Hepatitis C virus replication systems

Bachelor’s Thesis

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TARTU 2008
Abbreviations

CFU – colony forming unit
DMSO – dimethyl sulfoxide
ECF – efficiency of colony formation
EMCV – encephalomyocarditis virus
FCS – fetal calf serum
HBV – hepatitis B virus
HCV – hepatitis C Virus
HFHs – human fetal hepatocytes
HIV – human immunodeficiency virus
HTLV-1 - human T-lymphotropic virus-1
IFN-α – Interferon-α
IFN-β – Interferon-β
IRES - internal ribosome entry site
IU – international unit
LDLR - low-density lipoprotein receptors
luc – firefly luciferase
NANBH – non-A, non-B hepatitis
npt - neomycin phosphotransferase
nt - nucleotides
ORF – open reading frame
PBMCs – peripheral blood mononuclear cells
PEG – polyethylene glycol
PV - poliovirus
RT-PCR – reverse-transcription polymerase chain reaction
SCID - severe combined immunodeficiency
SOD – superoxide dismutase
uPA – urokinase-type plasminogen activator
UTR – untranslated region
**Introduction**

Hepatitis C virus (HCV) genome was cloned in 1989 (Choo et al., 1989). HCV is a major causative agent of chronic liver disease, which can develop into chronic active hepatitis, liver cirrhosis and hepatocellular carcinoma (Moradpour et al., 2001). It has been estimated that approximately 170 million people are infected with HCV worldwide (Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium, 1999). Hepatitis C virus is a member of Flaviviridae family and it is assigned to a separate genus, Hepacivirus (Robertson et al., 1998). HCV is a small enveloped RNA virus with a diameter of ~50 nm. HCV genome is ~9600 kb, single-strand RNA of positive polarity, which contains an open reading frame (ORF) flanked by 5’- and 3’-UTR sequences. ORF encodes a single precursor polyprotein of ~3000 amino acids (Choo et al., 1989). Translation is initiated by internal ribosome entry site (IRES) located in the 5’ UTR. HCV polyprotein is cleaved co- and posttranslationally into at least 10 products of two type: structural (core, E1, E2 and p7) and nonstructural (NS) proteins (NS2, NS3, NS4a, NS4b, NS5a, NS5b) (Suzuki et al., 2007).

Since the discovery of the virus it has been intensively studied. One of the primary requirements for the progress in the HCV research field was the establishment of the HCV replication model. Hard work and some luck have ultimately led to the development of efficient and reliable cell cultures for this pathogen, but it has been a long way to go. Soon after cloning the HCV genome and characterization of its proteins attempts have been undertaken to develop a robust cell culture system for HCV. Most often such systems are based on the inoculation of permissive host cells with virus present in a biological sample. However, in case of HCV this approach failed, because the replication rate in developed systems was too low. In parallel, other efforts concentrated on the identification of an infectious HCV clone, but these attempts have not been successful initially. First significant achievement in these studies was the construction of a functional HCV consensus genome with proven *in vivo* infectivity. Another important breakthrough was the establishment of the HCV replicon system based on a selection of cells that supported stable replication of subgenomic (or genomic) HCV RNAs. This system made the detailed studies of HCV replication, translation, etc. possible and allowed the generation of highly permissive cell clones. Unfortunately, these replicons did not support production of infectious virus.
However, very recently a particular HCV clone designated JFH-1 was isolated and used to develop a system, which supported generation of infectious HCV upon transfection of cultured human hepatoma cells. This new cell culture system represents a major breakthrough in the HCV field and should provide a wide range of possibilities for studies of the HCV life cycle.

The aim of this work is to give an overview of some *in vitro* HCV replication models and their progress from cell cultures infected with sera of patients to the development of a JFH-1-based systems producing virions that are proven to be infectious *in vitro* and *in vivo*. 
1. Overview of HCV genomic organization.

Fig. 1. HCV genome organization. HCV coding region is shown as a bar with vertical lines indicating the positions where polyprotein cleavage occurs. The 5'- and 3'-UTR structures are schematically drawn with HCV IRES, X-tail and poly(U/UC) tract indicated. The functions of viral proteins are specified in the bottom. The core+1 protein expressed in alternative reading frame is given in the upper left. The role of this protein in the viral lifecycle is still obscure, but it is known that the protein is not essential for HCV replication (McMullan et al., 2007). (U/UC), poly(U/UC) tract.

HCV genome consists of a single strand of positive-sense RNA of approximately 9.6 kb which contains an open reading frame (ORF) coding for a polyprotein precursor of approximately 3000 residues (Choo et al., 1989). ORF is flanked by untranslated regions (UTRs) at both ends (Fig. 1). The 5'-UTR, which is \( \approx 341 \) nucleotide (nt) in length, contains an internal ribosomal entry site (IRES), which is essential for cap-independent translation of viral RNA (Bukh et al., 1992; Brown et al., 1992; Tsukiyama-Kohara et al., 1992; Honda et al., 1999). The 3'-UTR varies between 200 and 235 nt in length, including a short variable region, a poly(U/UC) tract with an average length of 80 nt, and a virtually invariant 98-nt X-tail region (Tanaka et al., 1995; Kolykhalov et al., 1997; Ito and Lai, 1999). The X-tail region forms three stable stem–loop structures that are highly conserved among all genotypes and, as a result, the HCV genome likely ends with a double-strand stem structure. It appears that the 3' X region, as well as the 52 nt upstream of the poly(U/C) tract, are crucial for RNA replication, while the remainder of the 3'-UTR plays a role in enhancement of replication (Friebe and Bartenschlager, 2002; Yi and Lemon, 2003). To date, hepaciviruses are divided into 6 principal genotypes of HCV that differ in their nucleotide sequences by 31–34%, and in
their amino acid sequences by ~ 30%. An important feature of the HCV genome is its high degree of genetic variability (Martell et al., 1992; Pawlotski et al., 2006). Mutation rates, however, vary in different regions. The E1 and E2 regions are the most variable, while the 5′-UTR and terminal segment of the 3′-UTR have the highest degree of sequence conservation among various isolates. Due to this variability, HCV, like many other RNA viruses, is present in infected individuals as a population of diverse but closely related variants referred to as quasispecies (Martell et al., 2002).

The precursor polyprotein is cleaved into at least 10 different proteins: the structural proteins Core, E1, E2 and p7, and the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (Choo et al., 1989). Interestingly, an additional protein is translated from a coding sequence overlapping with the HCV core protein coding sequence in +1 reading frame (core+1 ORF) (Branch et al., 2005). However, role of this protein in the HCV life cycle is not yet known. Organization of polypeptide and functions of different HCV proteins are shown in Fig. 1.

2. Non-A, non-B hepatitis – unknown virus

For many years a disease called non-A, non-B hepatitis (NANBH) remained a puzzle for scientists. The disease was first described in 1975 (Feinstone et al., 1975) and further proven not to be associated with previously discovered hepatitis A and hepatitis B viruses (Choo et al., 1989). Numerous attempts have been undertaken to identify the causative agent of NANBH, however, despite intensive investigation it took almost 15 years until this agent was eventually isolated.

Conventional immunological methods available at that time were not successful in identifying specific viral antibodies and antigens (Choo et al., 1989). Failure of these methods was associated either with a lack of viral antibodies or insufficient concentrations of NANBH viral antigens. Taking this into consideration, Houghton and colleagues used a “blind” immunoscreening approach for the identification of viral antigens. First, in order to increase viral antigen concentrations, a cDNA library derived from the infectious material was constructed in the bacteriophage λgt11, which allowed the efficient expression of cDNA-
encoded polypeptides. The library was derived from a chimpanzee plasma containing relatively high infectious titer (Choo et al., 1989). This plasma was ultracentrifugated in order to ensure the pelleting of a small virus and nucleic acids were extracted from the pellet. Since the nature of the viral genome was unknown at that time, the extracted nucleic acids were denatured before synthesizing cDNA in order to allow both DNA and RNA to serve as template. Then the library, which contained about $10^6$ recombinant λgt11 phages, was screened for clones expressing viral antigens with serum from a chronic NANBH patient as it was supposed to contain viral antibodies. The screening resulted in identification of positive cDNA clone 5-1-1. By analysis of a larger overlapping clone (isolated from the same library and designated 81), it was proven not to be derived from a host genome, excluding the possibility of human or chimpanzee DNA contamination. It also was demonstrated that the clone is derived from a single-strand RNA molecule of positive polarity, which consisted of approximately 10 000 nucleotides and appeared to be flaviviridae or togaviridae family related virus (Choo et al., 1989). The ORF of the clone was expressed in bacteria as a fusion polypeptide with human superoxide dismutase (SOD) in order to investigate the relation of encoded polyprotein to NANBH. Further immunoblot analyses demonstrated that the chronic NANBH patient serum reacted specifically with this fusion polypeptide, indicating that the polyprotein encoded by the clone 5-1-1 ORF is associated with NANBH infections.

“Thus, our data indicate that clones 5-1-1 and 81 are derived from the genome of a blood-borne NANBH virus that we now term the hepatitis C virus (HCV)” (Choo et al., 1989). With these words the era of NANBH ended and the era of HCV began. Cloning of HCV without any prior knowledge concerning the virus and its genome, without an in vitro culture system, without previous visualization of the viral particles was an extraordinary achievement that finished a search for the infectious agent responsible for NANBH. This study was a milestone event in HCV research field. It significantly accelerated the progress in the molecular biology of HCV and allowed to proceed to another important stage – development of a cell culture system for newly identified virus.
3. Infection of cells with infectious HCV-containing sera. The lack of replication model.

After the HCV genome was cloned and described, several research groups concentrated their efforts on the development of a cell culture system for HCV. A whole branch of studies was dedicated to establishing a cell culture system based on the infection of primary cells or immortalized cell lines with the sera of infected patients. Unfortunately, it turned out to be very difficult. In spite of this, a number of cell culture propagation systems for HCV has been described (Table 1) that are based on the infection of primary cell cultures or cell lines with pathogen-containing sera. However, these systems suffered from poor reproducibility and yielded low level of HCV replication. Some of these models will be described here.

