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Review

Novel cell culture systems for the hepatitis C virus

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Abstract

Infections with the hepatitis C virus (HCV) are a major cause of acute and chronic liver disease. The high prevalence of the virus, the insidious course of the disease and the poor prognosis for long-term persistent infection make this pathogen a serious medical and socioeconomical problem. The identification of the viral genome ~10 years ago rapidly led to the delineation of the genomic organization and the structural and biochemical characterization of several virus proteins. However, studies of the viral life cycle as well as the development of antiviral drugs have been difficult because of the lack of a robust and reliable cell culture system. Numerous attempts have been undertaken in the past few years but only recently a highly efficient cell culture model could be developed. This system is based on the self replication of engineered HCV minigenomes (replicons) in a transfected human hepatoma cell line. A summary of the various HCV cell culture models with a focus on the replicon system and its use for drug development is described.

Keywords: Hepatitis C Virus; HCV cell cultures; Antiviral therapy; Flaviviridae; HCV replication systems; HCV replicons

1. Introduction

Acute and chronic liver diseases that are caused by an infection with the hepatitis C virus (HCV) are a serious medical, social and economic burden. About 2% of the world population is infected with this pathogen that can cause a large variety of clinical symptoms (Poynard et al., 2000). In most cases these are benign or even subacute and many patients do not realize the infection, until after 1–3 decades chronic liver damages manifest that can be as severe as liver cirrhosis or hepatocellular carcinoma. As yet, the only available therapy for chronic hepatitis C is the treatment with interferon-alpha (Ifn-α) either alone or in combination with the nucleoside analogue ribavirin (for review see Foster and Thomas, 2000). Unfortunately, only ~40% of treated patients develop a sustained response that is defined by the absence of viral RNA for more than 6 months after cessation of therapy (Lavanchy et al., 1999). Moreover, side-effects such as flu-like symptoms, fatigue or suicidal depression that are due to

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interferon are common. In spite of the beneficial effect of ribavirin, the side-effects are even more severe with combination therapy, because ribavirin can cause haemolytic anaemia and due to its teratogenicity is excluded from treatment of HCV-infected pregnant women. These complications clearly document the need for more effective therapies, but unfortunately, their development has been severely hampered by the lack of appropriate experimental systems. The only animal that can reliably be infected with HCV is the chimpanzee but owing to ethical reasons, high costs and the inherent difficulties in working with large animals restricts their use for such purposes. However, the greatest limitation for HCV research is the lack of a cell culture system that supports the efficient and reliable propagation of this virus. Numerous attempts have been undertaken in the last ~ 10 years to establish such systems but only recently a significant breakthrough could be achieved with the development of subgenomic self-replicating HCV RNAs (replicons; Lohmann et al., 1999). This review, after a brief description of the virus and its replication cycle, will summarize some of the infection-based cell culture systems and then focus on the HCV replicon system, recent improvements and its usefulness for drug development.

2. Genome organization and replication cycle of HCV

Characteristic for a member of the family *Flaviviridae*, HCV possesses a positive strand RNA genome that is packaged into an enveloped particle. This RNA carries a single long open reading frame (ORF) that encodes a polyprotein with a length of ~ 3010 amino acids (for a review see Bartenschlager and Lohmann, 2000; Fig. 1). Translation of the ORF is mediated by the 5′ non-translated region (NTR) that constitutes an internal ribosome entry site (IRES) permitting the direct binding of the 40S ribosomal subunit at the

