiently enhances replication of CH5 replicon in Trf-1 cells, suggesting that a synergistic effect of both mutations was necessary for initiating high replication of HCV RNA in this cell line. The efficiency of colony formation observed with transfection of Trf-1 cells was lower than that observed with transfection of CH5 K1609E, R2884G into the wild type Huh7 cells (deriving from a HCV subgenomic replicon cured cell line).

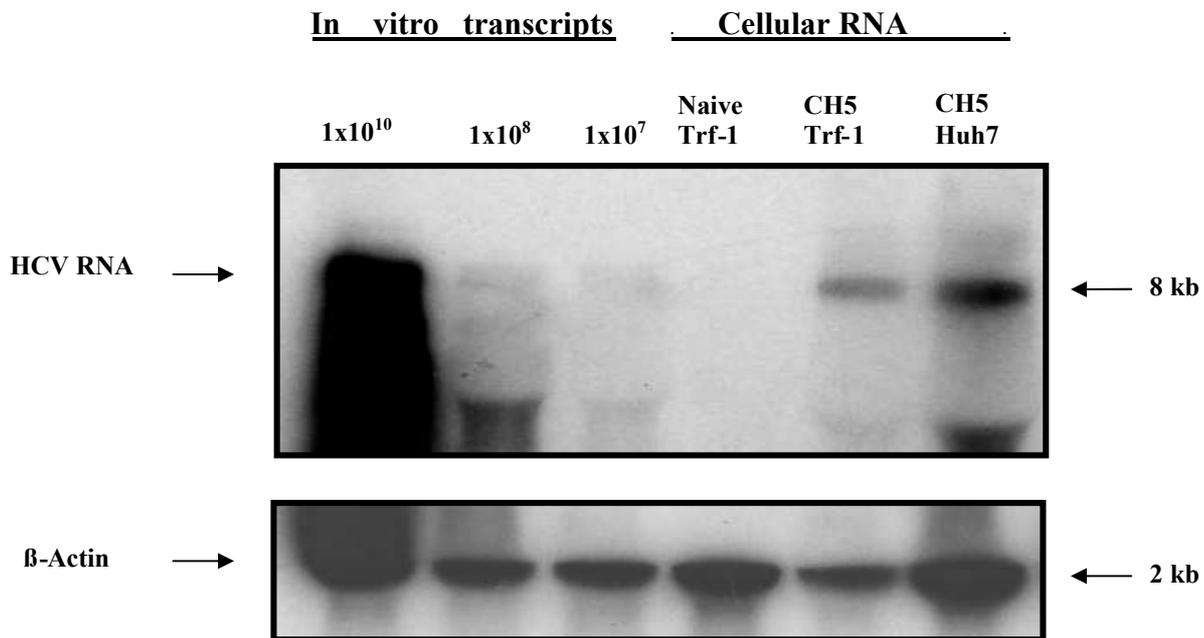
To confirm replication of CH5 K1609E, R2884G replicon variant in Trf-1 cell clone, cellular RNA was extracted from G418 resistant cell clone and analyzed by Northern blot (Fig. 54).

The level of HCV RNA in Trf-1 cells, obtained after two week of cultivation of the established cell line, was determined by real-time PCR, and was about  $4 \times 10^6$  copies genome/ $\mu$ g of cellular RNA, which was about 10 fold less than HCV RNA levels in Huh7 cells transfected with the same replicon.

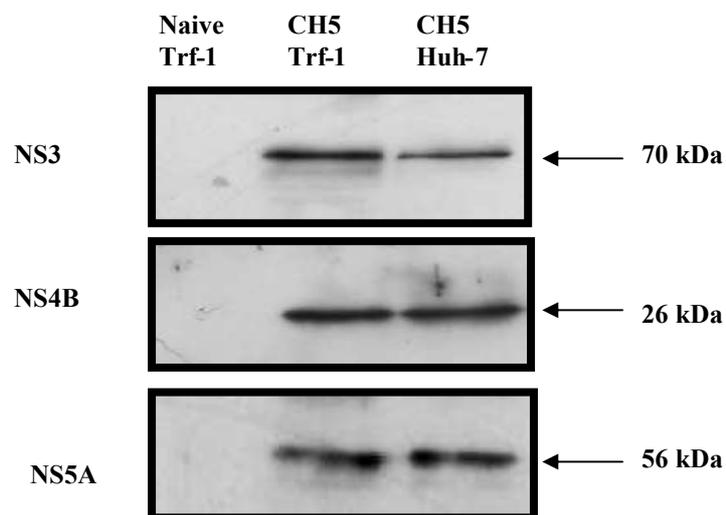
Expression of HCV non-structural proteins in Trf-1 cell clone was analyzed by Western blot. We could observe that all non-structural proteins of HCV were efficiently expressed (Fig. 55). Together these results suggest that Trf-1 cells support replication of HCV RNA, although they display some phenotypic differences compared with the wild type Huh7 cells.



**Fig. 53. Colony formation of Trf-1 cells transfected with CH5 variant replicons RNA.** Trf-1 cells were transfected with 1 $\mu$ g of in vitro synthesized RNA from CH5 K1609E, CH5 R2884G or CH5 K1609E,R2884G replicon. Transfected cells were cultivated for 3 weeks in presence of G418 (250  $\mu$ g/ml), and resistant colonies were stained with crystal violet. (A) Trf-1 cells transfected with CH K1609E RNA; (B) Trf-1 cells transfected with CH5 R2884G RNA; (C) Trf-1 cells transfected with CH5 K1609E., R2884G RNA.



**Fig. 54. Detection of HCV RNA in Trf-1 transfected cells.** Total RNA extracted from Trf-1(CH5 Trf-1) and Huh7 lunet (CH5 Huh7) cells bearing CH5 replicon were analysed by Northern blot. HCV RNAs and β-Actin mRNA were detected by hybridization to a riboprobe complementary to the neo gene or to β-Actin mRNA. Different amount of in vitro transcripts were used as standard. RNA isolated from naïve Trf-1 cells served as negative control

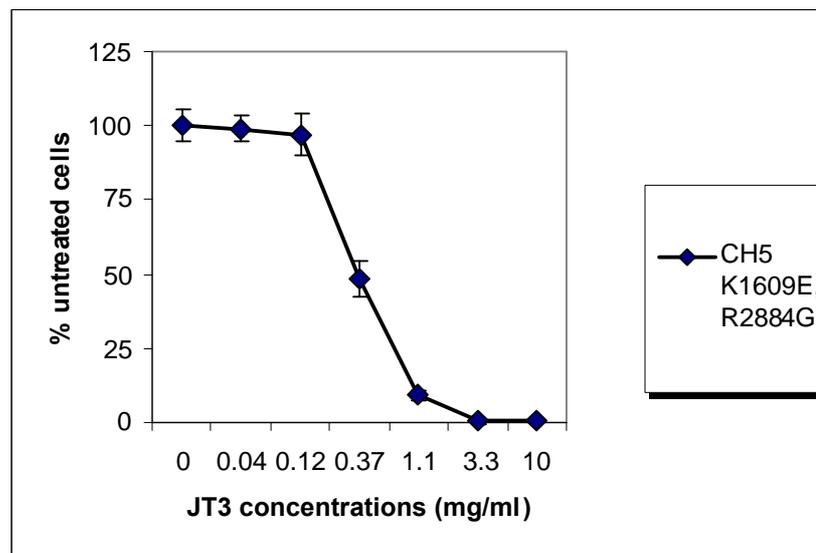


**Fig . 55. Detection of HCV expression in Trf-1 transfected cells:** Equal amount of supernatant from cell lysates of naive Trf-1 cells (Naïve Trf-1), Trf-1 bearing CH5 replicon (CH5 Trf-1), and Huh7 bearing CH5 replicon (CH5 Huh7) were separated by SDS-PAGE and analyzed using antibodies against NS3, NS4B and NS5A proteins of HCV.

### 3.1. Susceptibility of CH5 replicon in Trf-1 cells to inhibitors of HCV replication.

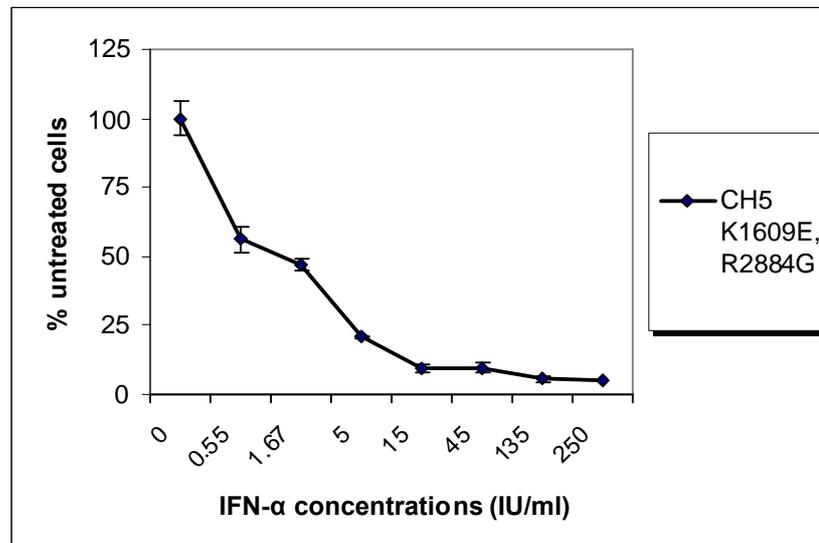
In a previous section we have shown that CH5 replicon was sensitive to interferons alpha and gamma and to JT3 in Huh7 cells. To see if the cellular environment in Huh7 Trf-1 cells may influence the characteristics of CH5 replicon, we assessed the sensitivity of CH5 to JT3 and IFN- $\alpha$  and IFN- $\gamma$  in Huh7 Trf-1 cells. As a result, CH5 was sensitive to JT3 (Fig. 56) and to IFN- $\alpha$  and IFN- $\gamma$  (Fig. 57) in a dose dependent manner. The IC<sub>50</sub> of JT3 ranged between 0.12 and 0.37 mg/ml. The IC<sub>50</sub> of IFN- $\alpha$  and IFN- $\gamma$  were 1.67 IU/ml and 10 IU/ml respectively.

Thus, these observations are concordant with our previous results obtained with Huh7 cells. Together, these results suggest that the cellular environment in Huh7 Trf-1 mutant cells is favorable for replication of CH5 and characteristics of the replicon, namely sensitivity to anti-HCV drugs, were conserved.

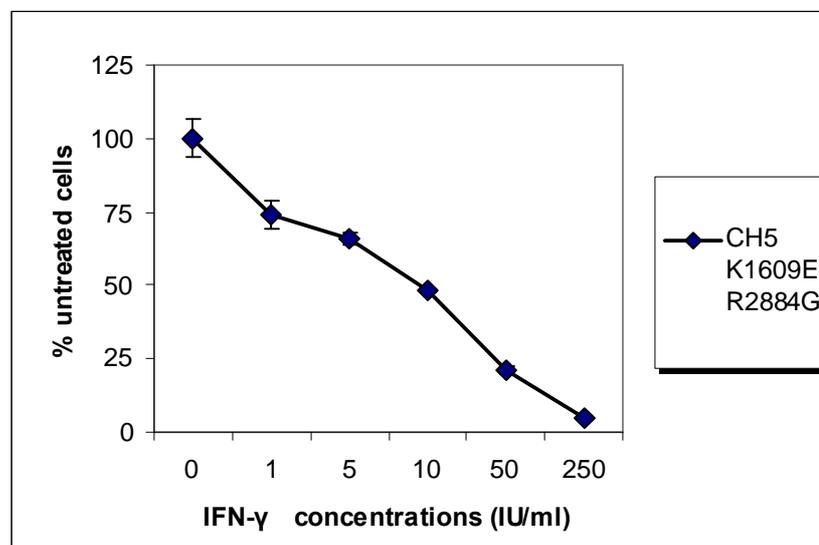


**Fig. 56. Inhibition of CH5 replication by JT3.** Trf-1 replicon bearing cell lines were treated with different concentration of JT3. HCV-RNA amounts in treated cells were determined 72 h after treatment with JT3 by real-time PCR. Experiments were performed in triplicates. Mean data and standard deviation bars are shown.