Since the main in vivo targets for hepatitis C virus are hepatic cells, they should have a greater potential for developing a cell culture system. Being guided by this assumption several groups used primary hepatocytes or hepatic cell lines in their studies.

In 1993, Iacovacci et al., using the reverse-transcription polymerase chain reaction (RT-PCR) technique, tested the susceptibility to the HCV infection of human primary fetal hepatocytes (HFH), maintained for over 3 months (Iacovacci et al., 1993). They observed that the developed cell culture system supported HCV infection and produced infectious virus (Iacovacci et al., 1993, 1997). Presence of the negative-strand HCV RNA was also detected in infected cells. However, only few HCV RNA molecules (0.001-0.01 genome equivalents per cell) were detected in cells and supernatants from infected HFH cultures (Iacovacci et al., 1993, 1997). In subsequent study by the same group the efficiency of this system was improved, resulting in approximately 0.065 HCV genomes per cell (Iacovacci et al., 1997). However, such replication level remained still too low for the efficient utilization of the system.

In another study (Zhao et al., 2002), hepatocytes originated from a tree shrew (Tupaia belangeri), a species closely related to primates, were infected with HCV. In this model, HCV RNA level reached about 1 genome equivalent per cell, which was the best result for analogous systems of that time. Unfortunately, 14 days after plating the viability of hepatocytes started to decline rapidly, precluding a meaningful HCV RNA analysis at later time points.
Another HCV cultivation system was established by culturing primary hepatocytes from patients with chronic hepatitis C virus by Ito and colleagues in 1996 (Ito et al., 1996). Hepatocytes were prepared from liver biopsies. Seven independent hepatocyte culture systems were used, and in five cases HCV RNA was detected in both cell culture and supernatants by

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Species</th>
<th>Persistence (days)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foetal human hepatocytes</td>
<td>Human</td>
<td>24</td>
<td>Iacovacci et al., 1993</td>
</tr>
<tr>
<td>Chimpanzee hepatocytes</td>
<td>Chimpanzee</td>
<td>25</td>
<td>Lanford et al., 1994</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Human</td>
<td>26</td>
<td>Cribier et al., 1995</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>Human</td>
<td>9</td>
<td>Müller et al., 1993</td>
</tr>
<tr>
<td>PBMCs (In vivo)</td>
<td>Human</td>
<td>28</td>
<td>Ito et al., 1996</td>
</tr>
<tr>
<td>Hepatocytes (In vivo)</td>
<td>Tree shrew (Tupaia belangeri)</td>
<td>14</td>
<td>Zhao et al., 2002</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>Human</td>
<td>25</td>
<td>Shimizu et al., 1992</td>
</tr>
<tr>
<td>HPB-Ma</td>
<td>Human</td>
<td>76</td>
<td>Shimizu et al., 1993</td>
</tr>
<tr>
<td>Hpb-Ma 10-2 clone</td>
<td>Human</td>
<td>&gt; 365</td>
<td>Nakajima et al., 1996</td>
</tr>
<tr>
<td>PH5CH (hepatocytes)</td>
<td>Human</td>
<td>30</td>
<td>Kato et al., 1996</td>
</tr>
<tr>
<td>MT-2</td>
<td>Human</td>
<td>15</td>
<td>Kato et al., 1995</td>
</tr>
<tr>
<td>MT-2C</td>
<td>Human</td>
<td>198</td>
<td>Mizutani et al., 1996</td>
</tr>
<tr>
<td>Daudi</td>
<td>Human</td>
<td>&gt; 2 years</td>
<td>Nakajima et al., 1996</td>
</tr>
<tr>
<td>HepG2, Huh-7, PK15, STE</td>
<td>Human, porcine</td>
<td>130</td>
<td>Seipp et al., 1997</td>
</tr>
<tr>
<td>MEG-01 megacaryoblastic</td>
<td>Human</td>
<td>continuous</td>
<td>Tagawa et al., 1995</td>
</tr>
<tr>
<td>leukemia cells</td>
<td></td>
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</tr>
</tbody>
</table>
RT-PCR up to the end of the observation period. In two cases (cultures from patients with liver cirrhosis) HCV was continuously released at a consistently high titer for 8 weeks. However, overall efficiency of this cell culture system was low and the system had a lot of limitations, for instance, due to the poor availability of infected primary human hepatocytes.

After almost 10 years of research, it was not still quite clear what factors affect the efficiency of the HCV replication in primary hepatocyte cultures. In 1999, Rumin and coworkers conducted a study using primary adult human hepatocytes (Rumin et al., 1999). The aim of the study was: to establish culture conditions that allow long-term replication of HCV in these cells, to define the features of infectious inocula and to analyze HCV quasispecies (heterogeneous viral population) selection in long-term cultures established from different liver donors. The HCV replication and passaging of infection to naïve cells were successfully shown. The highest viral titer was about 60 000 genomes per ml of supernatant (Rumin et al., 1999). Moreover, several important results were achieved: first, HCV replication turned out to be strongly dependent on the medium used for in vitro infection (dimethyl sulfoxide (DMSO) and normal human serum favoured long-term maintenance of highly differentiated hepatocytes). Second, under adequate culture conditions hepatocytes could survive in a differentiated state for 4 months without morphological changes. Third, it was indicated that neither the genotype, nor the viral titer or the anti-HCV antibody content in sera were reliable predictive factors of sera infectivity. And fourth, the obtained evidence demonstrated that different quasispecies were selected in cultures established from different livers and that these quasispecies were minor components of the inoculum (Rumin et al., 1999). Unfortunately, no significant improvement in viral replication efficiency was achieved in this and later studies based on primary hepatocytes.

Although primary human hepatocytes infected with sera of patients were the most physiological in vitro model at that time, there was a lot of issues inherent to the work with primary cell cultures. For example, it is difficult to obtain these cells and they are permissive to HCV for the very limited time during which these cells are usable (Iacovacci et al., 1993, 1997). That makes it hard to use them regularly and explains the limited number of studies based on this model. Thus, in parallel, efforts were made to develop an in vitro model based on immortalized hepatic cell lines. Seipp and colleagues have tested several approaches to establish reproducible in vitro infection systems for HCV using the human hepatoma cell lines Huh-7 and Hep2G, and porcine kidney cell line PK-15 (The list of the cell lines mentioned in
this work is given in Table 2) (Seipp et al., 1997). The effects of the polyethylene glycol (PEG) and DMSO have also been tested in order to improve in vitro infections. This group succeeded in establishing a long-term cell culture where positive-strand HCV RNA could be detected during 130 days. Negative-strand RNA was detectable after several weeks in culture, indicating persistent infection with low level of replication. The amount of viral RNA remained at low levels of maximally 0.03 genome copies per cell during the whole culture period. Although the results on nested RT-PCR were not always positive after prolonged culture, the production of the infectious viral particles was shown by passaging of infection to fresh cells of the same cell line, indicating that the reason for these negative results might be the lack of sensitivity of the detection system. The addition of PEG and DMSO turned out to have no effect in long-term cell cultures. Moreover, the best results were achieved mainly in unsupplemented HuH-7 cells grown in fetal calf serum (FCS)-free medium, because this medium increased expression of low-density lipoprotein receptors (LDLR), which were later shown to be involved at an early stage in infection of normal human hepatocytes by serum-derived HCV virions (Molina et al., 2007).

After it was reported that NANBH could be transmitted to chimpanzees via leukocytes derived from infected patients (Hellings et al., 1985), the hypothesis of the lymphotropism of its causative agent has risen, suggesting that lymphocytes may also be a host for it. To verify this hypothesis, Shimizu et al. tested a human T-cell line, MOLT-4, either uninfected or infected with murine retroviruses, for its ability to support HCV replication, inoculating them with virus-containing serum or plasma (Shimizu et al., 1992). Minus-strand HCV RNA and viral antigens were successfully detected. As MOLT-4Ma cells (those, preinfected with murine retrovirus) gave stronger detection signals for minus-strand viral RNA, they were chosen for further experiments, in which inoculated cells were maintained for ~ 4 weeks. In these cells HCV RNA was detectable for 24 days (Shimizu et al., 1992). Although the infection was transient and inefficient, this study was valuable, because it demonstrated the ability of HCV to replicate in human T-cell line. In another report (Shimizu et al., 1993), the same group presented the evidence that human T-cell line HPB-Ma (preinfected with murine retroviruses) supported HCV replication better than MOLT-4Ma. The HCV genome was detectable for 76 days, which was about 3 times longer than the duration reported for MOLT-4Ma cells (Shimizu et al., 1992). The production of virions in HPB-Ma cells was reported in subsequent study (Shimizu and Yoshikura, 1994). It was shown that the HCV-infected cells
were able to transmit the virus to new cells (neomycin resistant naïve cells, constructed specially for this study) after coculture (Shimizu and Yoshikura, 1994).