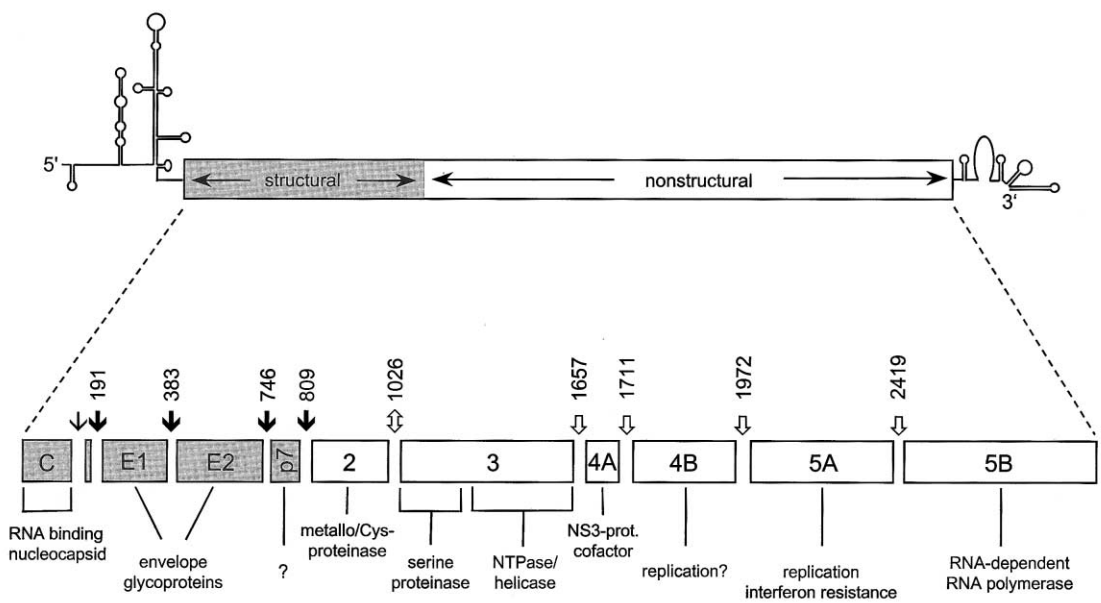

Fig. 1. A schematic presentation of the HCV genome is given at the top. The highly structured 5′ and 3′ NTRs are not drawn to scale. The individual cleavage products of the polyprotein are given below. Cleavage sites for (↓) host cell signalases, (⇳) the NS2-3 proteinase, (⇓) the NS3/4A proteinase complex and (↓) an unknown cellular enzyme are marked. Numbers above the arrows refer to the P1 positions of the corresponding cleavage sites. The functions of the different proteins are given in the bottom. The question mark below p7 indicates that it is of unknown function.

Table 1
A selection of cell culture systems for HCV

Cell type		Species	Validation methods[b]	Persistence[c]	Reference
Primary cells	Immortalized Line				
Hepatocytes (in vivo)[a]		Human	(+), (−) RNA; antigen (IF); HVR-sequence; transmission	28	Ito et al., 1996
PBMCs (in vivo)		Human	(+), (−) RNA; ^{3}[H] uridine incorporation	9	Müller et al., 1993
Foetal liver cells		Human	(+), (−) RNA	24	Iacovacci et al., 1993, 1997
Hepatocytes		Chimpanzee	(+), (−) RNA; Ifn-α	25	Lanford et al., 1994
Hepatocytes		Human	(+), (−) RNA	14	Fournier et al., 1998
Hepatocytes		Human	(+), (−) RNA; antigen (EIA); transmission; HVR-1 sequence	90	Rumin et al., 1999
PBMCs		Human	(+), (−) RNA; ISH; transmission	26	Cribier et al., 1995
	PH5CH liver cells	Human	(+) RNA; HVR-sequence	30	Kato et al., 1996
	PH5CH clones	Human	(+) RNA; Ifn-α	100	Ikeda et al., 1997, 1998
	HepG2 hepatoma	Human	(+), (−) RNA	<20	Tagawa et al., 1995
	WRL68 hepatoma			62	
	HepG2 & Huh-7 hepatoma, PK 15 porcine kidney cells	Human, porcine	(+), (−) RNA; transmission	130	Seipp et al., 1997
	HepG2, hepatoma	Human	(+) RNA; HVR-sequence	77	Clarysse et al., 2001
	JHH-1, −4, −6 hepatoma	Human	(+) RNA; (−) RNA only in JHH-4	Continuous[d]	Tsuboi et al., 1996
	CE B-cells	Human	(+) RNA; IF	65	Bertolini et al., 1993
	TOFE B-cells	Human	(+) RNA; 5′ NTR-sequence	~180	Valli et al., 1995
	Daudi B-cells	Human	(+) RNA, HVR-sequence, gradient centrifugation; transmission to chimpanzee	>2 years	Yoshikura et al., 1995; Nakajima et al., 1996; Shimizu et al., 1998
	MT-2 T cells	Human	(+), (−) RNA; HVR-sequence	15	Kato et al., 1995; Ikeda et al., 1997
	MT-2C clone	Human	(+), (−) RNA; Ifn-α; antisense	30/80	Mizutani et al., 1995, 1996a
	MT-2C clone	Human	(+) RNA, transmission; HVR-sequence	198	Mizutani et al., 1996b
	MT-2C clone	Human	complete genome sequence 12 days p.i.	n.a.	Sugiyama et al., 1997b
	MOLT-4 Ma T-cells	Human	(+), (−) strand; ISH; antigen (IF); Transmission	25	Shimizu et al., 1992
	HPB-Ma T-cells	Human	(+), (−) RNA; antigen (IF); titration	76	Shimizu et al., 1993