A



B

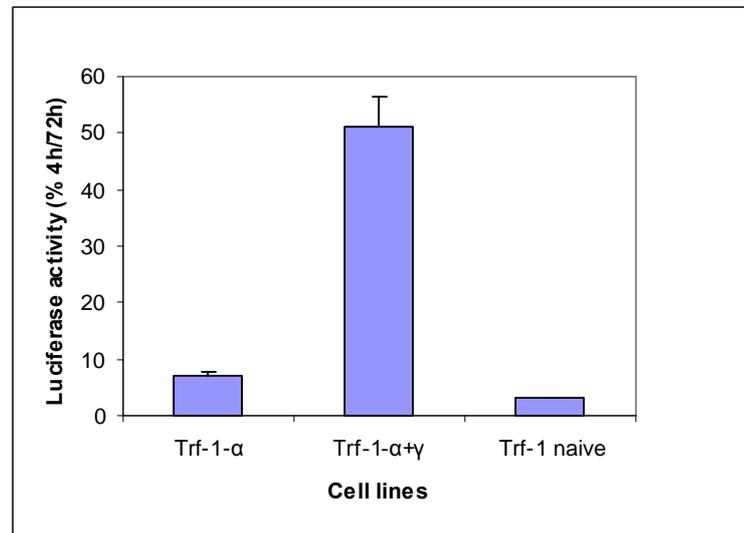


**Fig. 57. Inhibition of CH5 replication by Interferons.** Trf-1 replicon-bearing cells were seeded into 96 well plates. IFN- $\alpha$  OR IFN- $\gamma$  at different concentrations were added to the cells 24 h post seeding. The amounts of HCV RNA were quantified by real-time PCR 48 h after treatment with IFN- $\alpha$  OR IFN- $\gamma$ . Experiments were performed in triplicates. Mean data and standard deviation bars are shown. (A) IFN- $\alpha$  susceptibility curve of CH5 (B) IFN- $\gamma$  susceptibility curve of CH5.

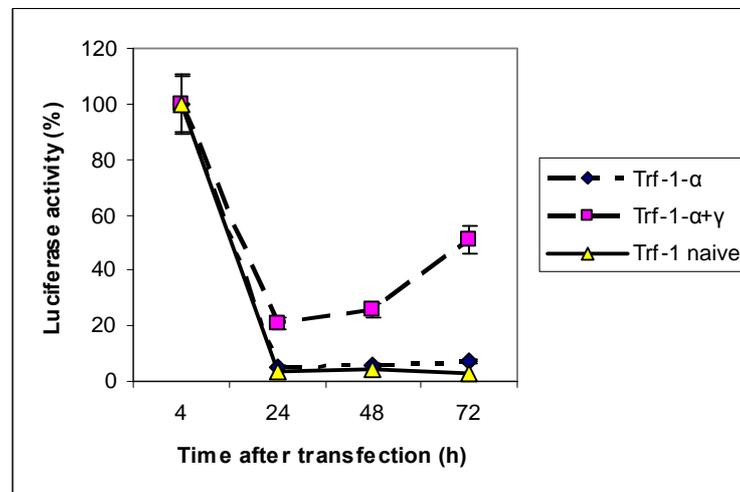
### **3.2. Cured Trf-1 cells support transient replication of HCV RNA.**

To generate a cured Trf-1 cell line, replicon-bearing cells were treated with IFN- $\alpha$  (200 IU/ml) or with combination of IFN- $\alpha$  (200 IU/ml) and IFN- $\gamma$  (200 IU/ml) for three weeks. Confirmation for the removal of HCV RNA from treated cells was obtained by transcription-mediated amplification “TMA” in independent experiments two weeks after treatment with cytokines. Permissiveness of the cured Trf-1 cells was assessed using a transitory transfection with Con1 replicon expressing luciferase as a reporter gene. Replication of Con1 in cured Trf-1 cells and in naïve Trf-1 cells was compared. As a result it was to observe that replication of Con1 was higher in cells treated with combination of cytokines than with IFN- $\alpha$  alone or than in naïve Trf-1 cells (Fig. 58). These observations suggest that Trf-1 cells cured by treatment with combination of IFN- $\alpha$  and IFN- $\gamma$  were more permissive than IFN- $\alpha$  cured cells and naïve Trf-1 cells.

A



B



**Fig. 58. Transient replication of HCV RNA in replicon-cured Trf-1 cells.** Naïve Trf-1 cells, IFN- $\alpha$ -cured cells (Trf-1- $\alpha$ ) IFN- $\alpha$  and IFN- $\gamma$ -cured cells (Trf-1- $\alpha$ + $\gamma$ ) were transfected with Con1 RNA, respectively. Luciferase activity in the lysates of transfected cells was measured at different time point and normalized for difference in transfection efficacy using the activity at 4 h after transfection. (A) Replication ability of Con1 at 72 h after transfection. (B) Replication ability of Con1 measured at different time points after transfection. Results are presented as mean  $\pm$ SD of the three independent experiments.

#### **4. Establishment of AD78/Con1 full-length, genomic replicon chimera.**

The development of a set of subgenomic Con1/AD78 chimeric replicons and AD78-based replicon reported in the previous sections open a possibility for extension of these studies, namely, for an attempt to establish a full-length selectable HCV genomic chimera of Con1/AD78 and full-length AD78-based genomic replicons.

In the previous sections we have demonstrated that CH5 replicon replicates efficiently in Huh7 and in Trf-1 cells. As a logical continuation of this research we also have tried to use the CH5 replicon as a basis for generation of the full-length replicon by insertion of the Core-NS2 fragment of HCV AD78 consensus sequence into the CH5 replicon. First, a Core-NS3 fragment of HCV AD78 consensus sequence was amplified from the plasmid pHCV, which contain the complete genome of HCV AD78. An intermediate plasmid was obtained by cloning of the amplified Core-NS3 fragment into pCR2.1Topo vector. The recognition site of NcoI (endonuclease) was found within the sequence of NS2 and NS3 genes of HCV AD78. Because we planed to use only the site located within the NS3 gene, we removed an NcoI site found within NS2 sequence of AD78 by site-directed mutagenesis. Subsequently, the Core-NS3 fragment was sub cloned into P4AD2.1Topo plasmid created previously, which contains the complete NS3 gene amplified from AD78-infected patient 4.

Two subsequent site-directed mutagenesis were made to remove BsrGI recognition sites located within E2 and NS2 genes of HCV AD78 in the obtained intermediate plasmid. Previously, we have created an intermediate plasmid that contained the sequence covering the EMCV- IRES to the first 300 nucleotides of NS3 gene amplified from CH4 replicon. Using NcoI and BsrGI restriction sites, the Core-NS3 fragment was sub cloned into the intermediate plasmid that contained the sequence of EMCV IRES to NS3 gene, and then transferred into the CH5 plasmid using PmeI and BsrGI restriction sites for obtaining CH5 FL replicon (Fig. 59).

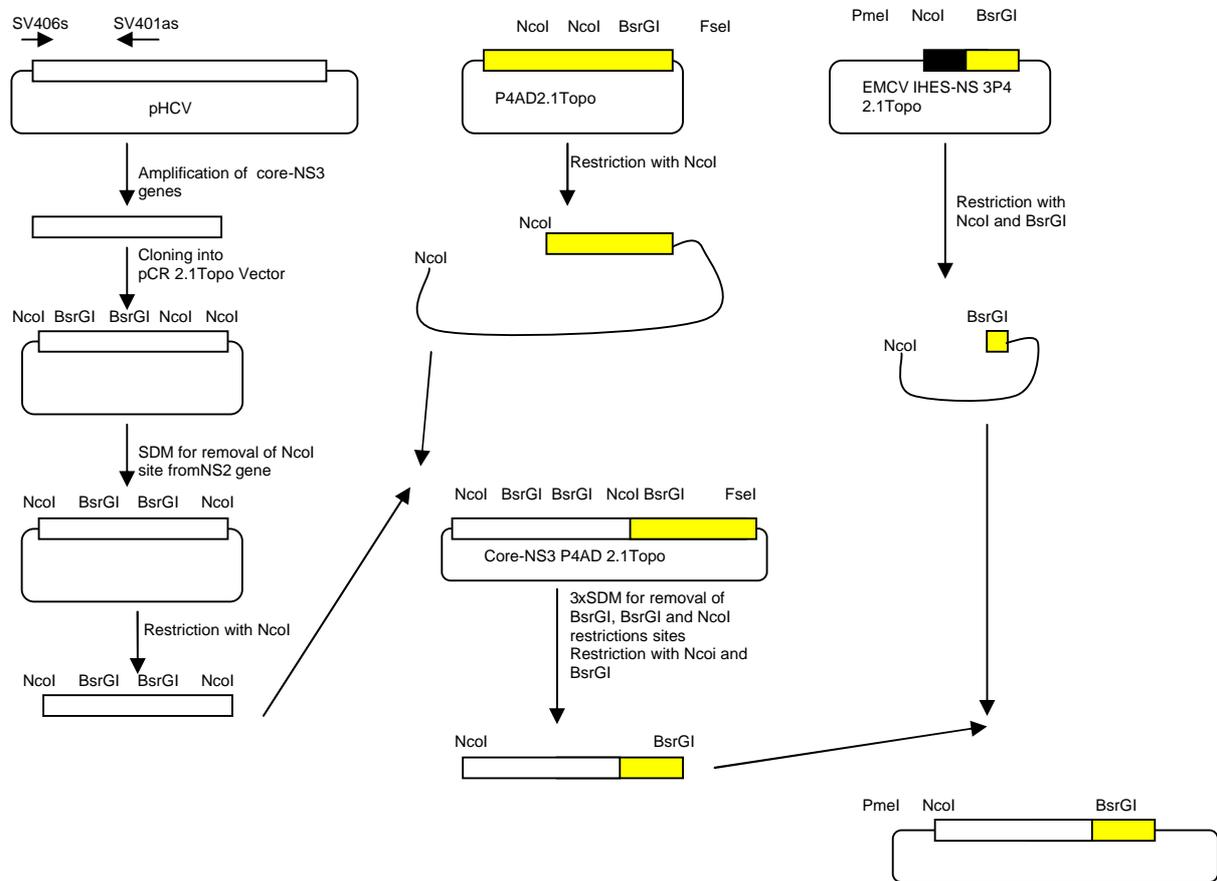
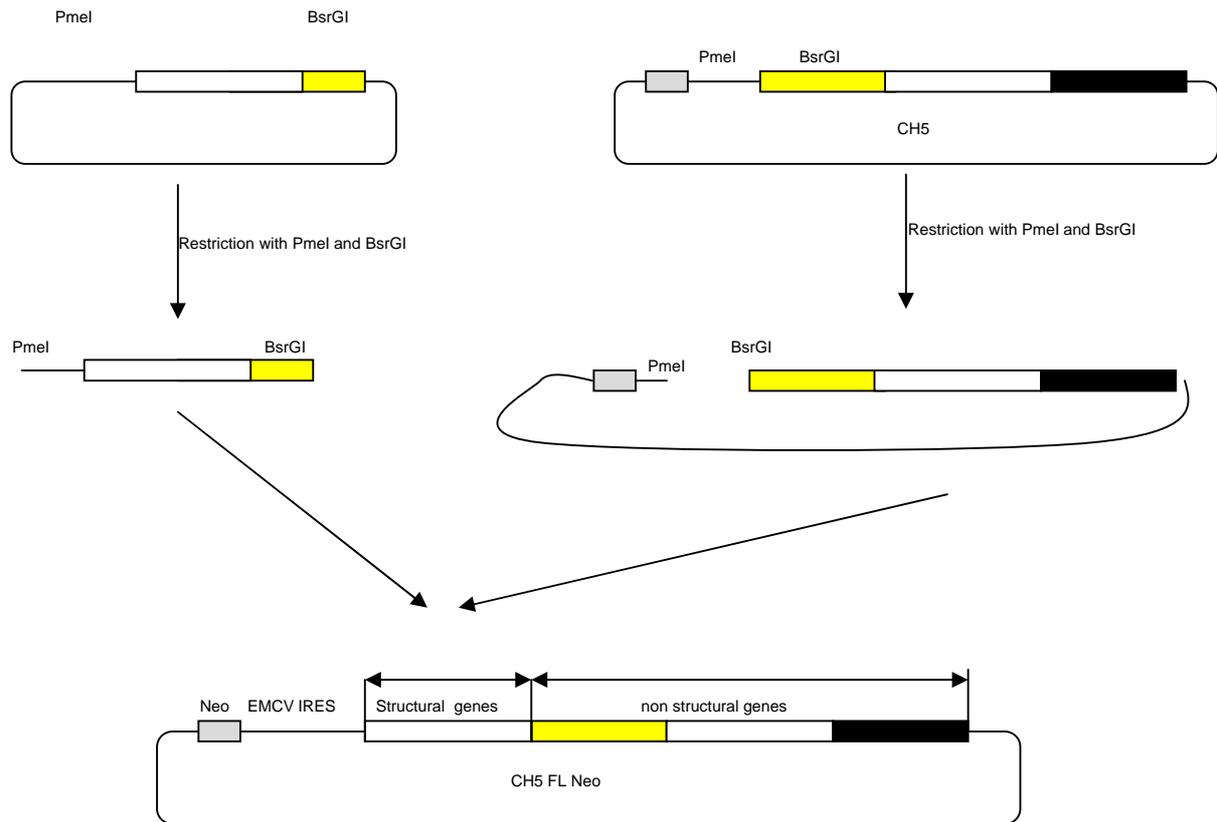


