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Replication of the hepatitis C virus in cell culture

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Abstract

Studies of hepatitis C virus (HCV) replication in cell culture have been greatly facilitated by the development of genetically engineered viral genomes that are capable of self-amplifying to high levels in a human hepatoma cell line. Since the original description of this 'replicon' model in 1999, important improvements have been made. Most notably, cell culture adaptive mutations were identified in various non-structural proteins that enhance RNA replication by several orders of magnitude. More recently, the permissiveness of the host cell was determined as an additional important factor contributing to efficient RNA replication. These discoveries allowed the development of transient replication assays, selectable full length genomes and a variety of novel replicons that will be useful for basic studies and facilitate the development of antiviral drugs. Ultimately, the replicon system may help to decipher the molecular basis of interferon-alpha (IFN-α) resistance.

Keywords: Hepatitis C virus; HCV; Replicons; Adaptation; RNA replication; Cell culture; Antiviral therapy; Positive strand RNA virus; Flaviviridae; Interferon-alpha

1. Introduction

About 15 years ago, the hepatitis C virus (HCV) was molecularly cloned and identified as the major causative agent of a disease that at that time was called non-A, non-B hepatitis (Choo et al., 1989). HCV is transmitted primarily by blood, blood products and parental risk factors but with the implementation of diagnostic tests to eliminate HCV-containing blood samples the incidence of newly occurring HCV infections has declined significantly (Donahue et al., 1992; Blajchman et al., 1995). Nevertheless, HCV is a serious medical problem, because in spite of a rather mild, and often asymptomatic initial cause of infection, the long-term consequences can be severe (Poynard et al., 1997). In most cases, the immune response is unable to clear the virus, resulting in its persistence. Patients with persistent infection are at high risk to develop chronic liver diseases with a wide spectrum of clinical manifestations including cirrhosis and hepatocellular carcinoma (HCC). Given the fact that currently about 170 million people are persistently infected with HCV, chronic hepatitis C has become the main indication for liver transplantation.

Current options for prophylaxis and therapy of chronic hepatitis C are very limited (Manns et al., 2001). Thus far, there is no vaccine that prevents infection with HCV. An acute self-limited course of infection does not confer protection against re-infection, but viral persistence and severity of subsequent HCV infections seem to be ameliorated (Mehta et al., 2002). Selective antiviral drugs that efficiently block virus production are not yet available in the clinic (Tan et al., 2002). Current therapy, which is costly and associated with significant side-effects including myalgia, arthralgia, headache, fever, severe depression and hemolytic anemia, is based on the combination of a conjugated form of interferon-alpha (IFN-α) and ribavirin. While the rate of sustained viral response in patients infected with HCV genotypes 2 and 3 is to 85–90%, success rates are only in the range of about 45% for patients infected with genotype 1 which is by far the most prevalent genotype. Thus, there is an urgent need for more effective therapeutics. Their development has been significantly delayed by the lack of appropriate cell culture models that allow the propagation of the virus in the laboratory. This roadblock has been overcome to some extent by the development of genetically modified HCV-minigenomes (replicons) that self-amplify in cultured hepatoma cells to very high levels (Lohmann et al., 1999). In the following review, we discuss this cell culture model and describe the most recent improvements and applications.

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2. Genomic organization of HCV and functions of viral proteins

Soon after the molecular cloning of the first HCV genome, it became clear that this novel virus is a distinct member of the family *Flaviviridae* to which the flaviviruses and pestiviruses belong (van Regenmortel et al., 2000). These viruses have an enveloped nucleocapsid in common that harbors a single stranded RNA genome of positive polarity. The RNA carries a single long open reading frame that in case of HCV encodes a polyprotein of about 3000 amino acids. Translation of this open reading frame is mediated via an internal ribosome entry side (IRES) that is located in the 5′ non-translated region (NTR; Tsukiyama-Kohara et al., 1992; Wang et al., 1993; Pestova et al., 1998). The resulting polyprotein is cleaved co- and post-translationally by concerted action of cellular and two viral proteases into at least 10 different products (Fig. 1; for review see Bartenschlager and Lohmann, 2000). To most of them distinct functions have been described. The structural proteins core (C), and the envelope glycoproteins E1 and E2 are the major constituents of the virus particle (Kaito et al., 1994; Yasui et al., 1998; Op De Beek et al., 2001). The small hydrophobic polypeptide p7 has recently been shown to be an ion channel that is sensitive to inhibition by amantadine, a drug that blocks ion channel function in influenza viruses (Griffin et al., 2003). It is speculated that the p7 protein is a viroporin and responsible for the flow of calcium ions from the endoplasmic reticulum into the cytoplasm. However, the exact role of this protein in the HCV life cycle remains to be established. NS2 and the amino terminal domain of NS3 constitute the NS2-3 proteinase that mediates cleavage between NS2 and NS3 (Grakoui et al., 1993; Hijikata et al., 1993). Although the exact mode of action of this enzyme has not been elucidated, recent evidence suggests that it is a cysteine-proteinase presumably activated via interaction with the cellular chaperone Hsp90 (Pieroni et al., 1997; Thibeault et al., 2001; Pallaoro et al., 2001; Waxman et al., 2001). The amino terminal domain of NS3 carries a serine-type proteinase activity that forms a stable heterodimeric complex with NS4A (Lin et al., 1994; Bartenschlager et al., 1994, 1995; Failla et al., 1994). This interaction is required for full activity of the enzyme. The carboxy terminal domain of NS3 harbors an ATPase/helicase activity capable to unwind double stranded nucleic acids (Suzich et al., 1993; Gwack et al., 1996; Kim et al., 1995). The role of this enzyme for RNA replication is not known but it is indispensable for infectivity in vivo (Kolykhalov et al., 2000). NS4B is a highly hydrophobic protein that appears to induce distinct membranous vesicles of unknown origin (Egger et al., 2002; Hugle et al., 2001). They accumulate in the cytoplasm of cells inducibly expressing either the complete polyprotein or only NS4B forming a structure called the membranous web which appears to be the site of HCV RNA replication (Gosert et al., 2003). Controversially discussed is the role of NS5A in the HCV life cycle. Several lines of evidence suggest that NS5A (as well as E2) interferes with the antiviral state in the cell that is induced by IFN-α (He and Katze, 2002). One molecular correlate appears to be the direct interaction