**Table 2.** List of cell lines mentioned in this work with a short description.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Species</th>
<th>Origin</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>Human</td>
<td>Hepatocellular carcinoma</td>
<td>Epithelial in morphology cells, derived from the liver tissue of a patient with a well differentiated hepatocellular.</td>
</tr>
<tr>
<td>Huh-7</td>
<td>Human</td>
<td>Hepatocellular carcinoma</td>
<td>Well differentiated human hepatocellular carcinoma cell line. Can be grown in serum-free medium.</td>
</tr>
<tr>
<td>PH5CH</td>
<td>Human</td>
<td>Hepatocellular carcinoma</td>
<td>Cell lines from non-neoplastic liver and hepatocellular carcinoma tissue. Immortalized with SV40 large T antigen</td>
</tr>
<tr>
<td>PK15</td>
<td>Porcine</td>
<td>A porcine kidney epithelial cell line.</td>
<td>A porcine kidney epithelial cell line.</td>
</tr>
<tr>
<td>MOLT-4Ma</td>
<td>Human</td>
<td>T-cell leukemia</td>
<td>T lymphoblast cell line, preinfected with murine retrovirus.</td>
</tr>
<tr>
<td>HPB-Ma</td>
<td>Human</td>
<td>T-cell leukemia</td>
<td>T-cell line, infected with an amphotropic murine leukemia virus.</td>
</tr>
<tr>
<td>MT-2</td>
<td>Human</td>
<td>T-cell lymphoma</td>
<td>T-cell line, which is continuously human T-lymphotropic virus-1 (HTLV-1)-infected.</td>
</tr>
<tr>
<td>MT-2C</td>
<td>Human</td>
<td>T-cell lymphoma</td>
<td>A clone of MT-2 cell line.</td>
</tr>
<tr>
<td>MEG-01</td>
<td>Human</td>
<td>Megakaryoblastic chronic myelogenous leukemia cells</td>
<td>A megakaryoblastic cell line.</td>
</tr>
<tr>
<td>Daudi</td>
<td>Human</td>
<td>B-cell lymphoma</td>
<td>Human B lymphoblast cell line.</td>
</tr>
<tr>
<td>Huh7-Lunet</td>
<td>Human</td>
<td>Hepatocellular carcinoma</td>
<td>Highly permissive cell lines, derived from Huh-7 cell line</td>
</tr>
<tr>
<td>Huh7.5</td>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Huh-7.5.1</td>
<td>Human</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Another study, by Nakajima et al., involved two cell lines: the above mentioned human T-cell line HPB-Ma (a particular clone HPBMa10-2) and a human B-cell line Daudi (Nakajima et al., 1996). These cell lines supported HCV replication and production of infectious hepatitis C virus for more than 1 year (Nakajima et al., 1996). However, Daudi cells developed cytopathic effects upon virus inoculation and the cell culture could only be maintained by adding fresh Daudi cells after removing the old medium at each passage. In summary, this group was able to obtain HCV adapted to cultured lymphatic cells. In addition, sequence analysis of the HCV genomes recovered from those cell cultures and from inoculum (HCV-positive serum) used for infection, revealed that only specific subsets of the virus could replicate well in lymphocyte cell lines (Nakajima et al., 1996). Thus, it might be speculated that HCV with a particular sequence might have a replication advantage in lymphocytes both in vitro and in vivo. Further, HCV, contained in culture supernatant of Daudi cells, was inoculated into a chimpanzee in order to test its in vivo infectivity (Shimizu et al., 1998). Unfortunately, the efficiency of infection was very low. This data, as well as the analysis of genomic sequences from inoculum used for infection of Daudi cells, from culture supernatant and from the samples collected from the inoculated chimpanzee, indicated that the HCV variant which grew preferentially in the cultured lymphocytes did not replicate favorably in vivo, and the replication advantage in vitro did not correlate with the in vivo infectivity (Shimizu et al., 1998). Sugiyama et al. have obtained and analyzed cDNA clones covering the whole viral genome from HCV infected MT-2C cells (another cell culture system based on the human T-lymphocyte cell line that supported HCV replication) (Sugiyama et al., 1997). The results of this study supported the suggestion that only a limited HCV population in the HCV inoculum is able to adapt to replication in a cell line, meaning that there is a selection for particular virus variants, that either bind to or replicate more efficiently in cultured cells (Sugiyama et al., 1997).

A system based on the infection of primary peripheral blood mononuclear cells (PBMCs) had also been reported. In 1995 Cribier et al. demonstrated that PBMCs were permissive for HCV replication in vitro (Cribier et al., 1995). However, the replication level was very low. Cells from healthy donors (volunteers, who were not infected with human immunodeficiency virus (HIV), hepatitis B virus (HBV) or HCV) were incubated with serum containing HCV. In some cases, negative-strand HCV RNA was detected in the cells, but not in the supernatants and detection of plus-strand RNA was only occasionally successful. Slightly better results were
obtained when the cells from different donors were pooled (mixed together). However, overall efficiency of the system was very low, HCV replication was weak and the HCV RNA concentrations in cultured cells did not exceed HCV RNA concentrations in the cells of chronically infected patients (Cribier et al., 1995)

In summary, use of HCV-containing sera to reconstitute the entire life cycle of HCV in vitro has proved to be very difficult if not impossible. Although described systems were quite valuable for in vitro investigation of some aspects of the HCV replication cycle, low replication efficiency and technical problems inherent to work with primary cells have limited their use. Studies described in this chapter indicated that HCV replication was possible in hepatocyte and lymphocyte cell lines. However, these systems were not reliable and did not result in robust HCV replication, making it impossible to use them as models for studying the viral life cycle in detail or for screening antiviral drugs.

4. HCV clones. Intrahepatic inoculation of genotype 1a Hepatitis C Virus: a chimpanzee model

The infection of cell cultures with cloned viral genome (cDNA) or its in vitro transcripts would give a lot of benefits in studying viral life cycle, compared to cultured cells infected with HCV using HCV containing sera. First, in case of this approach inocula do not contain extraneous material and are well defined. Second, the genome can be synthesized in large quantities. And third, it can be manipulated in any desirable way permitting analysis of different aspects of HCV molecular biology. Regardless of these obvious benefits, initial attempts to propagate HCV in cell culture using cloned HCV genome were not successful. Only in two studies cell culture systems with inefficient and transient infection were established (Yoo et al., 1995; Dash et al., 1997). In a study by Yoo et al. differentiated human hepatoma cell line HuH-7 was transfected with HCV RNA transcripts generated from a cDNA clone of HCV type 1 (HCV-1) (Yoo et al., 1995). Negative-strand RNA was detected and transmission of the infection to naïve cells was also shown. However, intracellular expression of viral antigens was not demonstrated (Yoo et al., 1995). Dash et al. used another human hepatoma cell line Hep2G for transfection with infectious HCV genome (Dash et al., 1997). In
this study expression of HCV proteins was also detected (Dash et al., 1997). Culture supernatants of infectious HCV RNA transfected cells were later shown to be infectious at low level in vivo (Dash et al., 2001). However, overall, these systems were rather an exception than a rule. Thus, most efforts in the field of HCV molecular clones at that time concentrated on the identification of an infectious clone. Problems with construction of a functional HCV cDNA included the highly variable “quasispecies” nature of this virus (presence of numerous “subsets” of viral genomes in infected organism), the small quantities of viral RNA present in clinical samples, which required in vitro amplification (producing extra errors) before cloning and the lack of a simple and verified transfection assay for infectivity (Kolykhalov et al., 1997).

The first infectious cDNA was successfully obtained in two independent studies from HCV genotype 1 in 1997 (Kolykhalov et al., 1997; Yanagi et al., 1997). The HCV genome was cloned from the high-titer serum of a patient called “H77“. Initially, HCV RNAs transcribed from cDNA clones of these genomes did not result in infection in chimpanzees after intrahepatic inoculation (Kolykhalov et al., 1997; Yanagi et al., 1997). That might be caused by low RNA transfection efficiencies in vivo and errors introduced during cDNA synthesis or PCR amplification (Kolykhalov et al., 1997). To test the latter concern, some clones were sequenced and the sequences were aligned in order to determine a consensus sequence for the H77 isolate (Kolykhalov et al., 1997). This analysis revealed that the sequenced clones contained nonconservative nucleotide sequence changes (resulting either in amino acid substitutions in predicted polypeptide or in frame shifts, or introducing stop-codons into the genome) throughout the genome, which could be deleterious and would explain the negative results (Kolykhalov et al., 1997). Thus, directed by this information, a full-length clone was constructed, which reflected the consensus sequence of H77, and in which all undesired mutations that introduced either amino acid changes into predicted polypeptide or frameshift mutations, or stop-codons, were excluded. Mutations were considered undesired if they were present in the minority of analyzed genomes. In order to test the infectivity of the constructed genome, it was inoculated into chimpanzees (the only reliable animal model that supports replication of HCV) by intrahepatic injection at multiple sites of the liver. In both studies chimpanzees developed an infection, indicating that genotype 1a H77 isolate consensus genomes were infectious in vivo, and that failure of initial attempts was caused by the presence of deleterious mutations in the utilized genomes (Kolykhalov et al., 1997; Yanagi et
al., 1997). These studies identified for the first time functional HCV genomes and demonstrated that HCV infection is sufficient to cause liver disease.

5. The HCV replicon system: cell culture access

5.1 Subgenomic HCV replicons

5.1.1 Establishment of the subgenomic replicon system

Since the construction of consensus genome some infectious HCV cDNA clones have been established, but none of them have been adapted to cell culture. At the same time, all HCV cell culture systems that have been reported appeared to have very low efficiency of viral replication. It became obvious that very sensitive methods for a detection of viral replication were required in order to utilize these systems. However, in case of cell culture systems based on the infection of cell lines with HCV clones, detection of a low-level replication is very difficult due to the high amounts of RNA used for transfection and its stability. To overcome these problems, a novel cell culture system that allowed the monitoring of HCV replication using conventional methods was developed. This system based on the replication of HCV replicons (subgenomic constructs expressing the viral replicase complex and capable of autonomous replication) that contained G418 resistance gene, which conferred resistance to this aminoglycoside. (Lohmann et al., 1999). G418 was chosen in this case, because it suited well for long-term selection, considering the low replication efficiency of HCV. G418 acts slower than, for example, puromycin, allowing replicons replicate to a level sufficient for conferring resistance to the cell. Such selectable constructs made possible detection of RNA replication by direct counting the number of selected cell colonies that supported replication of the HCV subgenomic RNAs. First replicons were derived from the HCV consensus genome Con-1 (genotype 1b), that was cloned from the liver of a chronically infected patient (Lohmann et al., 1999). Based on results with some plus-strand RNA viruses like flavi- and pestiviruses, which demonstrated that the structural proteins were not required for RNA replication (Khromykh and Westaway, 1997; Behrens et al., 1998), bicistronic constructs were generated. These were composed of two variants of the HCV IRES (nucleotides 1 to 377 or 1 to 389), the neomycin phosphotransferase (npt) gene (neo) (conferring resistance to G418 and
acting as a selectable marker), the IRES of the encephalomyocarditis virus (EMCV), which directs translation of HCV sequences from NS2 or NS3 up to NS5B, and the 3'-UTR (Fig. 2A) (Lohmann et al., 1999). Thus, in these bicistronic constructs, expression of the G418 resistance gene was under control of the HCV IRES, whereas the gene encoding the viral replicase complex was under control of the EMCV IRES (which ensured correct and efficient translation of NS proteins and precluded synthesis of npt/NS3 fusion protein).