Fig. 59. (to be continued)



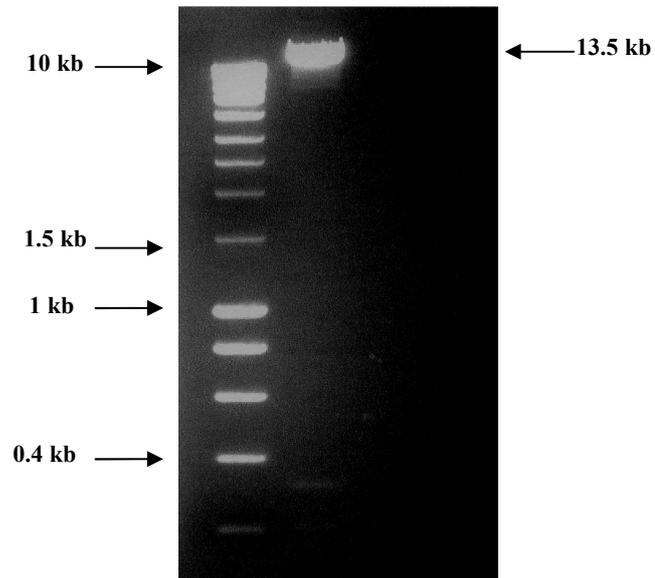
**Fig. 59. Creation of CHF5FL replicon variant.** A pHCV plasmid was generated after cloning of the complete genome of AD78 consensus sequence. Structural genes of the consensus sequence were amplified from pHCV and sub cloned in an intermediate vector before fusion with the nonstructural gene into CH5 subgenomic replicon. Sequences derived from the AD78 consensus sequence are depicted in white. Sequences derived from Con1 isolate and AD78 sequence from patient 4 are depicted in black and yellow, respectively.

To assess the replication ability of CH5FL replicon in cell culture, the CH5FL plasmid was linearized with SspI and ScaI. The correct size of the restricted plasmid was confirmed by agarose electrophoresis (Fig. 60A). The linearized plasmid was used for RNA transcription in vitro and the synthesized CH5FL RNA was submitted to electrophoretic analysis. As shown in Fig. 60B, the full-length RNA of CH5 replicon corresponded to an 11kb fragment. Huh7 cells were transfected with CH5FL RNA and submitted to selection with G418. Unfortunately, no colony formation was observed after several attempts.

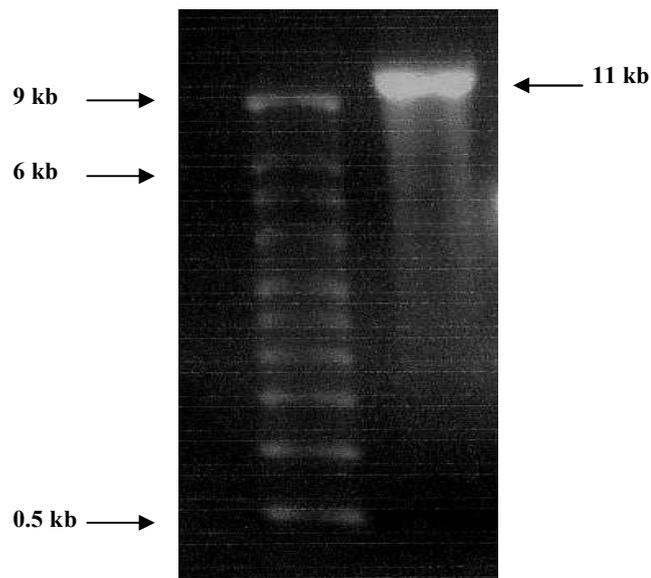
Considering the fact that the adaptive mutations K1609E and R2884G efficiently enhanced colony formation of CH5 replicon, we hypothesized that introduction of these mutations into CH5FL might also enhance its ability to replicate in Huh7 cells. To investigate this possibility, two replicon variants designated CH5FL R2884G and CH5FL K1609E, R2884G were generated. We assessed both replicons for their replication ability in Huh7 cells using different amounts of in vitro transcribed RNA (from 1 to 5 $\mu$ g of RNA). Cells were submitted to different concentrations of G418 (125, 250 and 500  $\mu$ g/ml) and cultivated for four weeks. Interestingly, we obtained G418-resistant cell colonies both with cells transfected with CH5FL R2884G RNA and with CH5FL K1609E, R2884G RNA. Colony forming efficiency observed with transfection of CH5FL R2884G replicon variant was very low (less than 1 colony/ $\mu$ g transfected HCV RNA) and lower than that after transfection of cells with the CH5FL K1609E, R2884G construct (more than 50 colonies/ $\mu$ g transfected HCV RNA) (Fig.61). This observation suggested that combination of K1609E and R2884G mutations enhances replication of HCV full-length RNA much more efficiently than R2884G alone.

For further analysis, we have tried to expand stable cell lines bearing CH5FL. As a result, only cells obtained from transfection of Huh7 cells with CH5FL RNA bearing both adaptive mutations were successfully established. However, we have observed that the established cell line grew slowly and failed to become completely confluent after four weeks in culture. After passage for an additional month of culture, these cells demonstrated improved growth properties.

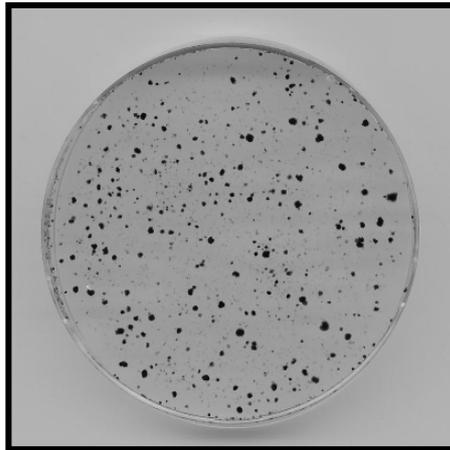
A



B



**Fig. 60. Gel electrophoresis of DNA and RNA of CH5FL.** (A) Plasmid bearing CH5FL replicon was sequentially restricted with SspI and with ScaI and analyzed on 1% agarose gel. A band of 13.5 kb corresponds to the plasmid containing the full-length replicon sequence. (B) Linearized CH5FL plasmid was used for in vitro transcription into RNA. Two  $\mu\text{g}$  of RNA were analyzed by electrophoresis under denaturing conditions. An 11 kb band corresponds to the sequence of CH5FL RNA.

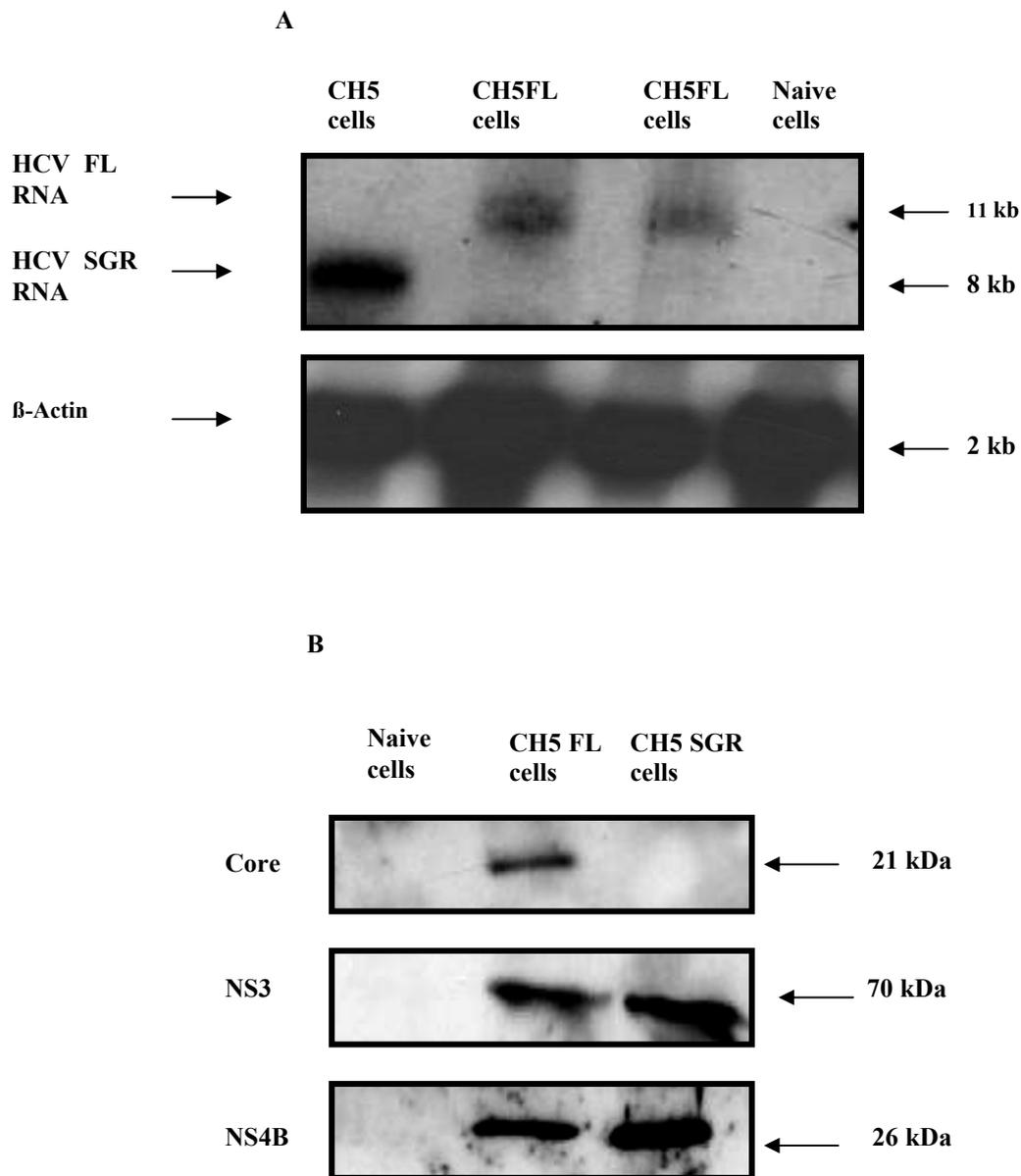


**Fig. 61. Colony formation of Huh7 cells transfected with CH5FL K1609E, R2884G variant replicon RNA.** Huh7 cells were transfected with 2  $\mu\text{g}$  of in vitro synthesized RNA from CH5 FL K1609E, R2884G replicon. Transfected cells were cultivated for 4 weeks in presence of 250  $\mu\text{g}/\text{ml}$  of G418, and resistant colonies were stained with crystal violet.