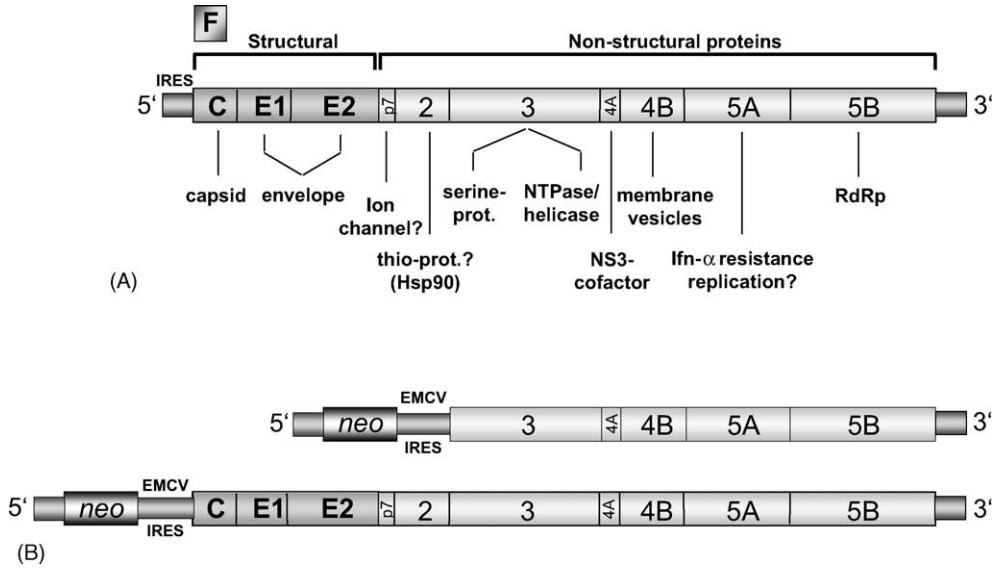

Fig. 1. Illustration of HCV genome and replicons. (A) Schematic representation of the HCV genome with the structural proteins C, E1 and E2 in the amino terminal quarter of the polyprotein and the non-structural proteins in the remainder. The F-protein (also designated 'alternative reading frame protein') generated by ribosomal frameshift is drawn above (Xu et al., 2001; Varaklioti et al., 2002). The functions of polyprotein cleavage products are given below the HCV genome. Activity of the NS2 proteinase appears to be activated by interaction with the cellular chaperone Hsp90. (B) The structure of the subgenomic HCV replicon carrying the selectable marker neo that confers G418 resistance is shown in the top panel, the structure of the selectable HCV full length genome below. For further details see text.

between NS5A and the protein kinase PKR (Gale et al., 1997). This kinase that is induced by IFN-α and activated by double stranded RNA is responsible, among others, for the phosphorylation of the translation initiation factor eIF2-α, thereby reducing RNA translation in the cell. Whether NS5A plays an additional, more direct role in RNA replication remains to be determined. NS5B is the RNA-dependent RNA polymerase (RdRp) that both in vitro and presumably also in vivo is able to initiate RNA synthesis de novo, i.e. via a primer-independent mechanism (Behrens et al., 1996; Lohmann et al., 1997; Luo et al., 2000; Zhong et al., 2000; O'Farrell et al., 2003; Sun et al., 2000; Kao et al., 2000).

3. The HCV replicon system

As alluded to above, development of HCV-selective antiviral drugs, as well as studies on virus replication, have been hampered primarily by the lack of efficient cell culture systems. A major breakthrough, therefore, was the development of HCV replicons that can be propagated efficiently in the human hepatoma cell line Huh-7. These replicons were derived from the consensus genome Con-1 that was cloned from the liver of a chronically infected patient (Lohmann et al., 1999). Based on the observation that smaller RNAs usually replicate more efficiently than longer ones (so-called defective interfering RNAs), we deleted the region encoding the structural proteins and inserted instead two heterologous elements (Fig. 1B). First, a selectable marker for which we chose the neomycin phosphotransferase (*neo*) gene, and second, the IRES of the encephalomyocarditis virus (EMCV). This resulted in a so-called selectable, bicistronic HCV RNA with the first cistron, the *neo* gene, being translated under control of the HCV IRES and the second cistron, the NS3-5B HCV coding sequence, being translated under control of the EMCV IRES. Upon transfection of Huh-7 cells with replicon RNAs generated by in vitro transcription of the cloned replicon sequences, and subsequent G418 selection, Huh-7 cell clones were generated that carried surprisingly high amounts of replicating HCV RNAs and viral proteins. In fact, compared to all in vitro infection systems that were available at that time, the HCV replicons amplified to levels that were about five orders of magnitude higher. By using strand-specific Northern blots, we calculated that on the average a single cell contained about 1000–5000 positive strand replicon RNA molecules whereas the number of negative strand RNAs was about 5- to 10-fold lower (Lohmann et al., 1999). Most importantly, the viral RNAs could be radiolabeled metabolically with [^{3}H]uridine in the presence of actinomycin D. Since this drug selectively blocks RNA synthesis from DNA templates but does not affect RNA production from RNA templates, this result unequivocally demonstrated that these HCV RNAs replicated autonomously to very high levels.