![Diagram](image)

**Fig. 2** Structure of HCV bicistronic replicons. (A) Subgenomic replicons composed of the 5'-UTR, the gene encoding neomycin phosphotransferase (npt), the encephalomyocarditis virus IRES, the region encoding HCV NS proteins (NS2 to NS5B or NS3 to NS5B) and 3'-UTR. In these replicons a part of the Core encoding gene is fused to the neo gene. The HCV IRES directs the expression of Core-neo fusion protein and the EMCV IRES directs the expression of NS proteins. (B) Full-length genomic HCV replicon with the bicistronic organization analogous to the subgenomic replicon, but encoding the complete HCV ORF downstream of the EMCV IRES. (C) Structure of a basic replicon construct used for transient replication assays (Friebe et al., 2001). It is composed of the complete HCV 5'-UTR, a 63-bp spacer (sp) element, the poliovirus (PV) IRES element, the firefly luciferase gene (luc), the EMCV IRES, HCV NS proteins NS3 to NS5B and HCV 3'-UTR.

In order to generate replicons with authentic 3'-end, the 3'-UTR of cloned replicon DNA was modified, which allowed to introduce a recognition sequence for the restriction enzyme ScaI at the end of the 3'-UTR. Then the plasmid (with cloned replicon) was linearized with ScaI (digestion with ScaI generates blunt ends, so that during in vitro transcription no additional
nucleotides can be added to RNA) and used for \textit{in vitro} transcription reaction. A 5'-flanking T7 RNA polymerase promoter and an engineered ScaI restriction site at the 3'-end allowed for production of RNA transcripts with authentic 5'- and 3'-terminal sequences. Upon transfection of the human hepatoma cell line Huh-7 with replicon RNAs generated by \textit{in vitro} transcription of cloned replicon sequences and subsequent G418 selection, only such cells in which the replicon amplified to sufficient levels survived. Though most of the cells died during the experiment, a low number of drug-resistant colonies could be isolated that supported a surprisingly high level of HCV RNA replication. By using strand-specific Northern blot assays, it was calculated that on the average a single cell contained about 1000-5000 positive strand replicon RNA molecules and about 5- to 10-fold lower number of negative strand RNAs. This was at least three orders of magnitude higher than the most efficient infection-based cell culture system available at that time. In order to ensure that replicons were capable of autonomous replication, cells were incubated with \(^3\)H]uridine in the presence of actinomycin D (selectively inhibits RNA synthesis from DNA, by forming stable complexes with double-stranded DNA, but not from RNA templates) and resulted radiolabeled RNAs were analyzed. Results demonstrated that replication of HCV RNA (carried out by HCV NS5B RNA-dependent RNA polymerase) was not affected by actinomycin D, whereas synthesis of cellular RNAs was blocked (Lohmann \textit{et al.}, 1999). All HCV proteins were also detected (Lohmann \textit{et al.}, 1999). Thus, this study defined the structures of selectable HCV RNAs replicating autonomously and to high levels in a human hepatoma cell line.

5.1.2 \textit{Characterization of cell lines carrying self-replicating hepatitis C virus RNAs}

Further, a detailed characterization of two cell lines carrying NS3-5B replicon was performed in a study by Pietschmann \textit{et al.} (2001). Among the analyzed properties were: stabilities of HCV RNAs under different conditions of cell passage, polyprotein processing kinetics, the half-lives of the cleavage products and their subcellular localization (Pietschmann \textit{et al.}, 2001, ref is the same) etc. When cells were passaged under continuous selective pressure (in the presence of G418) no significant reduction in the amount of the replicon RNA was found even 1 year of culturing. In the absence of selective pressure the amount of replicon RNA gradually reduced over time. However, even in this case replicons persistently replicated in Huh-7 cells.
for ~ 10 months. From this result it was concluded that replicon-bearing cells might be a good reflection of viral persistence *in vivo* (Pietschmann *et al.*, 2001). Finally, no morphological changes or alterations in growth properties were observed in cells with a replicon suggesting that these HCV RNAs and the viral nonstructural proteins (at least NS3 – NS5B) are not cytotoxic (Pietschmann *et al.*, 2001). A correlation was observed between the level of replicon RNA and cell growth (Pietschmann *et al.*, 2001). While actively dividing cells carried the highest amounts of HCV RNA, these amounts decreased dramatically in the non-dividing cells. The level of replicon RNA increased approximately twofold during the first 4 days of culture, but dropped sharply thereafter to ~ 1/40 of the original amount (Pietschmann *et al.*, 2001). This observation could be explained by the availability of host cell factors being high in actively growing cell cultures, but low in resting cells (Pietschmann *et al.*, 2001).

5.1.3 Identification and application of cell culture adaptive mutations

In spite of the high level multiplication of replicons within a selected cell clone, the replicon system had several limitations in its initial stage. One of them was the low number of cell clones obtained after transfection with replicon RNAs: only ~1 of $10^6$ HuH-7 cells supported HCV replication. Two possibilities might have been responsible for this effect. First, only a few cells in the transfection reaction were permissive and allowed persistent replication of HCV RNA, or, second, during selection, adaptive mutations were generated within the replicon which enhanced replication levels to an extent sufficient to establish a G418-resistant cell colony (Lohmann *et al.*, 1999, 2001).

To verify the concept of adaptive mutations, the sequences of HCV RNAs isolated from different selected cell clones were analyzed (Blight *et al.*, 2000). The analysis revealed that each clone contained replicons with mutations in defined region of NS5A (Blight *et al.*, 2000). In addition to an amino acid change in NS5A, mutations in NS3 and NS4B regions were also found (Blight *et al.*, 2000). In total, 9 different substitutions in NS5A, localizing to a region of about 30 amino acid residues, and a deletion of 47 amino acids were discovered (Blight *et al.*, 2000). Further, these mutations were introduced into the replicon. After transfection of mutant RNA transcripts into HuH-7 cells and G418 selection, each RNAs established replication in 0.2 to 10% of transfected cells, as compared with 0.0005% for the original replicon (a ~ 500 to 20 000-fold difference) (Blight *et al.*, 2000). All of the discovered changes in NS5A
enhanced the ability of HCV replicons to replicate (Blight et al., 2000). This finding suggested that NS5A might be important for the establishment of HCV replication in vitro. An important outcome of this study, besides the discovery of adaptive mutations, was the establishment of a more efficient replication system based on cell culture adapted HCV replicon. In another study, conducted by Lohmann et al. (2001), the efficiency of colony formation (ECF) of replicons from a selected cell clone (designated 9-13) and original replicons was tested. Clear difference was evident when a reduced G418 concentration was used: the ECF obtained with HCV replicons from selected cell line was several orders of magnitude higher compared to the original replicons (Lohmann et al., 2001). The most probable explanation for this observation was the presence of adaptive mutations. Therefore, sequences of HCV replicons isolated from the cell line 9-13 were determined. In all of them several amino acid changes in NS3 – NS5B region and one conserved mutation in NS5B were discovered. To analyze whether these mutations conferred an adapted phenotype the replicon was constructed, which contained almost all amino acid substitutions found with each clone. Unfortunately, this construct was not able to replicate in HuH-7 cells. This fact was thought to be related to a highly conserved mutation in NS5B. Thus, in order to determine which of discovered mutations were responsible for adaptation and to analyze for their possible synergism or additive effect, each substitution was introduced individually (or in different combinations) into the original replicon (Lohmann et al., 2001). The results showed that most mutations gave either ~ 2-10-fold increase in ECF or no increase at all. The greater improvement was achieved with a construct bearing a single mutation in NS5A, which increased the ECF ~ 100-fold (Lohmann et al., 2001). Despite this progress, the ECF obtained with the replicon from cell line 9-13 was still ~ 30-fold higher compared to the replicon with adaptive NS5A mutation. However, after some additional experiments it was found that replicons bearing single highly conserved mutation in NS5B (glycine-for-arginine substitution in amino acid position 2884, which was previously thought to be inactivating), combined with 4 mutations in NS3 showed ~ 1000-fold higher ECF compared to non-adapted replicons, whereas the mutation in NS5B alone was able to increase ECF ~ 500-fold (Lohmann et al., 2001). Thus, the most important adaptive mutation was identified. However, individual mutations were found to be synergistically lethal or beneficial, depending on the combination of specific mutations introduced. For instance, the highly adaptive mutation in NS5B was incompatible with some other adaptive mutations (for example, in NS5A or in NS4B) (Lohmann et al., 2001). Thus, cell culture adaptation could be achieved by many different mutations in different NS proteins, although there were
significant variations in the level of adaptation. In summary, these studies proved the hypothesis that adaptive mutations confer efficient replication to HCV replicons harboring them in vitro and identified the sites of some important mutations, providing a possibility of further improvement of the replicon system. In 2001 Krieger and colleagues developed a novel highly adapted HCV replicon that harbored two synergistic mutations in NS3 and one in NS5A and that showed an ECF of ~ 500 000 colony forming units (CFU) per µg of RNA, which was ~ 20-fold higher than that of the best adapted replicon previously described (Lohmann et al., 2001). Thus, this fact demonstrated that the mutation in NS5B was not the only responsible for high-level cell culture adaptation. At first, replicon RNA was passaged several times: total RNA was isolated from replicon-harboring cells and transfected into naïve HuH-7 cells. After selection, a fast-growing clone was used for preparation of total RNA and transfection into parental HuH-7 cells. After three successive passages nearly full-length replicon RNAs were amplified from obtained cell line. Then, almost the complete HCV ORF was inserted into the parental replicon construct. These constructs were further used for in vitro transcription reaction, and obtained transcripts were transfected into naïve HuH-7 cells to determine their ECFs. Four clones of 82 analyzed turned out to be more efficient than wild type (Krieger et al., 2001). The most efficient of them was the above mentioned (designated 5.1). The development of highly adapted subgenomic replicons allowed to adopt a new method – transient-transfection assay. In this approach cells are cultured only for some days after transfection and the replication efficiency is measured by detection of viral antigens or by the expression level of a reporter gene. Thus, there was no more need for selection when using this method and it saved a lot of time.