## **5. Characteristics of the cell line bearing the full-length replicon.**

### **5-1. Evidence for replication of the full-length CH5FL RNA in the G418-resistant cells.**

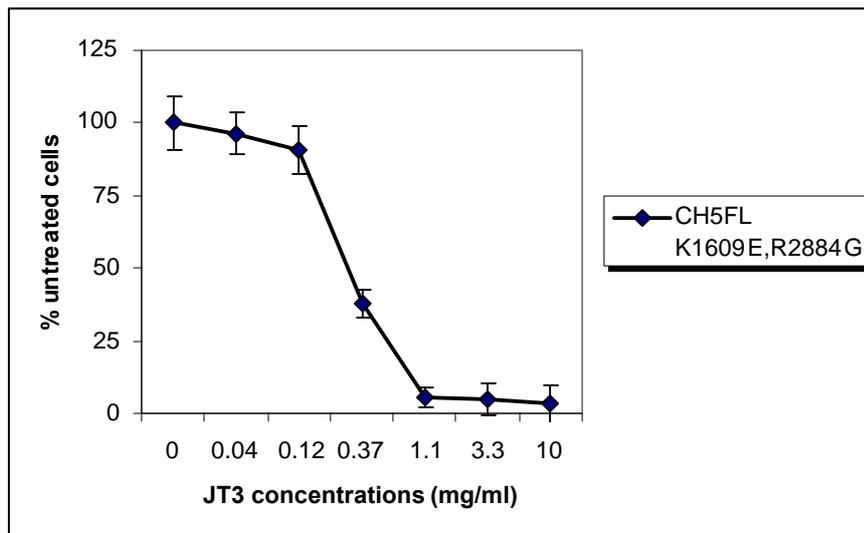
Transfection of the full-length CH5FL RNA bearing NS3 and NS5B adaptive mutations (K1609E and R2884G) into Huh7 cells has produced G418 resistant colonies, which were expanded as a polyclonal cell line. In order to demonstrate replication of HCV RNA in the established cell line, HCV RNA and HCV proteins were detected in the cells by Northern blot and Western blot, respectively. Results of the Northern blot analysis, as shown in Fig. 62 A, revealed a hybridization signal corresponding to an 11 kb molecule, both with the in vitro transcribed RNA of CH5FL and with the total RNA extracted from G418-resistant cells. This result confirms the presence of CH5FL RNA in the established cell line. Additionally, quantification of HCV RNA in cellular RNA by real-time PCR indicates a ratio of  $2 \times 10^{-6}$  HCV RNA copies/ $\mu\text{g}$  of cellular RNA. Moreover, expression of HCV proteins in the established cell lines, as shown in Fig. 62B, was demonstrated. The expression of HCV core protein was only observed in cells transfected with the full-length replicon RNA. Together these results provide a strong argument for an efficient replication of the full-length replicon CH5FL in Huh7 cells.



**Fig. 62. Detection of HCV full-length replicon RNA and proteins in G418-resistant cell line.** (A) Total RNA extracted from Huh 7 cells bearing CH5 full-length replicon (CH5FL cells) from two different cell culture passages and CH5 subgenomic replicons (CH5 SGR cells) were analysed by Northern blot. HCV RNAs and  $\beta$ -Actin mRNA were detected by hybridization to a riboprobe complementary to the neo gene or to  $\beta$ -Actin mRNA. RNA isolated from untransfected Huh7 cells (Naïve cells) was used as a negative control. (B) Equal amount of supernatant from cell lysates of naive Huh7 cells, CH5 full-length replicon cells (CH5FL cells), and CH5 subgenomic replicon cells (CH5 SGR cells) were separated by SDS-PAGE and analyzed by using antibodies against Core, NS3 and NS4B proteins of HCV.

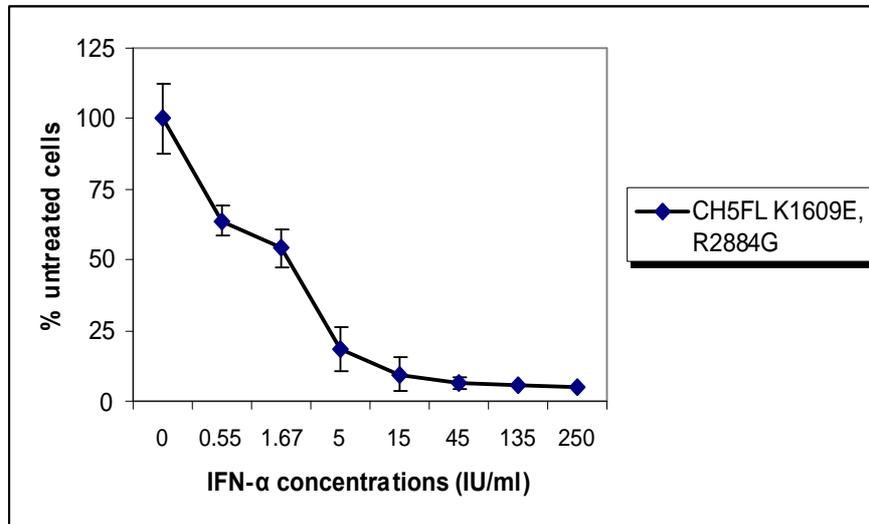
## 5-2. Sensitivity of the full-length CH5FL RNA to interferons and inhibitor of polymerase.

To further characterize our full-length replicon system, the established cell line was tested for sensitivity of the HCV RNA to JT3, IFN- $\alpha$  and IFN- $\gamma$ . The full-length replicon RNA was sensitive to JT3 (Fig. 63) and both cytokines (Fig. 64) in a dose dependant manner. The IC<sub>50</sub> of the polymerase inhibitor was less than 0.37 mg/ml and comparable with what observed with the subgenomic RNA. The IC<sub>50</sub> of IFN- $\alpha$  and IFN- $\gamma$  was found to be less than 5 and 10 IU/ml respectively. The effect of IFN molecules observed with the full-length RNA was comparable with what we observed with the subgenomic RNA.

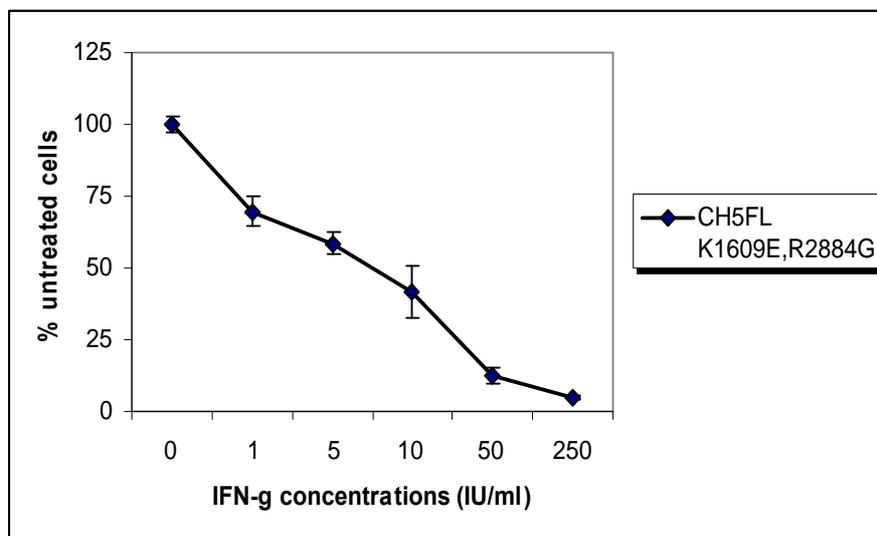


**Fig. 63. Inhibition of CH5FL replication by JT3.** Huh7 replicon bearing cell lines were treated with different concentration of JT3. HCV-RNA amounts in treated cells were determined 72 h after treatment with JT3 by real-time PCR. Experiments were performed in triplicates. Mean data and standard deviation bars are shown.

A



B



**Fig. 64. Inhibition of CH5LL RNA by IFN molecules.** Huh7 CH5FL-bearing cells were seeded into 96 well plates. IFN- $\alpha$  OR IFN- $\gamma$  at different concentrations were added to the cells 24 h post seeding. The amounts of HCV RNA were quantified by real-time PCR 48 h after treatment with IFN- $\alpha$  OR IFN- $\gamma$ . Experiments were performed in triplicates. Mean data and standard deviation bars are shown. (A) IFN- $\alpha$  susceptibility curve of CH5FL (B) IFN- $\gamma$  susceptibility curve of CH5FL.

## Discussion.

One of the major breakthroughs in HCV research in the last years was the development of subgenomic and genomic RNA replicons (Lohmann et al, 1999; Blight et al., 2000). The cells transfected with these RNA represent a very reliable and reproducible model to study different stages of HCV replication and to search for specific antivirals (Wakita et al., 2005; Zhong et al., 2005; Lindenbach et al., 2005). To a significant extent the progress in this field was achieved by using a very powerful methodical approach – construction of the hybrid or chimeric replicons. Indeed, the engineering of different intragenotypic and intergenotypic hybrids (Gates et al., 2004; Gu et al., 2004; Graham et al., 2006; Lanford et al., 2006; Pietschmann et al., 2006) provided a set of replicons that are being used both for fundamental and applied HCV research. It should be noted, however, that the potency of the chimeric replicon approach has not been fully explored so far. Thus, for many aspects of HCV research would be very useful to have a set of replicons, in which different genome regions are swapped by corresponding fragments from isolates of the same virus strain that demonstrate different biological characteristics, e.g. sensitivity or resistance to interferon. The isolates of the same HCV strain that are necessary for generation of such hybrid replicons might be obtained from a single-source outbreak of HCV infection. One such an outbreak caused by HCV AD78 strain, as has been mentioned above, occurred in Germany due to infection of women by contaminated anti-D globulin (Wiese et al., 2000, 2005).

The major aim of the current study was to create a panel of chimeric HCV subgenomic and genomic replicons, in which different fragments of HCV AD78 sequence were inserted into the back-bone of the Con1 replicon, as well as subgenomic replicon, which would contain protein encoding sequences derived only from AD78 strain.

### **1. Subgenomic replicon based on the consensus AD78 sequence is deficient for replication in cell culture.**

The first attempts to establish a functional HCV subgenomic replicon based on the consensus sequence of HCV AD78 isolate were unsuccessful. In order to identify the defective fragments of the consensus sequence a genetic approach was developed by swapping the fragments of AD78 consensus sequence into the backbone of Con1 replicon with generation of a set of chimeric Con1/AD78 replicons (Fig. 11). Using this approach we could identify that NS3 and NS5 genes of AD78 consensus sequence were defective. First, we focused our interest on the NS3 gene of AD78 in order to identify the deleterious mutations or motifs. Given that the NS3 gene of HCV is a multifunctional protein, it would have been beneficial to analyse separately different domains of this protein. Analysis of two chimeric Con1/AD78 replicons allowed us to conclude that the defect in the NS3 gene of AD78 consensus sequence was located in its N-terminal region, which covered the complete serine protease domain and a 245 amino acids fragment of the helicase/NTPase domain. Sequence comparison of AD78 consensus sequence with that of Con1 replicon revealed a difference of 21 amino acid residues within this fragment. Such a high number of differences made the approach based on site-directed mutagenesis of all these substitutions technically difficult. Therefore, we have amplified the NS3 gene sequences from serum of patients infected with HCV AD78 strain and inserted them into the Con1 replicon. Two of the three tested NS3 gene sequences turned out to be defective and one chimeric replicon replicated efficiently in Huh7 cells.

Sequence alignment comprising NS3 genes of Con1, AD78 consensus sequence, and individual sequences amplified from three AD78-infected patients revealed a number of amino acids differences within the N-terminal region of NS3 protein. Among these amino acid residues, five were exclusively found within the sequences of defective NS3 genes at positions 1177, 1205, 1263, 1277 and 1386. Possible influence of these five mutations on HCV RNA replication was investigated using the Con1 replicon as a model. Interestingly, substitutions of methionine for threonine at position 1205 and lysine for arginine at position 1386 have shown no effect on the replication of Con1. This observation suggests that these amino acid residues do not act as lethal mutations, at least, in the context of the Con1 sequence.

Alanine residue, which was present at position 1177 of defective NS3 genes, somehow reduced the fitness of Con1 replicon but did not abolish its replication completely. This substitution is located within the serine protease domain of NS3 protein and, presumably, may affect the activity of this enzyme without blocking the HCV RNA replication.