4. Properties of cells carrying stably replicating HCV RNAs

With the availability of cell clones that stably support high level HCV RNA replication, its influence on host cell growth and metabolism could be studied (Pietschmann et al., 2001). In spite of a very efficient RNA replication, no overt signs of cytopathogenicity like reduction of growth rate or altered morphology could be observed. This result is congruent with the majority of data implicating that liver damage is due to the immune reaction towards infected liver cells rather than to direct impairment of hepatocytes (Pawlotsky, 1998). Nevertheless there is increasing evidence that direct cytopathic effects of HCV contribute to liver cell injury (Giannini and Brechot, 2003).

When cells were passaged under continuous G418 selection, the replicons were maintained stably in the cells for several years. In the absence of selective pressure, a gradual reduction of replicon RNA levels was found. The rapidity with which RNA levels dropped highly depended on the culture conditions. In cells that were passaged at high confluency, replicon RNA levels were reduced much faster compared to those in cells that were always kept in an exponential growth phase demonstrating that HCV RNA replication and/or translation depends on host cell proliferation (Pietschmann et al., 2001). Whether this reflects the requirements for particular host cell factors that vary in abundance or activity during the host cell cycle or particular host cell conditions remains to be determined.

More recently in Huh-7 cells harboring a stably replicating HCV replicon, a distinct intracellular cytoplasmic structure was found which was designated the membranous web (Gosert et al., 2003). This structure was originally observed in human osteosarcoma cell lines that inducibly express either the complete polyprotein or only NS4B (Egger et al., 2002). Interestingly, in cells infected with other positive strand RNA viruses like the poliovirus and the flavivirus Kunjin virus similar membranous structures have been described and shown to represent the viral replication complex (Bienz et al., 1992; Chu and Westaway, 1992; Mackenzie et al., 1996). Thus, a plausible role of HCV NS4B would be to induce the formation of cytoplasmic vesicles that build up the site where viral RNA replication occurs. In line with this assumption it was found that all viral proteins as well as nascent HCV RNA localize to the membranous web (Gosert et al., 2003).

5. Cell culture adaptive mutations

The hallmark of the HCV replicon system is the efficiency with which these viral RNAs amplify in Huh-7 cells. Intensive studies performed over the last three years identified two major reasons: cell culture adaptive mutations and the level of permissiveness of the host cell. Cell culture adaptive mutations were originally identified by

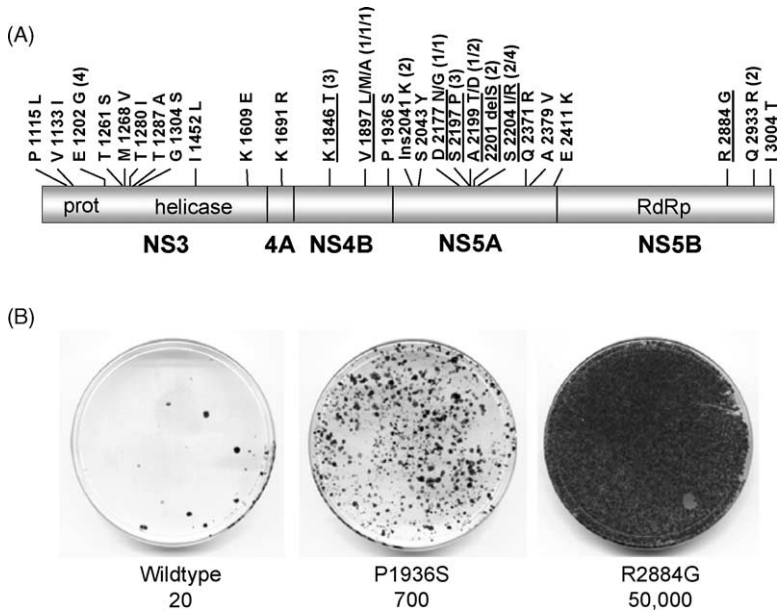

Fig. 2. Location of cell culture adaptive mutations and increase of G418 transduction efficiency. (A) Schematic representation of the NS3-5B coding region indicating the location of the NS3 proteinase (prot), the NS3 helicase and the NS5B RNA-dependent RNA polymerase (RdRp). Mutations that were found to be conserved in a total of 26 independent replicon cell clones are given above (Lohmann et al., 2003). Numbers in parentheses indicate the frequency with which a given mutation was found independently. The underlining is used to highlight those mutations that were found as single mutations in replicons whereas all the other mutations were only found in certain combinations. (B) G418 transduction efficiency observed after transfection of a non-adapted replicon (wild type), or a replicon carrying a mutation in NS4B (P1936S) or NS5B (R2884G) into Huh-7 cells and subsequent G418 selection. The number of resistant colonies obtained per microgram transfected HCV RNA is given below each corresponding replicon.

sequence analysis of HCV RNAs replicating in cell clones after G418 selection (Lohmann et al., 2001; Blight et al., 2000; Guo et al., 2001). In most cases, within the replicon RNA population replicating in a given cell clone, at least one mutation in the HCV coding region was found, resulting in an amino acid substitution that was absolutely conserved between all RNA molecules found in this cell clone. Although these ‘conserved’ mutations were found in every HCV non-structural protein, they cluster in certain areas. As exemplified in the comprehensive analysis of 26 replicons that were each cloned from an independent G418 resistant cell clone, most mutations map to a central region of NS5A (Fig. 2A; Lohmann et al., 2003). The major cluster is localized in the center of NS5A where serine residues are located that are required for hyperphosphorylation of this protein (Asabe et al., 1997; Kaneko et al., 1994; Tanji et al., 1995). Interestingly, several cell culture adaptive mutations lead to a removal of such serine residues (S2197, S2201, S2204) but so far it is not known whether and how alterations of NS5A phosphorylation affect RNA replication (Blight et al., 2000; Lohmann et al., 2003). A second cluster of adaptive mutations was found at the carboxy terminus of the NS3 serine-type proteinase domain and at the amino terminus of the NS3 helicase (Lohmann et al., 2003; Blight et al., 2003). Within the 3D structure, most of the mutations are located on the solvent accessible surface of the molecule and within the helicase domain, the mutations appear to be confined to one particular surface (Lohmann et al., 2001; Blight et al., 2003). The third cluster maps to two very distinct positions in NS4B (Lohmann et al., 2003; Guo et al., 2001; Kishine et al., 2002). Based on a recent topology model of NS4B, these two positions reside on the cytoplasmic loop connecting transmembrane domain two and three in case of K1846T, and at the cytoplasmic carboxy terminus of transmembrane domain 4 in case of substitutions affecting amino acid 1897 (Lundin et al., 2003). Interestingly, a phenylalanine substitution for valine at position 1897 has also been found in a replicon derived from the ‘MT-2C passaged HCV genome’ (Kishine et al., 2002). This genome represents a consensus isolate that was cloned from MT-2 cells that had been infected with HCV and passaged for prolonged times suggesting that the same cell culture adaptive mutations are operating with different HCV isolates.