Table 1 (*Continued*)

Cell type		Species	Validation methods[b]	Persistence[c]	Reference
Primary cells	Immortalized Line				
	HPBMA10-2 clone	Human	(+) RNA; gradient centrifugation; genome sequences; transmission; EM	>365	Shimizu and Yoshikura, 1994; Nakajima et al., 1996; Shimizu et al., 1996
	MEG-01 megakaryoblastic leukemia cells	Human	(+), (−) RNA; IS RT-PCR; antigen (IF, WB); EM	Continuous[d]	Tagawa et al., 1995

[a] Isolation of primary cell cultures from infected patient tissues; in all other cases cells were infected ex vivo.

[b] Method used to monitor HCV replication; (+), plus strand RNA; (−), minus strand RNA; Ifn-α and antisense, inhibition of HCV replication by interferon-α or antisense oligonucleotides; transmission, transfer of HCV to naive cells by cocultivation with infected cells or incubation with medium from infected cells; IF or WB or EIA, detection of antigen by immunofluorescence or Western-blot or enzyme immuno-assay, respectively; ISH, in situ hybridization; EM, detection of virus-like particles by electron microscopy; HVR-sequence, determination of the nucleotide sequence of the hypervariable region; titration, correlation between infectivity titres in cell culture and experimentally inoculated chimpanzees; IS RT-PCR, in situ RT-PCR;

[c] Number of days between infection and the last day of detection of HCV plus strand RNA; n.a., no data available.

[d] Persistent HCV production.

site of the initiator AUG codon of the ORF (Pestova et al., 1998; Tsukiyama-Kohara et al., 1992; Wang et al., 1993). In addition to this function, most of the 5′ NTR is required for efficient RNA replication (Friebe et al., unpublished results). Downstream of the ORF is a 3′ NTR that has a tripartite structure composed of a variable region following the stop codon of the ORF, a poly (U/UC) tract with a length that varies between 30 and 150 residues and a highly conserved 'X-tail' sequence (Kolykhalov et al., 1996; Tanaka et al., 1995, 1996; Yamada et al., 1996). The latter has been discovered only recently and it was shown to be essential for RNA replication in vivo and in cell culture (Kolykhalov et al., 1996; Yanagi et al., 1999a; Friebe et al., unpublished results).

At least 10 different products are generated by co- and posttranslational cleavage of the HCV polyprotein (Fig. 1; for reviews see Bartenschlager, 1999; Neddermann et al., 1997; Reed and Rice 1998). The structural proteins core, E1 and E2 are the major constituents of the virus particle (Kaito et al., 1994; Yasui et al., 1998) whereas the nonstructural proteins NS3-5B are essential for RNA replication (Lohmann et al., 1999). To most viral proteins distinct functions could be ascribed (Fig. 1). From a therapeutic point of view, enzymatic activities encoded by the HCV genome are of highest interest because they provide targets for antiviral intervention. These are the NS2-3 and NS3/4A proteinases, the NS3 nucleoside triphosphatase(NTPase)/helicase and the NS5B RNA-dependent RNA polymerase (RdRp; Bartenschlager et al., 1993; Behrens et al., 1996; Grakoui et al., 1993a,b; Hijikata et al., 1993; Kim et al., 1995; Lohmann et al., 1997; Suzich et al., 1993; Tomei et al., 1993). They are all essential for virus replication in vivo and with the exception of NS2-3, the crystal structures of these enzymes have been resolved (Ago et al., 1999; Bressanelli et al., 1999; Cho et al., 1998; Kim et al., 1996, 1998; Kolykhalov et al., 2000; Lesburg et al., 1999; Love et al., 1996; Yan et al., 1998; Yao et al., 1997). These results have been very informative because they nicely explained on a structural basis several of the biochemical properties that had been observed during studies of the recombinant HCV enzymes. For instance, the remarkable stability of the NS3/4A interaction can easily be explained by the tight intercalation of NS4A into the amino terminal NS3 domain. Thus, NS4A forms an integral component of the enzyme. This interaction crucially contributes to proper folding of NS3, its stability, association with intracellular membranes and catalytic activ-