A further determination and analysis of adaptive mutations were also performed. For that purpose the neo genes of the replicons were replaced by the luciferase encoding reporter gene (luc). The basic construct for transient-transfection assays utilizing luciferase reporter gene was originally described in Friebe et al., 2001. It contained the complete HCV 5’-UTR, a 63-bp spacer element, the poliovirus (PV) IRES element, a gene coding for firefly luciferase (luc), the EMCV IRES and HCV NS proteins NS3 to NS5B and HCV 3’-UTR (Fig. 2C). Using the luc-constructs it was possible to directly monitor the replication of HCV RNA by measuring the luciferase activity and analyze the effect of the mutations that were present in replicons (Krieger et al., 2001; Lohmann et al., 2003). By analyzing different RNAs with different levels of cell culture adaptation a clear correlation between the ECF and RNA
replication was found (Krieger et al., 2001). These results demonstrated that cell culture-adaptive mutations increased HCV RNA replication. However, correlation between ECF and the copy number of replicon molecules per cell after selection was not demonstrated. These numbers varied only slightly in cell lines obtained after transfection of replicons with different levels of cell culture adaptation. Thus, these results suggested that permissiveness of host cells played an important role in determining the replication level of HCV replicons (Krieger et al., 2001). In addition, despite the development of adapted subgenomic replicons, proportion of transfected HuH-7 cells, in which HCV replication could be detected remained relatively low (~ 10%), suggesting that cellular environment was another major determinant of HCV replication efficiency (Blight et al., 2000, 2002).

Fig. 3. Conserved mutations in HCV Con1 (isolate of genotype 1b) replicons identified by the clonal analysis of cells in which the HCV replication was induced (Lohmann et al., 2003). A schematic representation of HCV nonstructural proteins from NS3 to NS5B is shown. Clusters of adaptive mutations are highlighted in orange. Numbers refer to the amino acid position in the complete polyprotein (Core to NS5B) of Con1 isolate. Depicted are the amino acid residue changes detected by clonal analysis of G418-selected cell-lines generated by the transfection with HCV subgenomic replicon (Fig. 2A, upper). Single-letter code is used for designation of amino acid residues. Del, deletion; ins, insertion; prot., protease.

In 2003 Lohmann and colleagues performed a detailed analysis of cell culture adaptation. In total, 26 independent, selected replicon cell clones were examined and a large number of mutations was identified. Their effects on HCV RNA replication were quantified using replicons with luciferase-reporter gene. They also obtained a replicon, which was ~6 time more efficient than the best replicon previously described (rep5.1 (Krieger et al., 2001)) (Lohmann et al., 2003). This effect was achieved by combining a highly adaptive mutation in NS4B and with two NS3 mutations (E1202G and T1280I). All conserved mutations found in those 26 Con1 replicons, and their effects are shown in Fig. 3. and Table 3, respectively.
Table 3. Effect of adaptive mutations on replication efficiency of the HCV replicons.¹

<table>
<thead>
<tr>
<th>Mutation(s)</th>
<th>Increase in replication efficiency compared to wild-type (-fold) (only mean values)</th>
<th>Specific designation of replicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0</td>
<td>wild-type (wt)</td>
</tr>
<tr>
<td>D2737N</td>
<td>0.2</td>
<td>GND</td>
</tr>
<tr>
<td>E1202G</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>T1280I</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>G1304S</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>K1846T</td>
<td>30.9</td>
<td></td>
</tr>
<tr>
<td>V1897A</td>
<td>21.4</td>
<td></td>
</tr>
<tr>
<td>V1897L</td>
<td>19.8</td>
<td></td>
</tr>
<tr>
<td>V1897M</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>ins2041K</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>D2177G</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>S2197P</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>A2199D</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>del2202S</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>S2204R</td>
<td>12.7</td>
<td></td>
</tr>
<tr>
<td>S2204I</td>
<td>13.2</td>
<td></td>
</tr>
<tr>
<td>R2884G</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>Q2933R</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>I3004T</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>E1202G + T1280I</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>E1202G + T1280I + K1846T</td>
<td>191.7</td>
<td>ET</td>
</tr>
<tr>
<td>E1202G + T1280I + V1897A</td>
<td>76.9</td>
<td></td>
</tr>
<tr>
<td>E1202G + T1280I + S2197P</td>
<td>33.2</td>
<td>5.1</td>
</tr>
<tr>
<td>E1202G + T1280I + G1304S + S2197P</td>
<td>4.7</td>
<td>5.1 + G1304S</td>
</tr>
<tr>
<td>E1202G + T1280I + 2202delS</td>
<td>78.7</td>
<td></td>
</tr>
<tr>
<td>E1202G + T1280I + S2204I</td>
<td>96.0</td>
<td></td>
</tr>
<tr>
<td>E1202G + T1280I + S2204R</td>
<td>44.6</td>
<td></td>
</tr>
<tr>
<td>E1202G + T1280I + R2884G</td>
<td>24.0</td>
<td></td>
</tr>
<tr>
<td>E1202G + T1280I + Q2933R</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>K1846T + V1897A</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>2202delS + S2204R</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>S2204R + S2197P</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>V1897A + S2204R</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>V1897A + 2202delS</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>V1897A + R2884G</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>K1846T + R2884G</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>S2197P + R2884G</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

¹Based on the results obtained by Lohmann et al., (2003) and Krieger et al., (2001)
5.1.4 Role of the host cell line permissiveness. Generation of highly permissive cell lines.

Although adaptive mutations were the major determinant of efficient replication for HCV subgenomic replicons in HuH-7 cells, replication could be detected only in a subpopulation of transfected cells. Thus, it was concluded that permissiveness of the host cell plays an important role in efficient HCV RNA replication. Blight and colleagues identified several HuH-7 cell clones, which harbored replicons without adaptive mutations (Blight et al., 2002). It was suggested that these clones might be more permissive for HCV replication and provide a system to study HCV replication in the absence of adaptive changes (Blight et al., 2002). On the basis of this suggestion an attempt was undertaken to establish highly permissive cell lines. Several HuH-7 lines harboring subgenomic HCV replicons were “cured” of HCV RNA by prolonged treatment with IFN-α (Blight et al., 2002). From the treated clones were successfully cured cellular clones HuH-7.5 and HuH-7.8, bearing replicons with no amino acid changes within the HCV NS region (Blight et al., 2002). To test the ability of these clones to support HCV replication three G418-selectable subgenomic replicons were used. Two of them contained adaptive mutations and had replication efficiency ~ 500 (SG-Neo/5Adel47)- and 20,000 (SG-Neo/S2204I)-fold higher compared to wild type (Blight et al., 2000) and third was the wild type replicon. These replicons were transfected into the IFN-α-cured cells. After G418 selection it was observed that ECF of HuH-7.5 cells transfected with the most adapted replicon (SG-Neo/S2204I) was ~ 3-fold higher than that of parental HuH-7 cells. For cell lines HuH-7.5 and HuH-7.8, the number of G418-resistant colonies obtained after transfection of another mutated replicon (the less adapted one – SG-Neo/5Adel47) were significantly higher than those for parental HuH-7 cells (~ 33- and 9-fold increase, respectively). For the wild type replicons ECF in HuH-7.5 and HuH-7.8 increased ~ 10- and 2-fold, respectively (Blight et al., 2002). For IFN-α-cured HuH-7 cells no difference in ECF was noted compared to parental HuH-7 cells, indicating that IFN-α treatment alone did not alter the ability of HuH-7 cells to support HCV replication (Blight et al., 2002). Since the cured HuH-7.5 line was the most permissive of those analyzed, HCV replication was examined in this line compared to parental HuH-7 cell line. The results confirmed the suggestion that a larger fraction of HuH-7.5 cells supported detectable levels of HCV replication (Blight et al., 2002). Thus, this study highlighted the importance of the host environment for the HCV replication and provided a valuable, highly permissive cell line (HuH-7.5) for research on HCV (Blight et al., 2002). Another highly permissive HuH-7 cell
line (designated Huh7-Lunet) established by “curing” was described in Friebe et al., 2005. In this case a selective drug was used for treating the replicon-harboring cells. Compared to naïve HuH-7 cells, these “cured” cells supported higher RNA levels. (Friebe et al., 2005).

The importance of host cell permissiveness for efficient HCV RNA replication was also supported by another finding: passages of HuH-7 cells derived from the same progenitor culture varied significantly in their ability to support HCV replication (Lohmann et al., 2003). In fact, in the most permissive HuH-7 passage even wild-type replicon replicated more efficiently than the highly adapted replicons in a poorly permissive passage (Lohmann et al., 2003). It was suggested that these differences in permissiveness might be due to variations in abundance or activities of some cellular factors (this suggestion was later corroborated by several studies; for example, CD81 receptor expression level is one of such factors (Koutsoudakis et al., 2007)) (Lohmann et al., 2003). Thus, identification of these factors and their contribution to RNA replication became another challenging task in HCV field.