The impact of these mutations on HCV RNA replication was initially tested by inserting some of them into the parental replicon construct and determining the number of G418 resistant colonies after transfection of a defined amount of RNA. Depending on the particular mutation an increase in the efficiency of colony formation was found (Fig. 2B) that in case of some NS5A mutations could be as high as 10,000-fold compared to the original replicon construct (Blight et al., 2000). However, by using a functional screening assay rather than a sequence analysis, the most efficient replicons turned out to carry more than one adaptive mutation. In fact the combination of a single mutation

in NS5A (S2197P) with two in NS3 (E1202G, T1280I) was superior to any other single mutation in terms of efficiency of RNA replication when using replicons derived from the Con-1 HCV isolate (Krieger et al., 2001; Lohmann et al., 2003). Similarly, in case of replicons derived from the infectious H77 genotype 1a isolate, the combination of a mutation in the helicase domain (P1496L) with S2204I in NS5A led to a cooperative increase of RNA replication (Blight et al., 2003).

With the availability of highly efficient HCV replicons, it was possible to develop transient RNA replication assays that avoided the cumbersome and time-consuming selection of G418 resistant cell clones and that allowed a rapid and more direct analysis. One such assay is based on the transfection of Huh-7 cells with cell culture adapted HCV replicons and measurement of RNA levels after 48 h by using quantitative RT–PCR (Blight et al., 2000). Another approach is using HCV replicons that carry instead of the selectable marker *neo* the reporter gene luciferase from the firefly (Fig. 3A; Krieger et al., 2001). In this case, Huh-7 cells are transfected with a HCV replicon that harbors one or several cell culture adaptive mutations in parallel to a replication defective RNA that carries an inactivating mutation in the NS5B RdRp active site. Transfected cells are harvested at 4, 24, 48 and 72 h post-transfection and luciferase activities are measured. Results are corrected for transfection efficiency by using the 4 h value that only reflects translation from the input RNA (Fig. 3B). A rapid decline of luciferase activity is observed with the replication defective HCV RNA. In contrast, luciferase activity obtained with the replication competent RNA is kept at a high level resulting in an about 100-fold difference between this RNA and the negative control at 48–72 h post-transfection. This difference is also found when replicon RNA levels are compared by Northern blot (Fig. 3C) demonstrating that luciferase activity is an accurate marker for viral RNA replication.