Of note is the fact, that the alanine residue at position 1177 was not found in the NS3 gene sequences included into the HCV database (The Los Alamos HCV Sequence Database). Interestingly, a substitution of valine with alanine at position 1177 was observed in chimpanzee inoculated with RNA transcripts from Con1 full-length genome 40 weeks post inoculation, and at this time point the viral titer was even higher than titre observed during weeks 15 to 25 post-inoculation (Bukh et al., 2002). This observation suggests that the appearance of alanine instead of valine at position 1177 of HCV serine protease is possible *in vivo*. Because substitution V1177A was tolerated in the context of Con1 sequence both in Huh7 cells and in Con1-infected chimpanzee, we assumed that such a substitution most probably did not act as a lethal mutation in the AD78 sequence.

Serine residues observed at positions 1263 and 1277 of defective NS3 affected the fitness of Con1 replicon, without abolishing its replication. Both amino acids residues were located in the helicase/NTPase domain of NS3 gene. Results of our search in a HCV data base have shown that serine was found at position 1263 within NS3 genes of a number of HCV genotype 1b sequences, whereas at position 1277 serine residue occurred only occasionally and only in genotypes and subtypes other than 1b. Because each of these amino acids residues significantly reduced the fitness of Con1 replicon, it is logical to assume that simultaneous presence of these two serine residues in the AD78 sequence might contribute to the inability of AD78 replicon to replicate in Huh7 cells.

Since introduction of alanine and serine, which were present in the defective NS3 genes of AD78 at positions 1177, 1263 and 1277, into the Con1 replicon clearly affected its fitness, we hypothesized that the dual combinations of these mutations would cause a synergistic effect and would lead to a complete abolishment of the replication of Con1 replicon in cell culture. Contrary to our expectations, the replication of Con1 was not completely inhibited after introduction of dual amino substitutions (G1263S + N1277S, V1177A + N1277S, or V1177A+G1263S).

However, these combined substitutions caused evident reduction of the replicative efficacy of the Con1 replicon, as had been previously observed with single amino acid substitutions. These data suggest that even combinations of G1263S with N1277S, V1177A with N1277S or V1177A with G1263S might demonstrate relatively low influence on the activity of HCV helicase protein. Overall, our experiments did not demonstrate the lethal effect of the “uncommon” amino acids residues found in all defective NS3 sequences of AD78 isolate. One possible explanation of these findings might be the presence of additional uncommon amino acid residues not found in all defective NS3 genes. The NS3 protein plays an essential role of in the replication of HCV being the part of HCV replication complex (Penin et al., 2004; Quinkert et al., 2005). Biochemical analysis of NS3 helicase of HCV revealed a number of domains with conserved amino acids residues, whose substitutions impair the enzymatic activities of this protein (Lin et al., 1999; Tai et al., 2001; Mackintosh et al., 2005; Lam et al., 2005). Because all rare amino acids residues (T1205, A1177, S1263, S1277 and R1386) observed in the sequences of defectives AD78 NS3 genes were located outside of these conserved domains, one might speculate that these mutations might not have a profound negative impact on HCV helicase activity that would lead to complete inhibition of HCV RNA replication. On the other hand, we could not exclude that in a particular genetic context, namely in the AD78 sequence, some of these substitutions might work in concert and somehow would influence either the enzymatic activity of proteins encoded by these NS3 genes, which activity is required for replication of HCV, or the protein-protein interaction required for the formation of HCV replication complex, making these proteins incompatible with other non-structural proteins and/or host cell proteins involved in the replication of HCV. A special study, involving the site-directed mutagenesis of all these substitutions individually and in combinations, would answer this important question.

## **2. Conserved motif within the CTL NS3-1395 epitope is required for replication of HCV.**

Influence of amino acid substitutions, which are observed in the NS3 sequence of natural isolates of HCV, was investigated further by site-mutagenesis approach. This fragment of our investigation deals with an important aspect of HCV research, namely analysis of a role of cellular immune responses in virus evolution. There is a growing consensus that cellular immune responses against hepatitis C virus (HCV) play an essential role in the control and clearance of infection (Bowen and Walker, 2005a; Neumann-Haefelin et al., 2005). In most of the infected patients, however, the virus is able to evade the immune surveillance and to establish a persistent infection. The mechanisms that lead to the failure of the virus-specific T cell responses to eliminate the infected cells and to clear the virus are still not very well understood. Several mechanisms are discussed, among them the selection of viral escape variants (Neumann-Haefelin et al., 2005; Bowen and Walker, 2005a). Indeed, the studies in acutely infected patients and chimpanzees have clearly demonstrated that HCV escape from CD8<sup>+</sup> T-cell responses might be associated with the development of persistent infection (Bowen and Walker, 2005a, 2005b; Neumann-Haefelin et al., 2005). Recently, a number of CTL-escape variants of HCV have been reported (Timm et al., 2004; Bowen and Walker, 2005b; Cox et al., 2005; Neumann-Haefelin et al., 2005; Spangenberg et al., 2005; Tester et al., 2005) and evidence of HCV adaptation to the HLA background of a population has been presented (Ray et al., 2005). One intriguing feature of these findings is that the number of amino acid substitutions present in any of the escape variants of HCV CTL epitopes delineated so far is rather low and usually is restricted to exchange of only one amino acid residue at a particular position. It is assumed that a relatively low level of genetic polymorphism within a viral sequence may reflect a high cost to viral replicative capacity incurred by many amino acid substitutions. However, only a limited number of studies have addressed this issue in the context of HCV infection (Söderholm et al., 2006). The aim of the current study was to use the HCV replicon system as a tool to investigate the influence of a series of amino acid mutations in a previously described CTL-epitope, including the known escape-mutations, on HCV RNA replication in cell culture.

We have observed that introduction of two described escape mutations at positions 1397 and 1398 of NS3 gene of Con1-based replicon (pFKI–rep PI luc/ 5.1), reduced the replicative ability of this replicon. Moreover, randomly substitution of lysine with asparagine, methionine, theonine, glutamine or glutamic acid at position 1398 of the NS3 gene of pFKI–rep PI/luc 5.1 impaired its replicative ability. The obtained results provide support for a notion that CTL escape mutations might be costly to the virus. Both escape mutants of the NS3-1395 CTL epitope, which emerged during infection of patients expressing HLA-B8 allele, unfavourably influenced the replicative potentials of HCV replicon.

These data also demonstrated that the ability of HCV to accommodate sequence variation within this epitope is limited. Sequence data from all genotype 1b isolates from the Los Alamos HCV database demonstrate that the NS3-1395 epitope region is highly conserved and sequence variations are restricted to a few sites. Moreover, both observed escape mutations of this epitope are confined only to conservative exchange of amino acid residues, namely, of substitution of lysine by arginine at position 1397 and 1398. No other amino acid substitutions are observed in natural HCV isolates in these positions and replicon constructs with other substitutions in the NS3-1395 epitope demonstrated the impairment of HCV RNA replication. The NS3 -1395 epitope is a part of the helicase domain of the protein that is indispensable for HCV replication (Penin et al., 2004). It seems that this epitope is located within a structurally and functionally important region of the protein that cannot tolerate significant variability of the primary structure.

Reduction of virus fitness associated with the escape mutation K1397R and K1398R of the NS3-1395 epitope also explain why upon a transmission of the mutated virus into a host, which does not have the restrictive HLA allele, a reversion to the original prototype sequence that confers replication advantage occurs with a high frequency (Timm et al., 2005; Ray et al., 2005).

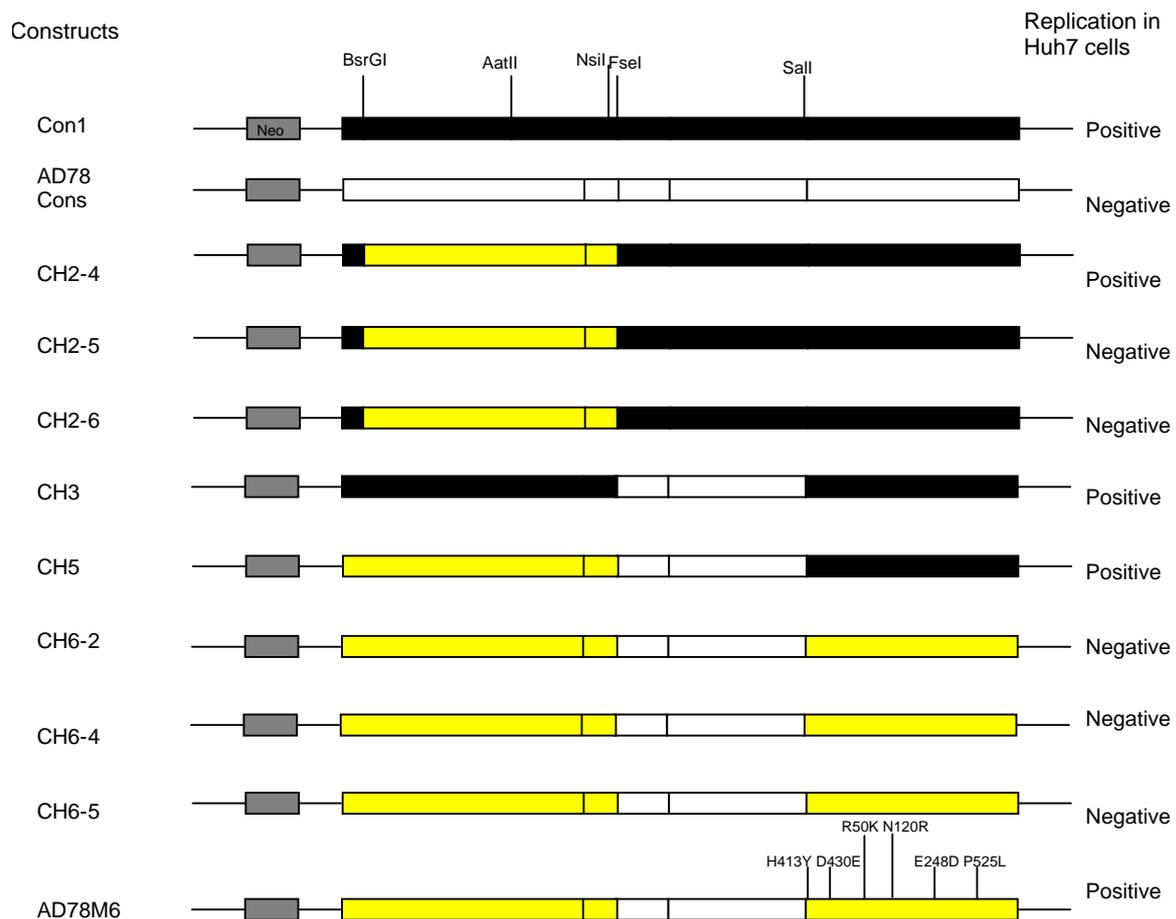
### 3. *Trans*-complementation of the defective NS3 genes cannot be achieved in vitro.

Recently, the persistence of the defective viral genomes in some HCV-infected patients was reported (Martell et al., 1992; Chau-Ting et al., 1997; Vallet et al., 2005). Although, the mechanism, by which these defective genomes persist, remains unclear, it is considered that such defective viruses might be complemented by wild type “complete” viruses, which provide in *trans* the elements essential for replication, enabling a reproduction of defective viruses. In the current study, we tried to explore if the *trans*-complementation can work in HCV replicon system. To this end, we assessed the in vitro rescue of defective replicons by *trans*-complementation using RNA of replication competent HCV subgenomic replicon as helper. All our attempts remained unsuccessful, although, a cell line bearing a persistent replicating RNA might offer the advantage of a functional HCV replication complex. Our results are concordant with data reported by Appel and coworkers (Appel et al., 2004) and Tong and coworkers (Tong et al., 2006). These two groups demonstrated that defective NS3 and NS5B genes of HCV cannot be complemented in *trans* by a functional proteins expressed from the endogenous replicons, although efficient *trans*-complementation of defective NS5A gene of HCV was observed. *Trans*-complementation was described for other members of the *Flaviviridae*, including bovine diarrhea virus (Grassmann et al., 2001) and Kunjin virus (Khomykh et al., 1999a; Khomykh et al., 1999b; Liu et al., 2002). In a Kunjin virus model *trans*-complementation of defective NS3 and NS5 genes was possible, whereas, only a defective NS5A gene was *trans*-complemented in a bovine diarrhea model system. The reasons for the absence of NS3 *trans*-complementation in HCV replicon system (as well as in Kunjin virus model) are not clear. One might suggest that the HCV replication complex is strikingly autonomous, and that HCV viral proteins, namely NS3 (presumably as a NS3/4A complex), NS4B, and NS5B are strictly *cis*-acting within a higher-order complex. Recently, it was reported that HCV RNA replication takes place within a membranous compartment (Miyanari et al., 2003; El-Hage et al., 2003; Quinker et al., 2005) that probably restricts access for exogenous RNAs and proteins. The formation of such a compartmentalized structure may limit the possibility for *trans*-complementation. Still, the question, how the defective HCV genomes, bearing deletions in NS3 genes (Yeh et al., 1997; Vallet et al., 2005), persist in patients, remains unanswered. Additional experiments with HCV replicons both in Huh7 and other cell systems supporting the HCV RNA replication might help to elucidate this important issue.