5.1.5 Efficient replication of various HCV genotypes

Another important issue relating to the replicon system was that initially subgenomic HCV RNA replicons had only been constructed from Con1 viral sequence (Lohmann et al., 1999, 2001). Attempts to develop analogous subgenomic RNA replicons from the sequence of a genotype 1a chimpanzee infectious clone H77 failed (Blight et al., 2000), so it still remained unclear whether only the Con1 replicons by some unknown reasons could replicate in HuH-7 cells. However, in 2002, a successful construction of selectable replicon from the infectious clone of Japanese genotype 1b virus (HCV-N) was reported (Table 4) (Beard et al., 1999; Ikeda et al., 2002). This clone was capable to replicate without the need for common adaptive mutations and it was also observed that the wild type clone replicated with approximately the same efficiency as the clones containing several adaptive mutations (Ikeda et al., 2002). However, when mutations (previously described by Blight et al. (2000) and Lohmann et al. (2001)) were introduced, their adaptive effect was observed (Ikeda et al., 2002). In the course of further experiments it turned out that the enhanced replication capacity of HCV-N RNA was due to a natural 4-amino-acid insertion in NS5A (Ikeda et al., 2002).
Table 4. HCV replication systems based on subgenomic and genomic replicons.

<table>
<thead>
<tr>
<th>Type of replicon</th>
<th>HCV isolate</th>
<th>Genotype</th>
<th>Cell line</th>
<th>RNA replication</th>
<th>Virion production</th>
<th>in vivo infectivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV subgenomic replicons</td>
<td>Con1 wild type</td>
<td>1b</td>
<td>HuH-7</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>Lohmann et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Con1/adapt</td>
<td>1b</td>
<td>HuH-7</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>Blight et al., 2000</td>
</tr>
<tr>
<td></td>
<td>HCV-N</td>
<td>1b</td>
<td>HuH-7</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Ikeda et al., 2002</td>
</tr>
<tr>
<td></td>
<td>H77</td>
<td>1a</td>
<td>HuH-7.5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Yi and Lemon, 2004</td>
</tr>
<tr>
<td></td>
<td>JFH-1</td>
<td>2a</td>
<td>HuH-7</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>Kato et al., 2003</td>
</tr>
<tr>
<td>HCV genomic replicons</td>
<td>HCV-N</td>
<td>1b</td>
<td>HuH-7</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Ikeda et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Con1/adapt</td>
<td>1b</td>
<td>HuH-7</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>Pietschmann et al., 2002</td>
</tr>
<tr>
<td></td>
<td>H77</td>
<td>1a</td>
<td>HuH-7.5</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>Blight et al., 2003</td>
</tr>
</tbody>
</table>

The HCV-N strain based model was valuable, because it provided a possibility to conduct studies with naturally occurring viral genome, without the need for additional mutations. However, ability of clones derived from other HCV genotypes to replicate in cell culture was not still demonstrated. Isolation of a highly permissive cell line HuH-7.5 (Blight et al., 2002) helped to change the situation: soon, establishment of a system utilizing replicons derived from HCV genotype 1a clone was reported (Blight et al., 2003). A Con1 highly adaptive Ser-to-Ile substitution in NS5A (S2204I) was incorporated into these replicons (Blight et al., 2003). After transfection into HuH-7.5 cells (chosen for their high permissiveness) it was observed that replicon bearing the mutation did replicated in HuH-7.5 cells (although with very low efficiency), while wild-type replicon did not. After a further analysis it was found that subgenomic RNAs within every individual cell clone acquired an additional adaptive mutation in NS3, and at the same time, S2204I change in NS5A was maintained (Blight et al., 2002). Thus, replication of genotype 1a RNAs in HuH-7.5 cell line required adaptive amino acid substitutions in both NS5A and NS3. The development of the genotype 1a based system was an important extension of the replicon system, which allowed to begin to study different genotypes of HCV, their properties and replication capabilities. It was followed by an establishment of subgenomic replicon system for the HCV of genotype 2a (Kato et al., 2003). This clone was isolated from a Japanese patient with fulminant hepatitis and designated JFH-1 (Kato et al., 2001). Fulminant hepatitis is an occurrence of hepatic encephalopathy (brain and
nervous system damage that occurs as a complication of liver disorders) within the first 8 weeks of the initial symptoms of hepatitis (Trey and Davidson, 1970). After transfection, this replicon could replicate without common adaptive mutations; however, some of the mutations found in the clones might be important in conferring higher replication phenotypes. This system provided a powerful new tool for conducting studies in HCV field. (Kato et al., 2003). Thus, the development of functional HCV genotype 1a and 2a HCV RNA replicons provided opportunities to study genotype-specific HCV replication. The most important replicon-based cell culture systems are represented in Table 4.

5.2 Genomic HCV replicons

Initially, HCV replicon system utilized only subgenomic replicons, therefore it was not useful for studying the whole life cycle of the virus or screening antiviral drugs targeting HCV structural proteins. Thus, the final goal was the establishment of a system allowing production of infectious particles in cell culture. However it was only possible with full-length HCV genome. For that purpose numerous HCV genomic replicons (Fig. 2B) were constructed and studied in parallel with subgenomic replicons (Ikeda et al., 2002; Blight et al., 2002, 2003; Pietschmann et al., 2002). Attempts to establish viable cell clones after transfection of cells with selectable HCV genome that was based on the original sequence of the Con1 failed (Pietschmann et al., 2002). Therefore cell culture adapted HCV genomes containing at least one adaptive mutation were further used. However after transfection of these cell culture-adapted into HuH-7 cells and G418 selection only a few number of colonies was obtained (3-4 orders of magnitude lower that for subgenomic replicons) (Pietschmann et al., 2002). Despite that, several independent cell lines could be established and analyzed. The average levels of HCV RNA in these cells was sufficiently high, only ~ 5-fold lower than that of subgenomic replicons (Pietschmann et al., 2002). Interestingly, no additional conserved mutations, which would contribute to the high replication level, were found after subsequent analysis. Thus, the reason for the low ECF was not clear. Despite the high replication level, attempts to demonstrate the release of viral particles failed (Pietschmann et al., 2002). In another study it was shown that replication of the full-length replicon in HuH-7.5 cells was not dependent upon EMCV IRES-driven translation of viral replicase complex (Blight et al., 2002). In fact, inclusion of heterologous elements, such as EMCV IRES or the neo gene decreased the
efficiency of replication (Blight et al., 2002). Replication of full-length genomic replicons in HuH-7.5 cells without a need for heterologous elements allowed the study of unmodified genomic RNAs. This point might certainly affect the virus assembly. However, none of studies that incorporated HCV genomic replicons demonstrated the release of viral particles (Blight et al., 2003, Ikeda et al., 2002, Pietschmann et al., 2002). This was a perplexing result, which, however, clarified that heterologous elements in the structure of replicons were not responsible for their inability to produce virions. Thus, there still were several possible explanations of this failure. For example, inability of HuH-7 cells to support viral particle assembly due to the lack of host cell factors required for virus formation or release. Another explanation was that cell culture adaptive mutations interfere with virion production. This possibility was supported by a study by Bukh et al. (2002). It was demonstrated that HCV RNA transcribed from the wild-type Con1 cDNA clone produced a typical HCV infection in chimpanzee whereas in vivo infectivity of cell culture-adapted HCV genomes (with high level of in vitro replication) was whether completely blocked or significantly attenuated (Bukh et al., 2002).