ity (reviewed in Bartenschlager, 1999). Unfortunately, it turned out that the substrate binding pocket of the enzyme is flat, shallow and solvent exposed, making the development of efficient and selective inhibitors very difficult. The crystal structure of the NS3 NTPase/helicase either alone (Cho et al., 1998; Yao et al., 1997) or complexed with a single-stranded DNA oligonucleotide has also been solved (Kim et al., 1998). The molecule carries three distinct domains with domains 1 and 2 involved in ATP binding. As inferred from the structure solved by Kim et al. (1998), the nucleic acid binds without sequence specificity in a groove that separates domain 3 from domains 1 and 2. The mechanism by which HCV NS3 unwinds double stranded nucleic acids is discussed controversially and three different models have been proposed (Cho et al., 1998; Yao et al., 1997; Kim et al., 1998). However, a detailed understanding of the mechanism by which ATP hydrolysis is coupled to RNA unwinding will be required for more efficient structure-based drug design.

Three groups independently solved the X-ray crystal structure of the NS5B RdRp (Ago et al., 1999; Bressanelli et al., 1999; Lesburg et al., 1999). Similar to other polymerases, NS5B can be subdivided into palm, fingers and thumb subdomains. Unique to this molecule are the intensive contacts between the thumb and fingers subdomains that are mediated by two loops originating in the fingers that pack against the thumb subdomain. Therefore, unlike the U-shaped structure found with other polymerases, HCV NS5B is a globular molecule with a fully encircled active site cavity. Another unique feature is a 12 amino acid residues long beta-hairpin in the thumb subdomain. This hairpin protrudes towards the active site and most likely interferes with productive polymerase binding to double stranded RNA molecules (Zhong et al., 2000; Hong et al., 2001). It was proposed that the beta-hairpin serves as a molecular 'gate' that prevents the slippage of the 3′ terminus of the template through the active site and thereby ensures de novo initiation of replication from the 3′ end of the genome (Hong et al., 2001).

A simplified outline of the HCV replication cycle can be summarized as follows: (1) attachment to the host cell and penetration; (2) release of the viral positive strand RNA; (3) translation of the ORF and processing of the polyprotein; (4) assembly of a replicase complex that contains at least NS3-5B, but most likely also cellular proteins; (5) production of positive strand RNA progeny via negative strand RNA intermediates; (6) formation of new virus particles that probably acquire their envelope via budding into the lumen of the endoplasmic reticulum; and (7) release of virus progeny presumably via transport through the Golgi compartment. All these steps take place in the cytoplasm where HCV proteins are found in close association with intracellular membranes (Pietschmann et al., 2001).

The primary host cell supporting HCV replication is the hepatocyte. In addition, the virus can also multiply in lymphoid cells both in vivo and after experimental infection of various cell lines (Cribier et al., 1995; Lerat et al., 1996, 1998; Mihm et al., 1996; Bouffard et al., 1992; Müller et al., 1993; Willems et al., 1994; Zignego et al., 1992). Although this observation is still discussed controversially (Lanford et al., 1995; Laskus et al., 1997; Takehara et al., 1992) a lymphotropism of HCV is consistent with the finding that relatively high genome titres can be found in the lymph nodes of infected patients (Sugiyama et al., 1997a). Moreover, clinical studies indicate a significant correlation between HCV infection and some lymphoproliferative disorders, especially the high prevalence of auto-antibodies, mixed cryoglobulinemia and non-Hodgkin lymphoma suggesting that HCV not only causes a liver disease but rather a multifaceted clinical syndrome (for review see Poynard et al., 2000).