#### 4. Efficient replication of the intragenotypic subgenomic replicon chimera in Huh7 cells

During the last years a series of subgenomic replicon based on sequences of HCV isolates from genotype 1a (Blight et al., 2003; Liang et al., 2005), 1b (Lohmann et al., 1999; Groblert et al., 2002; Kato et al., 2003; Ikeda et al., 2002), and 2a (Kato et al., 2003) was established. Intergenotypic subgenomic replicons chimeras composed on sequences of 1a and 1b isolates (Gu et al., 2003; Lemm et al., 2005) or 1b and 3a isolates (Lanford et al., 2006) were also reported. With exception to genotype 2a-based subgenomic replicon, all described replicons required adaptive mutations for efficient replication in cell culture. As has been discussed in the previous sections, we attempted to establish a subgenomic replicon using a consensus sequence of HCV AD78 genotype 1b isolate. All our attempts, including that with introduction of the potentially adaptive mutation S2204I remained unsuccessful. The mutation S2204I is located within the hyperphosphorylation region in the NS5A gene of HCV was described as one of the most potent adaptive mutation for HCV replication in Huh7 cells (Blight et al., 2000). Subsequent experiments using the genetic approach clearly demonstrated that the replicon based on the AD78 consensus sequence was not able to replicate due to some defects in NS3 and NS5B fragments. Because we could not identify lethal mutations in the defective NS3 gene of our consensus sequence, the sequences corresponding to NS3 to NS4A genome fragment were amplified from sera of individual AD78-infected patients, and were used for substitution of the homologous fragment of Con1 replicon. The obtained chimeras were assessed for their replication ability in Huh7 cells (Fig. 65). As a result, one such a chimera turned out to be replication competent. Since we have observed that swapping of the sequence of the NS4B to NS5A genes of the consensus sequence of HCV AD78 into the Con1 replicon also resulted in the formation of viable Con1/AD78 chimera, the next logical step was to generate a chimeric intragenotypic molecule that would be comprised of the AD78-derived sequences corresponding to NS3-NS5A sequence (NS3-NS4 genes of the individual isolate of HCV AD78 with NS4B-NS5A genes of the consensus sequence) and the small fragment of the Con1 sequence (NS5B polymerase gene). One such a chimeric construct was able to replicate in Huh7 cells. We have demonstrated that HCV non-structural proteins were expressed in cells bearing this replicon and HCV RNA levels in these cells were comparable to what was observed in cells supporting the replication of the Con1 replicon.

The original Con1 based replicon used in this work bears two adaptive mutations in NS3 gene and one adaptive mutation in NS4A gene. This combination of adaptive mutations improves drastically the ability of this structure to replicate in Huh7 cells. When NS3-NS4 sequence of the individual isolate of HCV AD78 were introduced into Con1 replicon sequence, we could observe that the colony forming efficiency of the obtained chimera was significantly lower than what observed with the original Con1 construct, most probably, due to the removal of adaptive mutations. However, combination of this NS3-NS4A sequence with NS4B-NS5A sequence of the consensus sequence in the Con1 replicon showed a significant improvement in colony formation efficiency, which was still lower than that observed with the original Con1 replicon. Although both the Con1 and AD78 sequences were subtype 1b sequences, we could observe a difference in colony formation efficiency between the Con1/AD78 chimeras bearing NS3 to NS5A or NS3 to NS4A sequence of AD78. This observation suggests that optimal HCV RNA replication occurs only when the replication complex is composed of proteins encoded by the sequence of one virus isolate and not by proteins of closely related viruses belonging to the same subtype. Most probably, this rule is not an absolute one, as suggested by the comparable levels of HCV RNA in cells, transfected with original Con1 replicon and chimeric Con1/AD78 replicon CH5, in which the NS5B sequence was derived from the Con1 replicon.



**Fig. 65.** Genetic approach used for generation of a subgenomic replicon based on the sequence of AD78 strain.

A set of chimeric Con1/AD78 replicons (CH2-4, CH2-5 and CH2-6) was generated by swapping a BsrGI-FseI fragment (corresponding to NS3 gene of HCV) of Con1 with homologous sequence derived from three individual patients infected with AD78 strain. CH2-4, CH2-5 and CH2-6 contain a NS3 sequence amplified from patients 4, 5 and 6, respectively. Sequences derived from Con1 and AD78 consensus sequence are presented in black and white, respectively. Sequences derived from patients infected with AD78 strain are shown in yellow. CH5 was generated by swapping a NS3 fragment of CH3 (chimera containing a NS5A sequence derived from AD78 consensus sequence) with the homologous sequence derived from patient 4. Complete AD78-based replicons CH6-2, CH6-4 and CH6-5 were generated by swapping a NS5B fragment of CH5 (derived from Con1) with homologous sequences amplified from infected patients 2, 4 and 5, respectively. AD78M6 was generated after a series of six mutagenesis within the NS5 sequence of CH6-5.

### 5. Identification of a „functional” AD78 NS5B sequence.

Our previous data demonstrated that the subgenomic replicon based on AD78 consensus sequence was partly defective due to its NS5B gene. To overcome this problem, we amplified the AD78 sequences, spreading from the terminal region of NS5A to the end of HCV PolyU/UC tract, from sera of three patients infected with this isolate. In our approach, we directly cloned the amplified sequence into pCR 2.1Topo vector and have chosen only one clone of each cloning reaction for subcloning the amplified sequence into the CH5 replicon. In other words, the remaining Con1 sequence in the CH5 replicon was swapped with its homologous part from AD78 isolates. Overall, three replicon variants, each containing NS3 to NS5B AD78 sequence, were obtained and tested (Fig. 65). Unfortunately, these replicons were not able to replicate, suggesting that the amplified sequences were defective. There might have been two reasons for these negative results. One reason might have been associated with the variability and/or defect in the 3'-UTR sequence (polyU/UC tract) of the amplified AD78 sequences. This possibility, however, has been ruled out in the special series of experiments on substitution of the 3'-UTR AD78 sequences of these three chimeric replicons with the same sequence fragment from the Con1 replicon. Another possible explanation was the occurrence of lethal mutations in the NS5B sequence of the amplified fragments of AD78 sequence. Due to the lowest number of potentially deleterious mutations in the P5 construct we have preceded with a mutational analysis of NS5B sequence amplified from AD78-infected patient 5. As we have already mentioned, eight “uncommon” amino acid residues were found within the sequence of P5. Five of these amino acids were present only in this particular sequence and the remaining three were common for all AD78 sequences and absent in the Con1 sequence. Sequential substitutions of the five amino acid residues, which were found only within the sequence amplified from P5, with their counterparts present in the Con1 sequence, did not result in the appearance of a viable replicon. Considering this observation, we assumed that these mutations were not directly associated with the malfunction of the polymerase encoded by NS5B sequence amplified from patient 5. Because these amino acids residues were not found in a HCV database we suppose that some of them might have appeared during the amplification of this sequence.

Thereby, the sequence obtained after exchange of these five amino acid residues, was used for an additional series of sequential site-directed mutagenesis, designed to remove the three residues, which were present only in AD78-derived sequences. As a result, functional replicons were obtained. The substitution of asparagine for arginine at position 120 alone or in combination with substitution of serine for lysine at position 209 within the polymerase sequence was sufficient for an efficient rescue of the polymerase activity of AD78 NS5B. Of note, the substitution of arginine for proline at position 566, when combined with both previous substitutions, reduced significantly the activity of the polymerase. This observation suggests that the asparagine residue at position 120, which is present in all AD78-derived sequences, including the consensus sequence, somehow downregulates the function of this protein in Huh7 cells and that the proline residue at position 566 is essential for its activity.

The polymerase of HCV consists of three typical subdomains termed finger, palm and thumb. Several conserved sequence motifs have been proposed in the palm (motifs A, B, C, D, E) and finger subdomain (motif F) (Ago et al., 1999; Behens et al., 1996; Bressanelli et al., 1999; Ferrari et al., 1999; Lohman et al., 1997, 1998). The residue asparagine found at position 120 of the polymerase of AD78 isolate is located within the finger subdomain. Whereas, the residues serine and proline at positions 209 and 566 are located within the palm and thumb subdomains, respectively. One of the three mutations (S209) is located within a NS5B CTL epitope (HCV database). Thus, these amino acid residues are located outside of the putative active centre of the polymerase. Nevertheless, these amino acid residues might somehow change the structure of NS5B product, which in turn, might lead to the reduction of the polymerase activity or/and to the inhibition of the interaction of NS5B protein with other constituents of the replicative complex. One cannot exclude a possibility that the NS5B protein, containing these three mutations, is still active but at the very low level. This low activity would possibly preclude the detection of low levels of AD78 P5 replicon replication in cell culture. In natural infection, however, such a low level of polymerase activity could be enough to support HCV AD78 replication. In this context, one should note relatively low levels of HCV RNA in the majority of women infected with the HCV AD78 strain (Wiese et al., 2005).

## 6. Role of adaptive mutations in replication of HCV subgenomic replicon in Huh7 cells

Cell culture adaptive mutations were found to be required for an efficient enhancement of replication of HCV subgenomic RNA in vitro. These adaptive mutations have been found to be scattered throughout the all non-structural sequence of HCV. Several adaptive mutations are clustered in the central region of NS5A, the amino terminus of the NS3 helicase domain, and at two distinct positions of the NS4B region (Blight et al., 2000; Lohmann et al., 2001; Krieger et al., 2001; Blight et al., 2003; Lohmann et al. 2003). Although the mechanisms by which these mutations increase replication of HCV RNA in cell culture remain unknown, these mutations can be subdivided into at least two groups by their effects on replication efficiency and cooperativity (Lohmann et al., 2003). The first group is associated with mutations in NS3 (e.g., E1202G, T1280I) that have a low impact on replication efficiency. However, these mutations enhance replication cooperatively when combined with highly adaptive mutations. The second group involves mutations in NS4B (e.g., K1846T, V1897A), NS5A (e.g., S2197P, S2204I), and NS5B (e.g., R2884G), and this group has a high impact on replication efficiency; however, these mutations are incompatible with each other (Lohmann et al., 2003). Genetic analysis of our intragenotypic replicon chimera, one month after establishment of the replicon-bearing cell line, has revealed only one substitution of arginine for glycine at amino acid position 2884 in NS5B gene (R2884G). We have investigated the influence of R2884G mutation on the replication of our chimeric replicon and could observe that colony formation efficiency of the replicon was improved by the presence of the mutation, suggesting that R2884G acts as adaptive mutation in the replicon. The difference in the colony formation efficiency between the wild type and the replicon bearing R2884G mutation was more evident when cells were selected with 500  $\mu\text{g/ml}$  rather than with 250  $\mu\text{g/ml}$  of G418. This observation suggests that the colony formation efficiency depends on the complex interplay between the replication of HCV RNA in the cells and the selective pressure, which depends upon G418 concentration.

The mutation R2884G was found to act as one of the most powerful adaptive mutation in the Con1 replicon. Analysis of the X-ray crystal structure of NS5B reveals that this adaptive mutation is located on the surface of the polymerase (Lohmann et al., 2001). Moreover, the mutation is close to the end of helix R of the thumb domain and far away from the active site of the polymerase. These observations suggest that R2884G substitution is not directly involved in catalysis, although a contribution of this residue to RNA template binding is possible (Bressanelli et al., 1999). How this substitution influences the initiation and fitness of HCV RNA replication remains still unknown. Furthermore, enhancement of HCV RNA replication was also observed with substitution of arginine for lysine at amino acid position 345 of HCV polymerase gene (R345K corresponding to R2764K in HCV polyprotein), although this substitution did not occur during selection (Cheney et al., 2002). The mechanism by which R345K substitution enhances the replication of HCV RNA remains also unclear and one should keep in mind that arginine at position 345 is conserved among all HCV isolates.