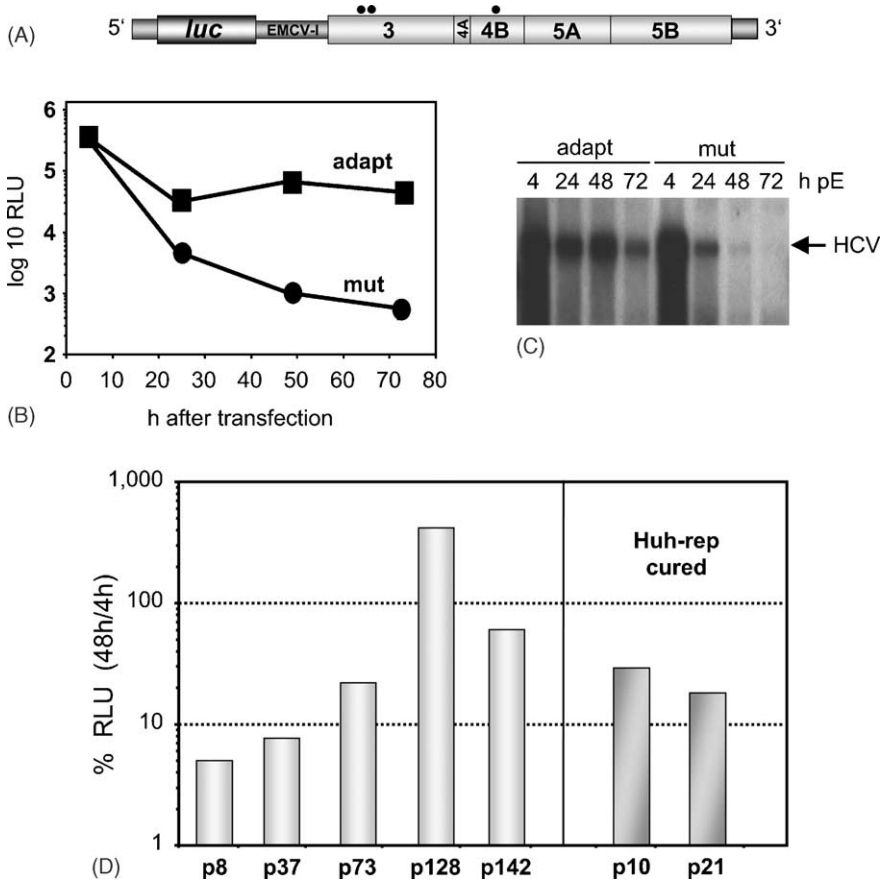

Fig. 3. Transient replication assay and importance of host cell permissiveness for HCV RNA replication. (A) The replicon used for this study carries a copy of the firefly luciferase gene (*luc*) that is translated under control of the HCV IRES. The location of cell culture adaptive mutations is indicated with dots above the NS3-5B coding region. (B) Huh-7 cells were transfected with the adapted replicon shown in panel A (adapt), or a defective replicon carrying an inactivating mutation in the NS5B RdRp (mut) and cell lysates were measured for luciferase activity or the amounts of replicon RNA by Northern blot (C) at 4, 24, 48, and 72 h post-transfection. The arrow indicates the position of HCV replicon RNA. pE, post-electroporation. (D) Influence of Huh-7 cell permissiveness on HCV RNA replication. Different passages of the same parental Huh-7 cells were transfected with the replicon shown in panel A and luciferase activities were measured at 4 and 48 h post-transfection. The percent ratio of the 48 h value versus the 4 h value that is used to correct for transfection efficiency is shown. In the right panel, 'cured' Huh-7 cells from which a selectable replicon RNA had been removed by treatment with IFN-α and that were cultivated for 10 or 21 further passages in the absence of G418, were transfected with the highly adapted replicon RNA shown in panel A. Replication was determined by measuring the amount of luciferase activity as described above.

By using this transient replication assay, the conserved mutations shown in Fig. 2A were tested individually and in combinations for their impact on RNA replication (Krieger et al., 2001; Lohmann et al., 2003). The following observations were made:

1. Mutations in NS4B at position 1846 have the highest impact on RNA replication.
2. Mutations in NS3 only lead to a very minor increase of RNA replication.
3. Mutations in NS3, especially E1202G, increase RNA replication cooperatively when combined with single adaptive mutations in NS4B, NS5A or NS5B.
4. The combination of highly adaptive mutations in NS4B, NS5A, and NS5B with each other reduces or in some cases completely abolishes RNA replication.

This result explains why the mutations in NS4B, NS5A, and a single one in NS5B were always found either as the only mutation that was conserved in a replicon RNA population, or in combination with one or several mutations in NS3 (Fig. 2A). In contrast, mutations in NS3 were always associated with at least one further mutation in the NS4B-5B region. Thus, the mechanism by which single mutations in NS4B, NS5A, or NS5B confer cell culture adaptation appears to differ from the one exerted by mutations in NS3.

Based on these observations, the following explanation for the emergence of cell culture adaptive mutations can be given. In cells transfected with the selectable non-adapted replicon, RNA replication is not sufficient to confer G418 resistance. Because of the high error rate of the HCV RdRp, during the initial low level replication, mutations arise that in rare cases confer cell culture adaptation. Only cells that carry such replicons harbor the *neo* gene in sufficient copy numbers to become G418 resistant whereas Huh-7 cells with non-adapted replicons will die because of the cytotoxicity of the drug. Only single adaptive mutations in NS4B, NS5A, or NS5B enhance RNA replication to a level required to confer G418 resistance whereas the mutations in NS3 are insufficient and always need some 'help' by highly adaptive mutations.

How these mutations increase RNA replication currently is not known. However, at least for the substitutions in NS3 and NS5B, they most often are located on the surface of the molecules and far away from the active sites of the respective enzymes (Lohmann et al., 2001; Blight et al., 2003). It is therefore assumed that cell culture adaptive mutations primarily affect directly or indirectly interactions with host cell factors.

In order to analyze the impact of cell culture adaptive mutations on infectivity in vivo, a panel of HCV genomes was constructed that were all derived from the Con-1 isolate that was also used to establish the replicon system (Bukh et al., 2002). Upon intrahepatic inoculation of chimpanzees with the parental HCV genome without adaptive mutations, a productive infection was established (Table 1). About one week after inoculation the animal became viremic and developed a persistent infection with average virus titers in the range of 10^5 genome equivalents per milliliter of serum. In contrast, infectivity was lost with the analogous genome into which two cell culture adaptive mutations in NS3 and one in NS5A had been introduced. Interestingly, when the genome carrying only the NS5A mutation was tested, the animal became infected but only after a delay of about one week. More importantly, all virus genomes recovered from the circulation corresponded to revertants. Thus, cell culture adaptive mutations lead to an attenuation in vivo with the level of attenuation presumably corresponding to the degree of cell culture adaptation at least with the Con-1 genome. In line with this observation, the HCV-N isolate that has a very low infectivity in vivo (Beard et al., 1999), could be used to establish HCV replicons that replicate rather efficiently in Huh-7 cells even in the absence of cell culture adaptive mutations (Guo et al., 2001; Ikeda et al., 2002). This is due to a four amino acid insertion in the center of NS5A that confers an adaptive phenotype and that appears to be tolerated in vivo. In contrast, the H77 isolate that has a very high infectivity in vivo (Kolykhalov et al., 1997) can only be replicated in cell culture upon the introduction of single adaptive mutations in NS5A and one cooperative mutation in NS3 and when using highly permissive Huh-7 cells (Blight et al., 2003).

Table 1
Effects of cell culture adaptive mutations on infectivity in vivo

HCV isolate	Genotype	Infectivity	Replication in cell culture
Con-1[a]	1b	++	+/−
Con-1/12102 + 1280 + 2197[b]	1b	−	+++
Con-1/2197[c]	1b	+/−	++
HCV-N[d]	1b	+/−	+
HCV-H77[e]	1a	+++	−

Infectivity data for the Con-1 isolate are described in Bukh et al. (2002).

[a] HCV isolate used to generate the first HCV replicon (Lohmann et al., 1999).

[b] Con-1 isolate carrying two cell culture adaptive mutations in NS3 (E1202G, T1280I) and one in NS5A (S2197P) (Krieger et al., 2001).