3. Cell culture systems for HCV

Since viruses are obligate intracellular parasites, the study of their multiplication cycle requires a permissive host cell. In the easiest case, such cells can be grown as a permanent cell line that is readily available in the laboratory. Numerous attempts have been undertaken to propagate HCV in cell culture but for unknown reasons, this turned out to be very difficult. Most experiments

were based on the infection of primary cell cultures or cell lines with HCV or the cultivation of primary cells isolated from tissues of persistently infected patients. However, these systems suffer from poor reproducibility and the low level of HCV replication demanding the use of highly sensitive detection methods. In many cases, replication was measured by the qualitative determination of the negative strand RNA intermediate by RT-PCR, but this method has some inherent technical problems that are difficult to overcome (Gunji et al., 1994; Lanford et al., 1994; Takyar et al., 2000). Therefore, several additional critera were used to demonstrate HCV replication in infected cells. These are the sequence analysis of HCV genomes or genome fragments to demonstrate genomic variability and selection of variants upon infection and cultivation, the transmission of cell culture-grown HCV to naive cells, the detection of viral antigens, the increase of positive strand RNA in infected cells, the inhibition of replication after treatment with interferon-α or antisense oligonucleotides and the long term persistence of HCV RNA. A compilation of cell culture systems for HCV is given is Table 1, but only some of them will be described here.

3.1. Infection of primary cell cultures and cell lines

Since infection and productive virus replication depends on host cell factors that are sometimes only expressed in highly differentiated cells, several groups used primary cells from humans or chimpanzees to propagate HCV in cell culture. For instance, Iacovacci et al. (1993, 1997) inoculated primary foetal human hepatocytes with HCV-containing sera. Starting on day 5 post infection, they detected an $\sim$20-fold increase of HCV positive strand RNA by RT-PCR during a 24 days cultivation period, but the overall efficiency of the system was apparently low (at maximum 20 000 HCV RNA copies per 10^6 cells). A similar course of infection was found by Lanford and coworkers after inoculation of primary hepatocytes from chimpanzees (Lanford et al., 1994). The positive strand RNA signal increased significantly from days 1 to 4 and remained at this level during the 25 days of culture, whereas negative strand RNA became first visible on day 4 and strongly increased thereafter. Interestingly, primary liver cells from baboons were not permissive supporting the notion that HCV has a narrow host range. However, the positive strand RNA in the inoculum used to infect the baboon hepatocytes could be detected up to 11 days post infection showing that great care must be taken to unambiguously differentiate between newly synthesized RNA and the input inoculum.

In a detailed study, Rumin et al. (1999) developed conditions that support the culture of primary human hepatocytes for at least 4 months without morphological changes. Upon infection of these cells with HCV, positive strand RNA was first detectable after 10 days and RNA titres in the supernatants gradually increased during 3 months of culture from $\sim$1000 to $\sim$60 000 genome equivalents per ml. However, the efficiency of the system was influenced by several parameters that were difficult to control. For instance, the infectivity of sera used for infection was poorly predictable because it did not correlate with the RNA titre and was influenced by cellular components like HCV-specific antibodies in the sera. Moreover, the infectability of the cultures depended on the quality of the hepatocyte preparation and on the particular donor.

In agreement with the lymphotropism of HCV described above, the infection of peripheral blood mononuclear cells (PBMCs) has also been reported (Table 1). For instance, Cribier et al. (1995) prepared independent cultures of fresh PBMCs from 10 different donors and infected these cells with two different sera containing high titres of HCV. Intermittently positive RT-PCRs were found in seven out of the 10 cultures both within the cells and in supernatants up to 28 days post inoculation. Similar results were obtained when pools of PBMCs were used for the infection studies, but in both cases replication levels were low and the amount of HCV RNA detected at the peak of replication hardly exceeded the amount of RNA that could be bound to the cells.