We have also investigated the genetic evolution of our replicon within a long-term culture system. Replicon bearing cell lines were continuously cultivated in presence of 500 µg/ml of G418, we therefore determined the sequence of HCV RNA (from NS3 to NS5B regions) after 12 months of culture. Surprisingly, the mutation R2884G was fixed within the RNAs population of the analysed cell line. This observation suggested that the R2884G mutation is required for an efficient replication of this particular replicon cell culture. Besides this mutation only one additional mutation – a substitution of lysine for glutamic acid at amino acid position 1609 (K1609E) within NS3 gene of the analysed sequence – was registered. The average mutation rate observed within the polyprotein of our replicon was  $3.9 \times 10^{-4}$  substitutions per amino acid per year. This result is in line with the published data, where the mutability of RNA viruses was found to range from  $10^{-1}$  to  $10^{-5}$  per base site per year (Holland et al., 1982). Moreover, using a full-length replicon cell culture, Pietschmann and co-workers found an average mutation rate of  $8.5 \times 10^{-3}$  substitutions per amino acid position per six months and they claimed that the coding region for structural proteins appears to be slightly more likely to acquire mutations than the non-structural portion of HCV genome with the exception of the NS5A protein (Pietschmann et al., 2002). Another group, using a subgenomic replicon cell culture system, found no change in the sequence of replicon over a 16-month culture period (Sumpter et al., 2004).

The replicon used in their study had a lysine insertion at HCV codon 2040 (K2040) within NS5A encoding region. Cell line bearing the replicon K2040 was shown to be refractory to transfected dsRNA and fails to activate IRF-1 or NF- $\kappa$ B due to an NS5A imposed block in PKR-dependent signalling. HCV RNA stability over a 16-month culture in this cell line was associated with the absence of IFN- $\beta$  and ISG expression.

Investigation of K1609E mutation in our study revealed an enhancement of the colony formation efficiency of the replicon, as has been also observed with the substitution R2884G. This result suggests that K1609E acts as an adaptive mutation in our replicon. The mutation K1609E was also reported to work as an adaptive mutation in HCV-O replicon (Ikeda et al., 2005). Although K1609E mutation was found in Con1 replicon, this mutation could not increase the efficiency of colony formation of Con1. The fact that K1609E mutation influences differently the colony forming efficiency of HCV replicons, we assume that this might be due to the differences in HCV strains. The mutation K1609E was found to be located within the helicase domain of HCV NS3 gene. An inspection of the X-ray crystal of the NS3 helicase reveals that the mutation K1609E is located on the surface of the molecule and far away from the active site (Lohmann et al., 2001). Because of its localization, K1609E is not directly involved in the catalytic activity of the helicase. The other explanation is that this mutation as well as R2884G might influence the protein-RNA or protein-protein interaction in the replication complex.

Because K1609E and R2884G occurred in the same RNA genome, we wondered whether both mutations work synergistically as adaptive mutations. To address this issue, K1609E and R2884G were combined into our replicon. Interestingly, we found that combination of both mutations increased significantly the colony formation efficiency of the replicon in comparison to what we observed with each of them individually. These observations suggest that K1609E and R2884G mutations are additive and improve the ability of HCV RNA to replicate in cell culture. Although, combination of K1609E with other NS3 mutations was found to enhance the colony formation efficiency of Con1 (Lohmann et al., 2001), the mechanism governing this effect is still unknown. In the current work we assume that combination of K1609E with R2884G enhances the replication of HCV RNA, because simultaneously these mutations might offer the advantage of an highly active helicase and polymerase proteins, facilitating so the replication of HCV RNA in cell culture.

### **7. Interaction between HCV RNA replicon and the host cell line.**

The performed experiments clearly demonstrated the production of HCV non-structural proteins in the established replicon-bearing cell lines. We have investigated the influence of HCV RNA replication and of HCV non-structural proteins on the rate growth of Huh7 cells. We have observed no difference in the cell growth rate between naïve and replicon-bearing cell lines. No morphological evidence for additional cell degradation was observed in replicon-bearing cells. These data suggest that HCV non-structural proteins do not induce cytopathic effects in culture of Huh7 cells. These observations are in line with data reported by Pietschmann and co-workers (Pietschmann et al., 2000), who have not observed signs of cytopathogenicity such as a reduced growth rate or structural alteration in replicon-bearing Huh7 cell lines. Thus, our data and the observations by Pietschmann and co-workers support the hypothesis that in HCV infection the liver cell damage is primarily due to the immune reaction targeted against infected cells rather than to a direct cytotoxicity of HCV. It should be noted, however, that recently Naka and co-workers reported that NS5B protein of HCV, individually expressed, causes the delay of cell cycle progression in PH5CH8 human immortalized hepatocytes cells by inducing interferon- $\beta$  via Toll-like receptor 3 signalling pathway (Naka et al., 2006). Because the TLR3 signalling pathway was found to be defective in Huh7 cells (Lanford et al., 2003), one can not exclude that the lack of cytopathic effects of HCV proteins observed in experiments with HCV replicons might be associated with this characteristic of Huh7 cells. Therefore, new cell lines, which would possess an intact TLR3 signalling pathway and which would be able to support HCV replication, are needed for a detailed investigation of the potential effects of HCV replication on the host cells.

We have also investigated the influence of Huh7 cells growth on replication of HCV replicon. It was demonstrated that HCV replication level depends on host cells growth, suggesting that HCV replication in cells might depends on the availability of host cells factors. These results confirm the data presented for the Con1 replicon earlier (Pietschmann et al., 2000). Recently, Windisch and co-workers reported that replication of the Con1 replicon in the human hepatoblastoma cell line HuH6 was independent from ongoing cell proliferation (Windisch et al., 2005). Furthermore, Honda and co-workers observed that the HCV IRES activity in Huh7 cells varied during the cell cycle (Honda et al., 2000). Together these observations suggest that dependence of HCV RNA replication on the cell density is a characteristic of Huh7 cells.

## **8. Evaluation of antiviral compounds in vitro.**

Sensitivity to interferon therapy has been reported to vary among the genotypes of HCV (Yoshioka et al., 1992) and even among different clones of the same genotype (Enomoto et al., 1996). HCV NS5A protein has been reported to be associated with the resistance of HCV to interferon therapy in vivo (Enomoto et al., 1996). Recently, the HCV NS3/4A protein was identified as a potent inhibitor of cytokine gene expression (Foy et al., 2003; Kaukinen et al., 2006). In the current work we have investigated the sensitivity of the chimeric Con1/AD78 replicon CH5 and the full-length chimeric replicon to interferon-alpha and -gamma. These replicons turned out to be as sensitive to both of these cytokines as the original Con1 replicon, despite the fact that the source of the amplified AD78 sequences was the sera from interferon-alpha non-responder patients. Our observations are consistent with data reported previously (Frese et al., 2001; Lanford et al., 2003; Aus em Siepen et al., 2005) and indicate that all available HCV replicons, irrespective of the source of the material (responder or non-responder patients), are sensitive to interferon-alpha and -gamma. The reasons for this effect remain unknown.

## **9. Attempt to establish a new cell line permissive for replication of HCV RNA.**

Recent data suggest that a replicon cured cell line “Huh7.5” (Blight et al., 2002) supports more efficiently the spread of infectious virions of HCV than another replicon-cured cell clone of Huh7 cells, “Huh7-Lunet” (Koutsoudakis et al., 2006). This difference observed between both clones of Huh7 cells was due to a high expression of CD81, a molecule previously implicated in HCV entry, on the surface of Huh7.5 cell clone. Moreover, Huh7.5 cell clone displays a high permissiveness for HCV RNA replication because carrying a mutation in the intracellular double-strand RNA sensor retinoic acid inducible gene I “RIG-I” resulting in an inactivation of innate immune responses usually suppressing viral replication (Sumpter et al., 2005). These observations suggest that a high replication and spread of infectious HCV virion in cell culture depend on the intrinsic properties of the host cell line. Pursuing the goal to establish a new cell line that might support high levels of replication of HCV RNA and robust spread of infectious virions, we have attempted to transfect a number of cell lines.

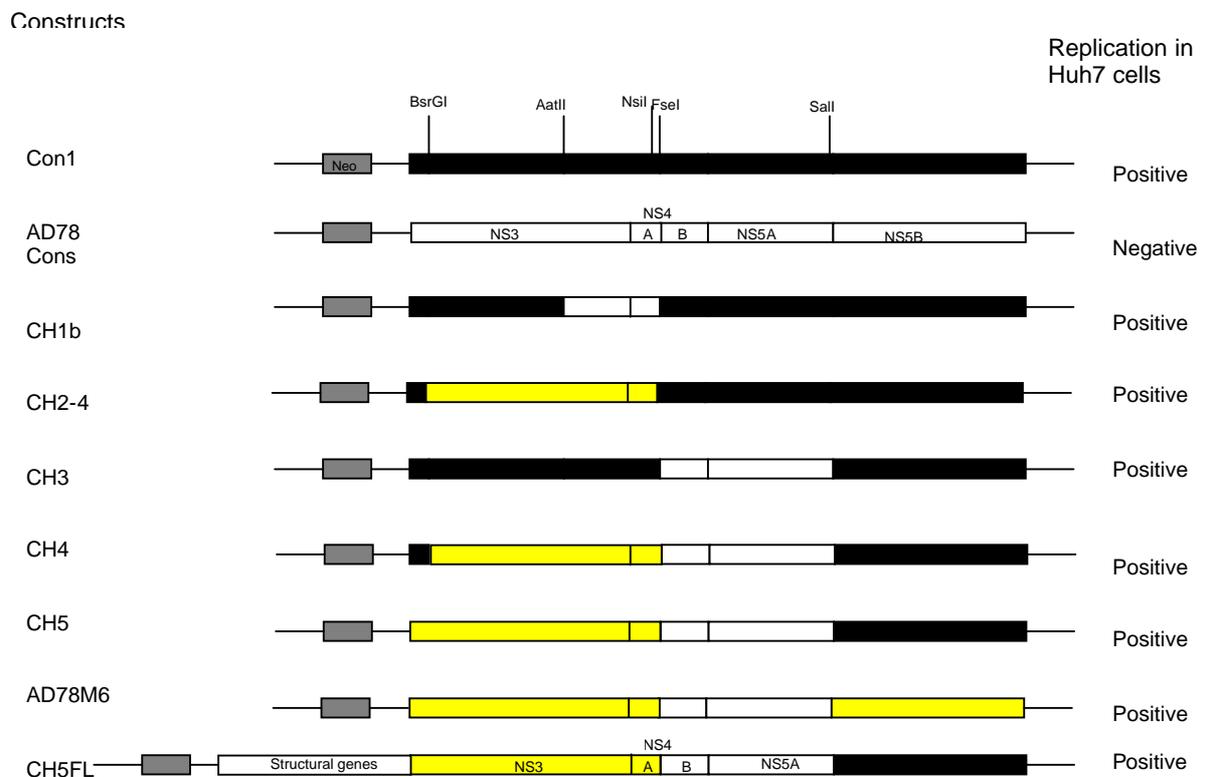
Among the transfected cell lines only a mutant Trf-1 of Huh7 cells could support replication of HCV RNA. Of importance is the fact that positive results were obtained only with the CH5 replicon bearing two mutations K1609E and R2884G. Transfection of Trf-1 cells with a highly adapted Con1 replicon or with the original CH5 replicon, or with CH5 construct bearing only each one of the adaptive mutations did not result in the appearance of G418-resistant colonies. Since only RNA carrying substitutions K1609E and R2884G was able to replicate in Trf-1 cells, we assume that these mutations were required for enhancing replication of CH5 RNA in this cell line as was already observed with Huh7 Lunet cells.