[c] Con-1 isolate carrying a single adaptive mutation in NS5A (S2197P) (Krieger et al., 2001).

[d] Beard et al. (1999), Guo et al. (2001), and Ikeda et al. (2002).

[e] Blight et al. (2000) and Kolykhalov et al. (1997).

6. Permissiveness of the host cell

In addition to cell culture adaptive mutations, the efficiency of HCV RNA replication is also determined by the host cell itself (Blight et al., 2002; Lohmann et al., 2003; Murray et al., 2003). By using the transient replication assay described above, up to 100-fold differences in the level of RNA replication were found between different passages of the same Huh-7 founder cell line (Fig. 3D). This difference was independent from the kind of adaptive mutation introduced into the replicon and also observed with the parental (non-adapted) HCV Con-1 RNA. However, the

level of permissiveness was difficult to control and subject to variations that could not be predicted. In a search for the reason of the permissiveness, variations in IRES-dependent HCV RNA translation and differences in RNA stability could be excluded (Lohmann et al., 2003). Thus, one explanation is that the level of permissiveness is determined by the amounts or activities of certain host cell factors that are required for RNA replication. In line with this assumption it was found that replication efficiency decreased with increasing amounts of replicon RNA transfected into Huh-7 cells suggesting that host cell factors in Huh-7 cells limit RNA amplification (Lohmann et al., 2003).

Another line of evidence stems from the observation that efficient HCV RNA replication can only be observed in a subpopulation of Huh-7 cells (Blight et al., 2002; Murray et al., 2003). Thus, the low number of G418 resistant colonies obtained after transfection of the non-adapted HCV replicon would not only be determined by the frequency with which a cell culture adaptive mutation is generated but also by the permissiveness of the particular host cell. Consequently, the cell clones obtained after G418 selection would contain cell culture adapted replicons and/or would have been selected for higher level of permissiveness. This assumption was confirmed by the following experiment: Huh-7 cell clones carrying stably replicating HCV RNAs were treated with IFN-α or a selective inhibitor that both efficiently block replicon RNA amplification (see below). After about two weeks most HCV RNAs had been removed resulting in a population of so-called 'cured' cells. Upon retransfection of these cells with selectable HCV replicons, the number of G418 resistant colonies was significantly higher compared to naive Huh-7 cells (Blight et al., 2002; Murray et al., 2003). Thus, 'cured' cells appear to be more permissive than the parental ones suggesting that during G418 treatment Huh-7 cells with higher levels of permissiveness are selected for. However, the difference in permissiveness between 'cured' and naive cells is difficult to make, because permissiveness is not a stable phenotype and therefore, naive cells that are used for comparison are subject to variations, too. As exemplified in Fig. 3D, 'cured' replicon cells are more efficient than naive Huh-7 cells of passage 8 or 37, but less permissive than naive cells of passage 128 or 142. Moreover, even with 'cured' cells, permissiveness is not a stable trait (compare p10 and p21 in the right panel of Fig. 3D).

7. Genomic HCV replicons and cell culture adapted full length genomes

Although subgenomics replicons are a valuable tool, the final goal was the establishment of a cell culture system that also allows the production of infectious HCV particles. Thus, Huh-7 cell clones carrying stably replicating genomic HCV replicons were generated (Pietschmann et al., 2002; Ikeda et al., 2002; Blight et al., 2003). These RNAs differ from the subgenomic replicons only by carrying the complete HCV open reading frame instead of the NS3-5B coding region (Fig. 1B). Moreover, cell culture adaptive mutations were introduced right from the beginning in order to increase the replication level. Upon introduction of these cell culture adapted, selectable HCV genomes into Huh-7 cells and G418 selection, only a low number of cell clones was obtained. However, within a given clone HCV genomes replicated at high levels which were only about five-fold lower compared to subgenomic RNAs. The low number of cell clones on one hand and the high replication levels on the other hand suggested that further cell culture adaptation took place but sequence analysis of complete genomes isolated from Huh-7 cells did not reveal such 'conserved' mutations (Pietschmann et al., 2002). Moreover, when these cells were 'cured' by treatment with IFN-α, they did not reveal an increased level of permissiveness (V. Lohmann and R. Bartenschlager, unpublished observation). Thus, the reason for the low G418 transduction efficiency observed with selectable HCV genomes so far is not clear.

Owing to the high replication level of selectable HCV genomes in a given cell clone, attempts to demonstrate production of virus particles were undertaken (Pietschmann et al., 2002; Ikeda et al., 2002; Blight et al., 2003). In the absence of cell lines that are readily infectable by HCV, this turned out to be problematic, because indirect criteria for virus production like the presence of nuclease resistant HCV RNA in cell culture supernatants is not a reliable marker. For instance, we found that Huh-7 cell clones carrying a stably replicating selectable HCV genome release substantial amounts of RNase-resistant HCV RNA into the cell culture medium that in sucrose density gradients has a buoyant density of about 1.04 g/ml (Pietschmann et al., 2002). While this density is analogous to the one observed for infectious HCV in patient serum, Huh-7 cells carrying stably replicating subgenomic replicons—without structural proteins—also release HCV RNA with identical properties into the cell culture supernatant suggesting that the liberation of these 'RNA-containing structures' is a non-specific process independent from the structural proteins. In line with this assumption, a very low G418 transduction efficiency was found when naive Huh-7 cells were incubated with culture supernatants from cells carrying a selectable genome or a subgenomic replicon (Pietschmann et al., 2002). The RNAs recovered from these transduced cells all corresponded to the input RNA used for the original transfection.