Owing to the technical and logistic problems that are inherent to the work with primary cell cultures, many attempts have been undertaken to develop an infection system with immortalized cell

lines. Two different cell types have been studied in detail: human hepatoma cell lines such as HepG2, Huh-7 or PH5CH and human B- and T-cell lines, in particular MOLT-4, MT-2, and Daudi (Table 1). With respect to liver cell lines, the most intensive analyses have been performed with the PH5CH line obtained by immortalization of human hepatocytes with the simian virus 40 large T antigen (Noguchi and Hirohashi 1996). Among several human hepatocyte lines analyzed, this cell line was found to be most susceptible to HCV infection (Kato et al., 1996). Subsequently, three clones were established that supported HCV replication more efficiently. Evidence for replication in these cells was based on: (a) an increase of positive strand RNA during the first 12 days of culture; (b) the prolonged detection of this RNA (up to 29 days p.i. when cells were kept at 37 °C and up to 100 days with cells at 32 °C); (c) the reduction of positive strand RNA upon incubation of infected cells with Ifn-α; and (d) a strong selection for HCV variants as determined by sequence analysis of the hypervariable region (Ikeda et al., 1997, 1998).

In an extensive study by Seipp et al. (1997) various cell culture conditions for the human hepatoma lines HepG2 and Huh-7 as well as the porcine cell lines PK15 and STE were tested to enhance infection with HCV. Addition of polyethylene glycol or dimethyl sulfoxide that were shown to increase the efficiency of in vitro infection with the hepatitis B virus (Gripon et al., 1998, 1993) had almost no effect with HCV. However, some increase and prolonged persistence of HCV RNA up to 130 days post inoculation was found in cells that were cultured in an FCS-free medium or in the presence of lovastatin. Both conditions stimulate the expression of low density lipoprotein receptors and it is assumed that this leads to enhanced binding of HCV particles associated with such lipoproteins (Agnello et al., 1999). However, even under the best conditions, HCV replication was low because only $\sim 10^4$ genome equivalents were detected in the culture supernatants of 10^6 cells (Seipp et al., 1997).

The T- and B-cell lines that are best characterized for infection with HCV are MT-2, MOLT-4 and Daudi. During a search for permissive cell lines, Kato and coworkers (1995) found that MT-2, a human T-cell line infected with the human T-lymphotropic virus-1 (HTLV-1), might be susceptible for HCV infection. The system could be improved by the establishment of 5 MT-2 cell clones (MT-2C) that supported a more persistent HCV replication as shown by the detection of HCV-RNA in these cells up to 198 days post infection. Further evidence for HCV replication was shown by: (a) the infection of naive MT-2C cells with cell culture supernatants from previously infected MT-2C; (b) the $\sim$10-fold reduction of positive strand RNA after a 2-day incubation of cells with 100 U/ml Ifn-α; and (c) the loss of viral RNA after a 4-day incubation of cells with 10 μM of an HCV-specific antisense oligonucleotide and the reappearence of HCV RNA 7 days after removal of the inhibitor (Mizutani et al., 1995, 1996a). Moreover, a comparison of the complete genome sequences isolated from infected MT-2C cells and from the inoculum revealed that only a limited virus population replicated in the cultured cells (Sugiyama et al., 1997b). This result indicates a selection for particular variants binding to or replicating more efficiently in the MT-2C cells.

The human T-cell lines MOLT-4 Ma and HPB-Ma, both infected with murine retroviruses were also shown to be susceptible to HCV infection (Shimizu et al., 1992, 1993). Moreover, a clone derived from HPB-Ma supported HCV replication for >1 year (Nakajima et al., 1996). By cocultivation of these cells with naive ones, the virus could be transmitted several times (Shimizu and Yoshikura, 1994). Inhibition of virus replication, but not of adsorption was observed after treatment of the cells with Ifn-α at concentrations between 200 and 2000 U/ml (Shimizu and Yoshikura, 1994). In addition to these cell lines, the human B-cell line Daudi supported HCV replication for >1 year, too (Nakajima, et al., 1996). However, upon virus inoculation these cells developed cytopathic effects presumably due to interferon that was induced by the infection (Yoshikura et al., 1995), and therefore the culture could only be maintained by the addition of fresh cells at each passage. HCV contained in culture supernatant of Daudi cells could be transmitted to

chimpanzees, although the infectivity was very low (Shimizu et al., 1998). Viral RNA became detectable in the serum of the animal 5 weeks after intravenous inoculation with $\sim 10^3$ genome equivalents and vanished after week 25. Interestingly, the major sequence of the hypervariable region in E2 that was found in the serum of the animal corresponded to the major species present in the patient serum used for infection of Daudi cells. However, in PBMCs of the chimpanzee the predominant variant corresponded to the one found in Daudi cells. Since this variant was not present in the inoculum patient serum, these results strongly suggest the selection of a lymphotropic HCV variant during passage in the Daudi cells.