In additional experiments, the HCV RNA was removed from Trf-1-replicon cell line by treatment with interferon-alpha interferon-gamma. Because an efficient transient replication of HCV RNA in cells is the best criterion of a replication permissiveness of a cell line, a highly adapted Con1 replicon expressing a fire luciferase was used to assess the sensitivity of the “cured” Trf1 cells for HCV RNA replication. Surprisingly, IFN- $\alpha$ -treated cells could support replication of Con1 replicon only at low level. However, an increased permissiveness was observed with cells cured by treatment with combination of IFN- $\alpha$  and IFN- $\gamma$ . The phenotype of IFN- $\alpha$ -treated cells observed in the current study was also reported in other studies (Blight, 2002; Murray, 2002). These observations suppose that curing of replicon-containing cell lines do not always yield a cell population that are more permissive for the replicon tested and that the phenotype of the cured-cells might reflect the feature of the original parental cell line. This assumption is supported by the observation that a highly permissive replicon-cured cell line Huh7.5, carrying a mutation in the intracellular double-strand RNA sensor retinoic acid inducible gene I “RIG-I”, was obtained from a G418-selected cell clone that harboured replicons without adaptive mutations. One should note that a replicon-bearing cell line used for establishing a cured-Trf-1 cell line in the current work derived from a polyclonal cell population obtained by mixing a number of G418-resistant Trf-1 cell clones. Considering the hypothesis above, we assume it might be possible that among these cells only a fraction of cells became permissive after removal of HCV RNA by treatment with combination of cytokines.

### 10. Replication of the full-length, genomic Con1/AD78 replicon in Huh7 cells.

So far, three HCV sequences of genotype 1b (Con1, HCV-N and HCV-O stains) were successfully used for generating a dicistronic full-length genome replicon of HCV (Pietschman et al., 2002; Ikeda et al., 2002; 2005). In the current study, we also have attempted to generate a full-length dicistronic replicon based on the sequence of AD78 isolate. To this end, our best replicating CH5 replicon was used for an additional insertion of the AD78 consensus sequence, encoding core-NS2 proteins. As a result, a full-length replicon chimera CH5FL was generated (Fig. 66). Efficient replication of the established replicon was observed only with the construct containing two adaptive mutations K1609E and R2884G. Our observations are in accordance with data providing by other groups (Pietschman et al., 2002; Ikeda et al., 2002), who reported that Con1 and HCV-O full-length genome replicons required adaptive mutations for an efficient replication in Huh7 cells. Our attempts to infect naïve Huh7 Lunet cells with filtered supernatant from full-length replicon bearing cells were unsuccessful suggesting that this culture released no or very low amounts of infectious HCV progeny. This observation is consistent with the data reported previously (Pietschmann et al., 2002; Ikeda et al., 2002). The lack of an infectious HCV progeny production in our cell culture system might be attributable to the presence of EMCV IRES and neomycin gene in the 5'UTR of the HCV genome that interfere with virion assembly and release. Additionally, Bukh et al. reported that cell culture adaptive mutations reduced or completely blocked the infectivity of Con1 genome in chimpanzee (Bukh et al., 2002). These data suggest that the presence of two adaptive mutations in our full-length construct somehow precluded the formation of the infectious particles in the current study. This suggestion is supported by the observation that a robust production of infectious HCV progeny in transfected Huh7 cells was restricted to only one isolate of HCV genotype 2a termed JFH-1 (Wakita et al., 2005; Lindenbach et al., 2005; Pietschmann et al., 2006), which replicate to high levels without requiring adaptive mutations. However, this assumption was more recently challenged by the observation that a highly adapted genome of H77 (Genotype 1a of HCV) also supports formation of infectious HCV virions in Huh7.5 cell lines, although the infectivity of these particles was much lower than those, produced by JFH-1 strain (Kyung et al., 2006). This finding provides support for the hypothesis that another cell culture-adapted full-length replicon of HCV might also produce infectious virions if a highly permissive cell line expressing on its surface a high level of HCV receptors is used for transfection.

Adaptive mutations that enhance replication of our replicon system have never been tested in the monocistronic full-length genome replicon of Con1, which failed to produce infectious virions (Pietschmann et al., 2002). Considering this assumption, in the future we plan to generate a monocistronic full-length replicon that bear both adaptive mutations and to use a Huh7.5 cell clone in attempt to establish an infectious cell culture system based on HCV AD78 strain of genotype 1b.



**Fig. 66.** A set of generated chimeric Con1/AD78 and AD78-based replicons and their ability to replicate in Huh7 cells. Sequences derived from Con1 and AD78 consensus sequence are shown in black and white, respectively. Sequences derived from individual patients infected with AD78 strain are presented in yellow.

## Summary

The major aim of the study was the development of a novel subgenomic and full-length, genomic replicon system based on the sequence of HCV AD78 strain, which caused a single-source outbreak in several thousands women in Germany. Availability of serial blood samples from this AD78 cohort together with the generated AD78-based replicons would allow developing and implementing a series of new experimental approaches to study several important aspects of HCV research, including the HCV evolution and role of HCV-specific humoral and cellular immune responses in resolution of HCV infection.

In the current work we have used a genetic approach consisting in sequential substitution of genes from Con1, a prototype replicon of genotype 1b that replicates efficiently in Huh7 cells, with homologous genome fragments of AD78 strain. A set of functional HCV subgenomic chimeric Con1/AD78 replicons was generated. These chimeric replicons contained the following AD78-derived genes in the context of the Con1 sequence: (a) NS5A, (b) NS4 + NS5A; (c) NS3 + NS4A, and (d) NS3 + NS4 + NS5A. Finally, a subgenomic replicon, containing all non-structural genes of AD78 strain was generated. Huh7 cells transfected with these replicons support high levels of HCV RNA replication. The obtained results demonstrated that a viability of the chimeric replicons very much depends on the genetic context of the back-bone sequence, and that interactions between non-structural proteins may represent a critical determinant of replication competence. These data demonstrate the existence of a very complex interplay between different non-structural HCV proteins and regulatory elements in a particular cellular environment.

Establishment of the Huh7 cell lines persistently supporting replication of the chimeric Con1/AD78 replicon was associated by the appearance of adaptive mutations in NS3 and NS5B proteins, which significantly increased the levels of RNA replication. This highly adapted chimeric replicon was used as a basis for the development of a full-length, genomic replicon, comprised of genes for all structural proteins and NS2 to NS5A non-structural proteins of HCV AD78 strain, and of NS5B sequence from HCV Con1 strain.

The developed and characterized new set of subgenomic and genomic replicons, including the intragenotypic chimeras, may be used as a tool to study the mechanisms of HCV RNA replication, mechanisms of HCV resistance to interferon and other antivirals. Generation of these novel replicons on the basis of AD78 sequence allows also to study the mechanisms of HCV interaction with host cells and to analyse the influence of mutations, including the B- and T-cell escape mutations on HCV replication. Use of this genomic replicon for the development of the HCV AD78-based cell culture system for quantification of virus-neutralization, and for establishment of AD78-specific cell targets for assessment of T cell-mediated immune responses, would provide a unique possibility to study the evolution of the HCV-specific immune responses in a cohort of women infected with HCV AD78 strain.

The other aim of the current study was the analysis of the influence of mutations in the NS3 region of HCV genome on HCV RNA replication. Measurement of HCV RNA replication in cells, transfected with subgenomic replicon bearing different mutations in a well-characterised CTL-epitope located in NS3 protein, have shown that both tested escape mutations impaired significantly the HCV RNA replication, thus suggesting that the CTL escape mutations in the HCV genome might be associated with considerable reduction of viral fitness. At least in some cases, the amino acid exchanges leading to escape mutations probably arise not randomly and represent the only possible amino acid substitutions in the particular epitope site. This, in turn, suggests that the low level of variability of HCV CTL epitope might be attributable, in part, to an excessive fitness cost of the majority of possible amino acid mutations. The observations on a limited degree of variability of some HCV CTL epitopes may also have important consequences for the selection of potential targets of the T cell-based HCV vaccine of the future.

Finally, a new variant of human cells able to support the replication of HCV RNA was found. These Trf1 cells represent a variant of Huh7 cells, which is defective in cell surface protein trafficking. The Trf1 cells, transfected with the subgenomic replicons, support high levels of HCV RNA replication and might be used as tool to study peculiarities of HCV interactions with the host cells

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**List of Abbreviations**

<b>ARFP</b>	Alternative reading frame protein
<b>BSA</b>	Bovine serum albumin
<b>b-DNA</b>	Branched DNA
<b>cDNA</b>	Complementary DNA
<b>CD81</b>	Human tetraspanin cell surface receptor
<b>CH</b>	Chimera
<b>CTL</b>	Cytotoxic lymphocyte
<b>DEPC</b>	Diethylpyrocarbonate
<b>DMEM</b>	Dulbecco's modified Eagle medium
<b>DMSO</b>	Dimethyl sulfoxid
<b>DNA</b>	Deoxyribonucleic acid
<b>DNase</b>	Deoxyribonuclease
<b>dNTPs</b>	Deoxynucleotide triphosphates
<b>DTT</b>	Dithioltheitol
<b>ECF</b>	Efficacy of colony formation
<b>ECL</b>	Enhanced chemiluminescence
<b>EDTA</b>	Ethyleneglycol-bis(aminoethyl)-N,N,N',N'-tetraacetic acid
<b>ELISA</b>	Enzyme linked immunosorbent assay
<b>EMCV</b>	Encephalomyocarditis virus
<b>ER</b>	Endoplasmic reticulum
<b>FBS</b>	Fetal bovine serum
<b>Fig.</b>	Figure
<b>FITC</b>	Fluorescein isothiocyanate
<b>G418</b>	Neomycin sulfate
<b>g</b>	Gramm
<b>h</b>	hour
<b>HEPES</b>	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
<b>HCV</b>	Hepatitis C virus
<b>HP</b>	Horseradish peroxidase
<b>HVR</b>	Hyper variable region
<b>IFN</b>	Interferon
<b>IgG</b>	Immunoglobulin G

<b>IRES</b>	Internal ribosome entry site
<b>IU</b>	International unit
<b>kb</b>	Kilo base
<b>kDa</b>	Kilo Dalton
<b>LB</b>	Luria-Bertani
<b>Luc</b>	Luciferase
<b>M</b>	Molar
<b>mM</b>	Millimolar
<b>min</b>	Minutes
<b>mg</b>	Milligramm
<b>ml</b>	Milliliter
<b>MMLV</b>	Moloney murine leukaemia virus
<b>MOPS</b>	3-(N-morpholino)-propane sulphonic acid
<b>MTT</b>	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide
<b>Neo</b>	Neomycin phosphotransferase
<b>nm</b>	Nanometer
<b>ORF</b>	Open reading frame
<b>PAGE</b>	Polyacrylamide gel electrophoresis
<b>PBS</b>	Phosphate buffered saline
<b>PCR</b>	Polymerase chain reaction
<b>PMSF</b>	Phenylmethylsulfonyl fluoride
<b>RNA</b>	Ribonucleic acid
<b>RNase</b>	Ribonuclease
<b>rpm</b>	Rotation per min
<b>RT-PCR</b>	Reverse transcription-polymerase chain reaction
<b>sec</b>	second
<b>SDS</b>	Sodium dodecyl sulfate
<b>TEMED</b>	N,N,N',N'-tetramethylenediamine
<b>T-PBS</b>	Tween-PBS
<b>TNF-<math>\alpha</math></b>	Tumor necrosis alpha
<b>U</b>	Unit
<b>UTR</b>	Untranslated region
<b>Wt</b>	Wild type

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Erklärung:

Hiermit erkläre ich, gem. 6 Abs. 2, Nr.7 der Promotionsordnung der der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema “ Development and characterization of subgenomic and full-length replicons based on the sequence of HCV AD78 isolate” zuzuordnen ist, in Forschung und Lehe vertrete und den Antrag von Herr “Oniangue-Ndza” befürworte.

Essen, 30.03.2007

(Prof. Dr. M. Roggendorf)

Erklärung:

Hiermit erkläre ich, gem. 6 Abs. 2, Nr.6 der Promotionsordnung der der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, 30.03.2007

(Cesar Oniangue-Ndza)

Erklärung:

Hiermit erkläre ich, gem. 6 Abs. 2, Nr.8 der Promotionsordnung der der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

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(Cesar Oniangue-Ndza)