In order to exclude the possibility that the lack of virus production is due to the use of genetically modified HCV genomes that carry heterologous elements (neo and EMCV IRES), transient replication assays were performed by using authentic HCV genomes with or without cell culture adaptive mutations in parallel to the corresponding subgenomic replicons (Pietschmann et al., 2002; Blight et al., 2003). However, also in this case nuclease-resistant HCV RNA-containing structures were released into the culture supernatant after transfection with subgenomic RNAs. The fact that the original HCV genome used to construct all HCV

replicons is infectious in vivo suggests that the structural proteins are fully competent for virion formation. Therefore, a specific block in virus production due to the lack of host cell factors required for virus formation or release seems to be a more plausible explanation. Alternatively, production of virus particles in Huh-7 cells is possible but the efficiency is so low that it can not be detected with the methods employed thus far and is masked by the release of HCV RNA-containing structures already in the absence of the structural proteins. Further studies will be required to discriminate between these possibilities.

8. Applications of the HCV replicon system

In spite of this limitation, the replicon system has become one of the most important tools to study HCV RNA replication, pathogenesis and persistence. For instance, HCV replicons have successfully been used to map the minimal region in the 5′ NTR of HCV required for RNA replication. It was shown that the first 125 nucleotides are sufficient for replication demonstrating that the region required for RNA translation (about nucleotide 40–355) and replication overlap (Friebe et al., 2001; Luo et al., 2003). A similar mapping study of the 3′ NTR revealed that the very last 98 nucleotides of the HCV genome (the X-tail) are indispensable for RNA replication (Friebe and Bartenschlager, 2002; Yi and Lemon, 2003a,b). In contrast, the variable region can be deleted with only minor reduction of replication whereas the poly(U/UC) region of variable length can be shortened significantly without affecting RNA replication. These results are remarkably similar to what was observed with HCV genomes carrying similar mutations in the 3′ NTR and tested for infectivity in the chimpanzee model (Yanagi et al., 1999). Apart from such studies, the replicon system will certainly be instrumental to identify host cell factors required for RNA translation and replication and to study the mechanism that is operating in regulating the switch from translation to replication and vice versa. Furthermore it is a useful tool to analyze the mechanism of replication complex formation (Bost et al., 2003; Shi et al., 2003; Mottola et al., 2002), the structure and function of this complex (Hardy et al., 2003; Lai et al., 2003; Ali et al., 2002), the induction of the membranous web as site of viral replication, the roles of NS4B and NS5A for RNA replication, or the identification of *cis* acting sequences in addition to the 5′ and 3′ NTRs required for replication, to list just a few areas of research that might benefit most from this cell culture model.

9. Inhibition of HCV replicons by interferon-alpha

Finally, the replicon system may help to unravel the mechanisms underlying the apparent resistance of genotype 1 HCV isolates to the antiviral activity of IFN-α. It has recently been shown that HCV replicons derived from the

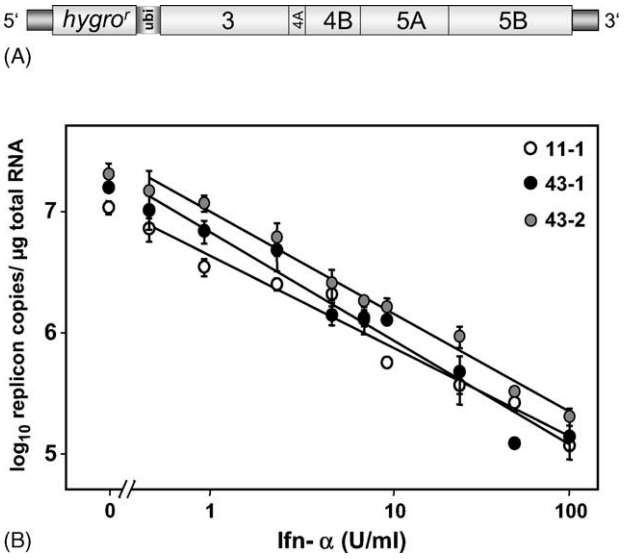

Fig. 4. Inhibition of HCV RNA replication by IFN-α. (A) Structure of the monocistronic HCV replicon RNA. The selectable marker hygromycin phosphotransferase (*hygro*^{r}) is fused at its amino terminus to the first 12 amino acid residues of HCV core and at its carboxy terminus to ubiquitin (ubi) followed by the HCV NS3-5B coding region. In this replicon, RNA translation and replication are exclusively controlled by HCV sequences. (B) Three independent cell clones (11-1, 43-1, 43-2) each harboring a stably replicating monocistronic replicon were incubated with increasing concentrations of IFN-α for 72 h and HCV RNA was determined by quantitative RT–PCR using the TaqMan technique. Values are corrected for total RNA by using a multiplex assay that allows the amplification of both the HCV 5′ NTR and the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Note the 100-fold reduction of replicon RNA amounts with an IC_{50} of about 1 U/ml.

Con-1 or the HCV-N isolate are highly sensitive to the antiviral state that is induced by treatment of Huh-7 cells with IFN-α (Blight et al., 2000; Frese et al., 2001; Guo et al., 2001; Lanford et al., 2003). However, given the bicistronic design of HCV replicons it was not clear whether the observed inhibition was due to a block of HCV functions or to an inhibition of the EMCV IRES. This latter possibility has been ruled out by using monocistronic replicons in which HCV RNA translation and replication are controlled exclusively by HCV sequences (Fig. 4). In fact, these RNAs appear to be even more sensitive to the antiviral state induced by IFN-α, because the IC_{50} is as low as about 1 U/ml in case of monocistronic HCV replicons but 2.5–5 U/ml in case of bicistronic RNAs (Kaul and Bartenschlager, unpublished results). Thus, one way by which IFN-α inhibits HCV replicons might be a block of RNA translation (Wang et al., 2003).