3.2. Cultivation of in vivo infected cells

Given the inherently low efficiency of the infection systems described above, several attempts have been undertaken to culture cells isolated from persistently infected patients. Two different cell types have been used for this approach: primary human hepatocytes and PBMCs. A system that is based on the cultivation of primary hepatocytes prepared from liver biopsies was described by Ito and coworkers (1996). In five independent cultures they detected variable amounts of HCV RNA in cells and supernatants by RT-PCR up to the end of the observation period that in two cases corresponded to 56 days post seeding. Although several additional observations supported the notion of productive HCV replication in this cell culture system (Table 1), its efficiency was low and the poor availability of infected primary human hepatocytes adds further to the limitation of this system. As a more readily available alternative, PBMCs from persistently HCV-infected patients have been used, but replication in these cells is also very low (Müller et al., 1993).

3.3. Transfection of cell lines with cloned HCV genomes

In comparison to the infection of cultured cells with HCV containing samples, the transfection of cells with cloned viral DNA or with in vitro transcripts generated from this template (cRNA) is superior for several reasons. First, the inoculum is homogenous and well defined; second, the genome can be synthesized in large quantities; and third, it can be manipulated at will permitting genetic analyses of a whole variety of different aspects of the HCV life cycle. However, in spite of the availability of cloned functional HCV genomes that are infectious in experimentally inoculated chimpanzees (Beard et al., 1999; Kolykhalov et al., 1997; Yanagi et al., 1997, 1998, 1999b), for unknown reasons this approach turned out to be very difficult. Thus far there are only two reports indicating the replication of HCV after transfection of synthetic RNA into the human hepatoma cell lines HepG2 and Huh-7 (Dash et al., 1997; Yoo et al., 1995). However, in both studies HCV genomes were used that lacked most of the 3′ NTR. This result contradicts the finding that both the poly(U/UC)-tract and the highly conserved X-tail at the very 3′ end of the genome are essential for replication both in vivo (Kolykhalov et al., 2000; Yanagi et al., 1999a) and in cell culture (Friebe et al., unpublished results). Furthermore, in the study by Yoo and coworkers (1995) HCV-RNA could be labeled metabolically with ^{3}[H] uridine, but viral antigens could not be detected using a variety of specific antisera. This result is surprising given the higher sensitivity of antigen detection compared to metabolic radiolabeling of RNA. For both studies, up to now neither a sequence analysis of the replicating genome nor a successful infection of chimpanzees with cell culture grown virus has been presented.

3.4. Transfection of cell lines with subgenomic replicons

The demonstration of HCV replication after transfection of cRNA has some inherent technical problems. First, HCV most likely is not cytolytic and therefore classical virological methods like plaque assays can not be used. Second, high amounts of input RNA are required for transfection. This RNA is very stable and even a non-replicating genome can be detected in cells for prolonged time (Lohmann et al., 1999). Third,

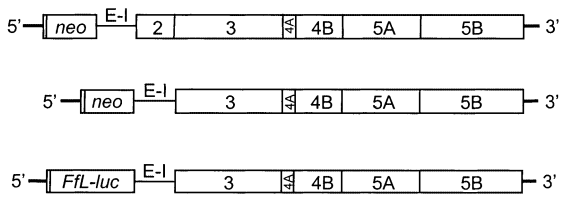

Fig. 2. Structure of the subgenomic selectable HCV replicons composed of the HCV 5′ NTR (thick lane), the gene encoding the neomycin phosphotransferase (*neo*) or the luciferase of the firefly (*FfL-luc*), the EMCV IRES (EI), the region encoding HCV NS2-5B or NS3-5B and the authentic 3′ NTR (thick lane). Note that for highest activity of the HCV IRES, the 12–16 5′-terminal codons of the core ORF were included resulting in the expression of a core-neomycin phosphotransferase fusion protein.