6. Replication systems that support production of the infectious virus.

6.1 The cloned viral genome of genotype 2a (JFH-1)

Despite the overall success in the field of HCV, studies of complete HCV life cycle and development of anti-HCV drugs remained still hampered by the lack of efficient, virion producing cell culture system. Although full-length HCV genomic replicons were constructed that efficiently replicated in cell culture, they could not produce infectious viral particles (Pietschmann et al., 2002; Blight et al., 2003; Ikeda et al., 2002) and were not infectious in vivo (Bukh et al., 2002). This was attributed to the effect of adaptive mutations that were present in the utilized genomes. Thus, there was a strong need for a viral genome that could replicate to high rate both in vivo and in vitro, and preferably without the need for adaptive mutations. Based on these criteria, scientists drew their attention to the HCV genotype 2a consensus genome cloned from a Japanese patient with fulminant hepatitis - JFH-1 (Kato et al., 2001). JFH-1 subgenomic replicon was previously described to be able to replicate
efficiently in cell culture without common amino acid mutations (Kato et al., 2003), so it was supposed that this clone might be a valuable tool in developing a novel cell culture system. The assumption turned out to be correct after appropriate experiments conducted by Takaji Wakita and colleagues (Wakita et al., 2005). Upon transfection of HuH-7 cells with JFH-1, it replicated to high level in ~ 80% of cells, and genome-length RNA remained detectable up to 72 hours (Wakita et al., 2005). Production of viral particles was shown by passaging of infection to naïve HuH-7 cells after inoculation with cell culture supernatants, and by electron microscopy (Wakita et al., 2005). Cell culture-grown virus was also infectious in chimpanzee, although virus titers were relatively low and infection was transient (Wakita et al., 2005). To determine whether JFH-1 RNA-transfected cells could sustain continuous HCV replication, the cells were serially passaged. However, infection did not persist for a long time – after day 12 HCV RNA level in cell culture medium began to decrease (Wakita et al., 2005). Nevertheless, despite the limited spread of infection in vitro and its transient course in vivo with low viral titers, this was the first report describing the production of HCV in cell culture, which could infect both cells and chimpanzees (Wakita et al., 2005). In addition, the fact that JFH-1 replicated in cell culture without adaptive mutations and was infective in vivo supported the hypothesis that these mutations could interfere with in vivo infectivity of HCV. Soon after Wakita’s report, another group published results of their work, where they demonstrated the establishment of a robust highly efficient in vitro infection system based on the HuH-7-derived cell lines and the JFH-1 consensus clone (Zhong et al., 2005). In this study a new, highly permissive cell line HuH-7.5.1 was established by “curing” a replicon-harboring HuH-7.5 cell line with IFN-γ (Zhong et al., 2005). During first 14 days after transfection of HuH-7.5.1 cells with JFH-1 genome, intracellular HCV RNA amount was subsequently decreasing, but then suddenly started to increase reaching maximal levels of more than $10^7$ per µg of total RNA (~300 molecules per cell on average). These levels were maintained until the experiment was terminated on day 26 (Zhong et al., 2005). These results suggested that HCV was actively replicating in HuH-7.5.1 cells (probably because of the emergence of cell culture-adapted variants). Further analysis revealed that the percentage of HCV-positive cells in the transfected cell cultures increased from 2% on day 5 posttransfection to almost 100% on day 24, which supposed that either the low percent of infected cells gained selective advantage or that the infection was spreading in cultured cells (Zhong et al., 2005). Inoculation of naïve HuH-7.5.1 cells with cell culture supernatants from the above-described transfection experiments resulted in a new infection, indicating that JFH-1 transfected HuH-7.5.1 cells
were releasing infectious virions (Zhong et al., 2005). Several additional cell lines were tested in that study for their ability to support replication of JFH-1, but only HuH-7 gave positive results. Interestingly, replication efficiency in this cell line was comparable to that of HuH-7.5.1 cells, although the kinetics of replication were delayed (Zhong et al., 2005). In summary, this research group established a simple yet robust system for propagating HCV, which provided a possibility to monitor the complete HCV infection process in vitro. Similar results (with respect to cell culture infectivity) were obtained with another JFH-1 based replication system (Cai et al., 2005). In this case HuH-7 cells were used, in which JFH-1 cDNA was stably integrated into chromosome. The transcription of this cDNA was under the control of cytomegalovirus (CMV) promoter at the 5’-end and a hepatitis delta virus ribozyme at the 3’-end. HCV RNA was expressed efficiently in the stable HuH-7 cell lines and infectious HCV virions were produced and secreted, that was proven by the infection of naïve HuH-7.5 cells (Cai et al., 2005).

6.2 The infectious JFH-1 inter- and intragenotypic chimeras.

Soon after the first reports related to the replication of full-length JFH-1 in cell culture were published, this HCV clone became the object of a great attention for scientists. In a different approach, Lindenbach and coworkers constructed full-length chimeric genomes (Fig. 4A) with the use of the core-NS2 gene regions from the infectious J6 (genotype 2a) and H77 (genotype 1a) virus strains (Lindenbach et al., 2005). Remainder of the genome originated from JFH-1 subgenomic replicon, that replicated efficiently in cell culture (Kato et al., 2003). These full-length chimeras were competent for RNA replication after transfection into the HuH-7.5 cell line (Lindenbach et al., 2005). However, after incubation of naïve HuH-7.5 cells with culture media supernatants, only with J6/JFH-1 the transfer of infection was obtained. indicating that genotype 1a/2a chimera was able to replicate but not to spread in cell culture. This finding suggested that some specific interactions between structural and NS proteins were important for virus assembly and release. Experiments showed that cell culture-produced J6/JFH-1 virus (referred to as HCVcc) was highly infectious and formation of HCVcc did not depend on the emergence of rare variants (Lindenbach et al., 2005). In another study by the same group the infectivity of HCVcc J6/JFH-1 was also investigated (Lindenbach et al., 2006). Two HCV-negative chimpanzees were inoculated with cell culture-grown J6/JFH-1. The course of
infection corresponded to typical course of acute HCV infection, with rapid rise of viremia, peak viral titers of $10^4$ to $10^5$ international units (IU; in this study 1 IU corresponded to 10.8 RNA copies) per ml of plasma, and induction of innate and acquired immune response (Lindenbach et al., 2006). Infectivity was also examined in uPA-SCID mice (immunodeficient transgenic mice transplanted with primary human hepatocytes, which are then permissive to HCV infection; this mouse model is described in Mercer et al., 2001). Within two weeks after infection with HCVcc plasma of mice contained high levels of viral RNA. Moreover, when an uninfected mouse was inoculated with plasma of infected mouse, it became infected (Lindenbach et al., 2006). Taken together, these data demonstrate that HCVcc is infectious in vivo and show that infection can be serially passed to a naïve animal (at least in case of using mouse model). Virus recovered from plasma of infected animals (both mice and chimpanzees) was further tested for infectivity in vitro and turned out to be infectious in cell culture (Lindenbach et al., 2006). Interestingly, specific infectivity (ratio of infectivity titer to RNA titer; infectivity titer is a number of infectious units per ml of inoculum and RNA titer is the number of RNA copies per ml of inoculum) of J6/JFH-1 recovered from animals was 10-100 higher than that of cell culture derived HCV (Lindenbach et al., 2006). In summary, these studies have shown that HCVcc is capable of sustained replication both in vivo and in vitro, and that in vivo produced virus remains infectious in vitro.

**Fig 4.** Structure of chimeric HCV genomes. (A) Design of chimera constructed by Lindenbach and coworkers (Lindenbach et al., 2006). Core to NS2 region is derived from J6(CF) isolate and all other sequences from JFH-1. (B) Structure of chimeric genomes used by Pietschmann and colleagues (Pietschmann et al., 2006). Viral genome segments were fused right after the first transmembrane domain of NS2.
However, despite this success detailed comparative studies between different HCV strains were not still possible because of the restriction to the JFH-1 and J6/JFH-1 chimera. Therefore a comprehensive panel of JFH-1-based chimeras was constructed, in which the JFH-1 coding region from core up to the first transmembrane segment of NS2 was replaced by the analogous region of several other infectious HCV isolates (Fig. 4B) (Pietschmann et al., 2006). At first, to determine whether it is possible to generate viable intergenotypic chimeras, a novel chimeric genome (designated Con1/C6) was constructed which carried the core to the NS2 region of genotype 1b Con1 isolate (Fig. 4A) (Pietschmann et al., 2006). This chimera was transfected into a highly permissive clone HuH-7 cellular clone designated HuH-7-Lunet in parallel to the JFH-1 genome. Although the efficiency of replication was equal, chimera demonstrated ~ 100 lower infectivity when naïve HuH-7-Lunet cells were inoculated with cell culture supernatants (Pietschmann et al., 2006). Given the low yield of infectivity achieved with this chimera a series of Con1/JFH-1 intergenotypic chimera with different crossover points were constructed in order to determine an optimal junction site. Crossover points varied from C terminus of E2 to NS2-NS3 cleavage site. To facilitate the analysis, a luciferase reporter gene was added to these constructs, allowing fast and accurate RNA replication measurement in transfected and infected cells. The best result was obtained when C3 junction (Fig. 4B) was used – more than 20-fold higher infectivity compared to the original chimera which used C6 junction (NS2-3 cleavage site; Fig. 4A) (Pietschmann et al., 2006). These results suggest, in addition, that NS2 plays an important role in virus assembly and release. Further, in order to determine whether the same strategy was applicable to other chimeras, hybrid genomes were constructed from the JFH-1 replicase and three other HCV isolates: genotype 1a (H77), 2a (J6CF) and a newly isolated consensus genotype 3a (HCV-452). With the exception of HCV-452, chimeras with C3 junction yielded the best results. In summary, obtained data suggested that the optimal junction for the construction of a chimeric HCV genome depends on the particular isolate that will be fused to JFH-1. Nevertheless, the data indicated that the C3 position in most cases is superior to NS2-NS3 cleavage site (Pietschmann et al., 2006). To compare the different properties of various chimeras, transfection into HuH-7-Lunet cells and further analysis were performed. Most efficient was a J6-JFH-1 chimera designated Jc1 that released virus titres of about $10^6$ infectious units per ml – at least ~10-fold higher than wild-type JFH-1, arguing that J6 structural proteins have an intrinsically higher capacity for virus assembly and/or release. It was followed by Con1-chimeras, H77-chimeras, and 452-chimeras. Overall, the HCV chimeras were almost as...
effective as JFH-1 (or even superior). Interestingly, despite comparable RNA replication, cells transfected with these RNAs released very different amounts of infectious virus particles, arguing that the structural proteins or p7 encoded by these genomes have a very different capability to assemble or support release of virus particles (Pietschmann et al., 2006).

Interestingly, mutations affecting E1, p7, NS2 and/or NS3, and contributing to enhanced yields of the cell culture-grown hepatitis C virus have recently been found in H77/JFH-1 chimera, suggesting that they compensate for incompatibilities between proteins of different genotypes (Yi et al., 2007). In summary, a panel of intragenotypic and intergenotypic chimeras was established (Table 5), which produced infectious viral particles. This achievement has broadened the scope of available replication systems and enabled the identification of the determinants of efficient virus production.

![Fig. 5](image)

**Fig. 5** Comparison of infection kinetics of ED43/JFH1 and J6/JFH1 (based on Scheel et al., 2008). Dark colored columns show supernatant infectivity titers and light colored columns show RNA titers. J6/JFH1<sup>1</sup> is the first-passage virus and J6/JFH1<sup>2</sup> is the third-passage virus.

In addition to available JFH-1-based systems of genotype 1 and 3 chimeras, a genotype 4a/JFH-1 cell culture system was developed very recently (Table 5) (Scheel et al., 2008). Three 4a/2a (ED43/JFH-1) intergenotypic recombinants were constructed, each having a different crossover point. HuH-7.5 cells were chosen for transfection. Interestingly, the chimera which had the junction that was used in the development of other intergenotypic chimeras (Pietschmann et al., 2006), was not infectious (Scheel et al., 2008). However, other variants spread rapidly, with infection kinetics similar to J6/JFH-1, but with lower peak infectivity titers (Fig. 5) (Scheel et al., 2008). The analysis of the viral genome, recovered
from culture supernatants after first, second and third passages, revealed that several mutations had taken place (Scheel et al., 2008). Subsequently, these mutations were investigated and most of them was found to be adaptive, however, only in certain combinations or depending on the junction site of a chimera (Scheel et al., 2008). Another important achievement of this study was the construction of a H77C/JFH-1 chimera producing infectious viral particles (Scheel et al., 2008).