As alluded to in a previous section, the rate of sustained response of infections with genotype 1 isolates is significantly lower compared to infections with genotype 2 or 3 variants. This has been ascribed to a direct interaction of NS5A with PKR that is induced by IFN-α and activated by double stranded RNA (Gale et al., 1997). NS5A from non-responder patients was found to interact with and thereby inhibit, PKR

more efficiently than NS5A of responders (Gale et al., 1998). It is therefore surprising that all HCV replicons derived from the Con-1 isolate have an ISDR sequence identical to a resistant variant but are highly susceptible to IFN-α. However, the reduction of HCV replicons by IFN-α per se is not a direct measure for therapy success. In fact, most treated patients have a biphasic decline in HCV RNA (Neumann et al., 1998). A first rapid drop of viremia that reflects the direct inhibition of HCV (e.g. RNA translation or replication), and a second much slower decline that appears to reflect the death rate of infected cells representing the cellular reservoir that produces HCV progeny. Although a rapid decline of viremia during the first phase is a good prognostic marker for therapy success (Rosen et al., 2002), most patients, even those that later on turn out to be non-responders or relapsers, react to IFN-α therapy with an initial drop of viremia. Since the replicon system only allows the analysis of this first phase, but not the second phase that is much more important for sustained therapy success, currently we do not know how the success of IFN-α therapy in a patient translates into the replicon system. Moreover, neither for the Con-1 (Koch and Bartenschlager, 1999) nor for the HCV-N (Beard et al., 1999) or the HCV-MT2 isolate (Sugiyama et al., 1997) the biological properties, especially the reactivities to IFN-α in vivo are known. Finally, about 45% of genotype 1b viruses can efficiently be treated with IFN-α. Therefore, comparative kinetic and quantitative studies are required by using HCV replicons that are derived from virus variants with clinically proven IFN-α resistance or sensitivity. Because of some unknown restrictions, this has so far not been possible. Most HCV isolates, including several with proven infectivity turned out not to replicate in Huh-7 cells. The reason(s) for this astonishing observation remains to be clarified.

10. Future perspectives

Since the first successful establishment of a HCV replicon amplifying in Huh-7 cells to very high levels, this system has been improved substantially. Thus far, there are four different HCV isolates available that are capable to replicate in Huh-7 cells and that belong to genotype 1b (Lohmann et al., 1999; Guo et al., 2001; Kishine et al., 2002) or genotype 1a (Blight et al., 2003). The discovery that efficient RNA replication is due to both cell culture adaptation and host cell permissiveness enabled the development of rapid and reliable, transient replication assays, the establishment of selectable HCV full length genomes, monocistronic replicons and replicons that constitutively express either a transactivator that induces the expression of a reporter gene or a reporter gene that is stably integrated into the replicon (Yi and Lemon, 2002; Murray et al., 2003; Frese et al., 2002). Since in these cases reporter gene activity is a direct correlate of RNA replication, these replicon systems are particularly amenable for high throughput tests. For instance, we have recently shown that a replicon carrying in addition to the selectable marker the gene encoding the firefly luciferase can be used to determine the amount of biologically active IFN-α in sera and plasma of patients under treatment (Vrolijk et al., 2003). Moreover, this system can be used for screening large collections of compounds for antiviral activity in 96- and even 384-well plate formats. Finally, the replicon system is a powerful tool to evaluate the antiviral activity of a given drug, for drug optimization, selection for antiviral drug resistance (and in this way confirmation of the viral target) and verification of resistance mutations for instance in transient replication assays (Trozzi et al., 2003).

In spite of these achievements, we are still faced with several limitations that need to be overcome in order to make the system more applicable for HCV research. First, we will need a system that allows the production of infectious virus particles. Unfortunately, in spite of efficient replication of complete HCV genomes in Huh-7 cells and expression of all viral proteins, this is still not or only very inefficiently possible. Second, thus far HCV replicons can only be propagated in Huh-7 cells. Attempts with other cell lines failed raising the question what makes Huh-7 cells so unique. Whatever the reasons are, they appear not to be unique to HCV, because replicons of the closely related GB virus B and the hepatitis A virus (HAV) could also only be propagated in Huh-7 cells (Yi and Lemon, 2002; De Tomassi et al., 2002). This result is perplexing because HAV can be propagated in other cell lines as well both after infection and after transfection of cloned full length genomes. One possible explanation is that only in Huh-7 cells HAV replication is efficient enough to allow detection of subgenomic replicon RNA replication. In case of transfection of full length HAV genomes infectious virus is generated that spreads throughout the culture and thereby amplifies which is not possible when using subgenomic replicons. In any case, the availability of cell lines other than Huh-7 that support HCV RNA replication would be important in order to broaden the scope of the replicon system and to exclude the possibility of cell line dependent effects. For instance it has recently been shown that Huh-7 cells have a defect in the activation of double stranded RNA-dependent pathways like activation of PKR and induction of apoptosis raising concerns whether the high sensitivity of HCV replicons to IFN-α observed in Huh-7 cells reflects the in vivo situation (Lanford et al., 2003). Although we found that the same is true for other hepatoma cell lines like HepG2 (A. Kaul and R. Bartenschlager, unpublished results), at least for those studies where host cell factors are—directly or indirectly involved—additional cell lines other than Huh-7 may be required to confirm observations made with the currently available HCV replicon system. Hopefully, the most recent improvements of HCV replicons made with Huh-7 cells will foster future studies to establish alternative cell lines that support HCV RNA replication with comparable efficiency.

11. Note added in proof

While this manuscript was in press, Zhu et al. reported the successful establishment of HCV replicons in the human cervix carcinoma cell line HeLa and in the mouse hepatoma cell line Hepa1–6 (Zhu et al., 2003) after transient transfection of total RNA prepared from replicon harbouring Huh-7 cells. Replicons in HeLa and Hepa1–6 cells carry in addition to the adaptive mutations identified in Huh-7 cells (E1202G, S22041) mutations in the HCV coding sequence that may confer cell type specifity.

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