In summary, the developed systems represent a significant step in the extension of the currently available JFH-1-based cell culture systems of genotypes 1, 2 and 3. Establishment of functional chimeric clones allows to test genotype-specific anti-HCV drugs targeted to structural proteins. It also allows for broad-scale comparative studies of different HCV strains. Another outcome is the possibility to study interactions between structural and nonstructural HCV proteins. Moreover, functionality of these chimeric clones underlines the uniqueness of JFH-1 genome and demonstrates that the key to an efficient replication in cell culture lies within NS region of this genotype 2a HCV clone.

Table 5. Systems that support production of infectious virus.

<table>
<thead>
<tr>
<th>HCV isolate</th>
<th>Genotype</th>
<th>Cell line</th>
<th>RNA replication</th>
<th>Virion production</th>
<th>In vivo infectivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JFH-1</td>
<td>2a</td>
<td>Huh-7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>Wakita et al., 2005; Zhong et al., 2005.</td>
</tr>
<tr>
<td>J6-JFH-1</td>
<td></td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>Pietschmann et al., 2006; Lindenbach et al., 2005</td>
</tr>
<tr>
<td>Structural chimeras of JFH-1</td>
<td>2a, 1b, 1a, 3a</td>
<td>Huh-7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Con1-JFH-1</td>
<td></td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>Pietschmann et al., 2006; Lindenbach et al., 2005</td>
</tr>
<tr>
<td>H77-JFH-1</td>
<td></td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>Pietschmann et al., 2006; Lindenbach et al., 2005</td>
</tr>
<tr>
<td>452-JFH-1</td>
<td></td>
<td></td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>Pietschmann et al., 2006; Lindenbach et al., 2005</td>
</tr>
<tr>
<td>ED43/JFH-1 (chimeric)</td>
<td>4a/2a</td>
<td>Huh-7.5</td>
<td>++</td>
<td>+</td>
<td>not reported</td>
<td>Scheel et al., 2008</td>
</tr>
<tr>
<td>JFH-1 adapt (JFH1mut1-6)</td>
<td>2a</td>
<td>Huh-7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>Kaul et al., 2007</td>
</tr>
<tr>
<td>H77-S</td>
<td>1a</td>
<td>Huh-7.5</td>
<td>++</td>
<td>+</td>
<td>n. r.</td>
<td>Yi et al., 2006</td>
</tr>
</tbody>
</table>
6.3 Cell culture-adapted JFH-1

Despite the efficiency of the original JFH-1 cell culture system (Wakita et al., 2005; Zhong et al., 2005) was much higher than that of H77-based system (Yi et al., 2006), in general it was rather low. In order to improve the JFH-1 cell culture system and to gain insight into the determinants of efficient virus production, Kaul and colleagues adapted the wild-type JFH-1 to HuH-7.5 cell line (Kaul et al., 2007). For that purpose, Huh-7.5 cells were infected with the plain supernatant of HuH-7-Lunet cells transfected with JFH-1 RNA genome, and passaged up to 11 times. As a result, a virus pool was obtained, in which titers were ~ 1000-fold higher compared to parental JFH-1 strain (Kaul et al., 2007). After a comparative analysis with wild-type JFH-1 (JFH1wt) it became clear that the adapted variant (JFH1/adpt1) spread much faster in cell culture. Likewise, cells infected with the adapted viruses produced about $10^6$ 50% tissue culture infectious doses (TCID$_{50}$) per ml at 4 days postinfection, whereas JFH1wt titers were undetectable with this assay (Kaul et al., 2007). Viral RNA levels were also about 1000-fold higher in JFH1/adpt1-inoculated cells (Kaul et al., 2007). For further analysis, HCV genomes were isolated and sequenced. Several mutations were found both in structural (in E1 and E2) and NS (NS5A and NS5B) regions. To determine the effect of these mutations, they were inserted separately and in several combination into the JFH1wt genomes, and genomes were transfected into the HuH-7-Lunet cells by electroporation (Kaul et al., 2007). A V2240L mutation in NS5A, either alone or in combinations, turned out to be a major determinant of enhanced amounts of released infectivity and accelerated kinetics of virus release of JFH1/adpt1 (~ 100-fold increase in infectivity titers) (Kaul et al., 2007). In order to clarify whether the mutation in NS5A is the only determinant of efficient virus production, a second adaptation experiment with JFH-1 and HuH-7.5 cells was performed. Surprisingly, none of the mutations identified in the first adaptation experiment reappeared in the second one. In addition, most mutations in JFH1/adpt2 resided in other proteins, namely, p7 and NS3. Only one mutation resided in NS5A (Kaul et al., 2007). In this case, a mutation in p7 turned out to be the most efficient one (the effect was comparable to V2240L in NS5A in the first experiment). In subsequent experiments it was found that the V2240L mutation in NSS5A increased virus titer of JFH-1 intergenotypic chimeras (those described in Pietschmann et al., 2006), especially of the less efficient 452/JFH-1 chimera (Kaul et al., 2007). In the light of recent results showing that in vivo replication of Con1 genomes carrying replication-enhancing mutations is whether completely block or highly attenuated (Bukh et al., 2002), it
was very important to know whether the highly cell culture-adapted JFH-1 variant (JFH1mut1-6) is viable in vivo. The uPA-SCID mouse model was chosen for analysis. Two mice were inoculated with the virus and at 2 weeks after infection the sera of both animals contained high loads of HCV RNA, which remained at high level until week 15 postinfection when the experiment was terminated (Kaul et al., 2007). These results suggested that the highly adapted JFH-1 genome was viable in vivo. However, after a detailed analysis it was found that the major adaptive mutation in NS5A was not maintained and reverted to wild type in nearly half of the sequenced viral genomes isolated from both mice. This finding suggests that in vivo fitness of this highly adapted JFH-1mut1-6 genome was impaired by adaptive mutations (Kaul et al., 2007). Nevertheless, the availability of a cell-culture adapted genome which does not fail to replicate in vivo provides a good possibility for establishment of a highly efficient and reliable cell culture system, suitable for screening antiviral drugs.
Summary

After the hepatitis C virus genome was cloned, the major factor hampering the development of methods for combating HCV was the lack of efficient in vitro system for propagating the virus. As infection of primary cells or immortalized cell lines with sera from infected patients seemed to never give a satisfactory result, efforts focused on alternative approaches. Identification of a functional cloned viral genome for infection of cell lines turned out to be problematic, if not impossible, because of highly variable nature of HCV. Construction of consensus genomes that were infectious in vivo was an important step forward, but it did not solve the problem. A significant breakthrough was the development of the replicon system, based on the efficient replication of subgenomic HCV RNA in cell culture and on the selection of cells supporting viral replication. Identification of adaptive mutations and isolation of highly permissive cell clones allowed to establish robust replication systems for HCV subgenomes of different genotypes. Involvement of new approaches, such as transient-transfection assay and utilization of reporter gene-carrying constructs allowed fast and precise measurement of viral replication, thus, greatly accelerating studies of HCV molecular biology and particularly of the interactions between viral replicase complex and host cells. However, it was not still possible to study the whole life cycle of the virus, and even construction of full-length genomic replicons did not lead to desired result, because of their inability to assemble infectious virions, probably caused by adaptive mutations. Nevertheless, this problem has been recently resolved – a genotype 2a clone (JFH-1), isolated from the serum of a patient with fulminant hepatitis, was used to establish cell culture systems that supported release of virus particles which were infectious both in vivo and in vitro. Further improvements of these systems led to isolation of cell culture-adapted JFH-1, which is much more efficient compared to wild type. Another important outcome is the construction of HCV intergenotypic and intragenotypic chimeras of genotypes 1-4 that allowed genotype-specific studies of at least a part of viral life cycle. The ability to study a genetically defined virus in the test tube and in living animals now allows the determinants of HCV infectivity, pathogenesis, neutralization and immune escape to be analyzed in detail at the molecular level. Moreover, culturing HCV from clinical samples should become possible now and a useful positive control can be provided to help to isolate additional virus strains that can grow in cell culture.
However, the main question that still remains unanswered is what makes the JFH-1 isolate so special? If the determinants of its efficacy and natural \textit{in vitro} growth potential can be found, it may be possible to develop efficient cell culture systems for other HCV genotypes. This, in its turn, will allow to study hepatitis C virus in its whole variety and develop the genotype-specific drugs and vaccines.
C hepatiidi viiruse replikatsioonisüsteemid.
Aleksei Suslov

Resüümee


Käesolev töö annab ülevaate C hepatiidi viiruse põhilistest in vitro replikatsioonisüsteemidest ja kirjeldab nende arenemise etappe alates viiruse genoomi kloneerimisest kuni tänapäevaste efektiivsete süsteemideni, mis on võimelised produtseerima funktsionaalseid virione ja lubavad uurida viiruse täieliku elutsükli ja molekulaabioloogiat.

Antud töös on käsitletud järgmised teemad:

1. C hepatiidi viiruse genoomi isoleerimine


5. Hiljutine funktsionaalse HCV JFH-1 genoomi isoleerimine ja sellele järgnenu mitmekesiste viirust produtseerivate süsteemide loomine.

6. JFH-1 genoomil põhinevate genotüüpidevaheliste kimääride konstrueerimine, mis võimaldas, vahemalt osaliselt, uurida nii viiruse struktuursete ja mittestruktuursete valkude omavahelisi interaktsioone kui ka HCV-d genotüübispetsiifiliselt.
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