owing to the low level of replication, highly sensitive detection methods are required that are difficult to control for (Lanford et al., 1994; Gunji et al., 1994). Fourth, the only molecule that is exclusively produced during HCV replication is the negative strand RNA intermediate. Its detection is very difficult when using RT-PCR because of the high amounts of transfected input RNA and the possibility of DNA contamination which is due to carry over from the in vitro transcription reaction.

3.4.1. Establishment of the HCV replicon system

To circumvent most of these problems, we have recently developed an alternative cell culture system that is based on the stable replication of subgenomic selectable HCV RNAs (replicons; Lohmann et al., 1999). These molecules were derived from an HCV consensus genome that we cloned from liver tissue of a chronically infected patient (Koch and Bartenschlager, 1999; Lohmann et al., 1999) by introducing the following modifications (Fig. 2): deletion of the structural region from core up to p7 or even NS2, and insertion of the gene encoding the selectable marker neomycin phosphotransferase as well as the IRES of the encephalomyocarditis virus (EMCV). The resulting replicons were bicistronic with translation of the first cistron (neo) being directed by the HCV IRES and translation of the second cistron (NS3-5B) by the EMCV IRES. Upon transfection of the human hepatoma cell line Huh-7 with these replicons and selection with neomycin sulfate (G418), a low number of colonies was obtained. Subsequent analyses of cell lines derived from these clones revealed that they contained autonomously replicating HCV RNAs (Lohmann et al., 1999). Most surprisingly, the amounts of replicon were high enough to allow detection by Northern-blot and even metabolic radiolabeling with ^{3}[H] uridine. In some cell lines we detected ~ 5000 positive strand RNA copies per cell and a 5–10 fold lower amount of the negative strand RNA intermediate. This corresponds to an $\sim 100\,000$-fold higher level of replication compared to the in vitro infection systems (see above). Viral proteins were found exclusively in the cytoplasm in close association with membranes of the endoplasmic reticulum suggesting that this is the site of RNA replication as is the case with many other positive strand RNA viruses.

3.4.2. Properties of replicon-harboring cell lines

During a long-term follow up study of replicon-harboring cell lines, we did not observe signs of cytopathogenicity (Pietschmann et al., 2001). The growth rates of these cells were comparable to those without a replicon and no gross structural abnormalities were visible. When passaged under continuous selection with G418, replicon RNAs could be stably propagated for >2 years. However, when this drug was omitted from the culture medium, replicon levels dropped with the kinetics being determined by the degree of senescence of the cells. When they were passaged under conditions of high confluency, replicon levels dropped by more than one order of magnitude within 1 month. This decline was much slower with cells that were regularly passaged at a much higher dilution and even after 10 months of culture under these conditions, $\sim 10\%$ of the cells contained the replicon. An explanation for this peculiarity was provided by the observation that HCV RNA replication or translation is tightly linked to host cell metabolism (Pietschmann et al., 2001). In actively dividing cells in the exponential growth phase, RNA and protein levels were highest and they dropped significantly when cells reached a confluent state. When such cells were passaged in

the absence of G418, replicon amounts increased in parallel to cell growth but they did not reach the level they had in the cells before confluency. Thus, after several passages under these conditions replicon RNA levels were very much reduced.

3.4.3. Identification of cell culture adapative mutations and improvements of the HCV replicon system

In spite of the high level multiplication of replicons within a selected cell clone, the culture system was somewhat limited by the low numbers of G418-resistant colonies obtained after transfection of the HCV RNA. Subsequent studies showed that the original replicon we generated from the cloned consensus genome replicated only at a low level and had to acquire cell culture-adaptive mutations (Blight et al., 2000; Krieger et al., 2001; Lohmann et al., 2001). These enhanced RNA replication to a level that was sufficient to confer G418 resistance to the transfected cell. Evidence for this conclusion came from an experiment that demonstrated a higher G418 transduction efficiency of replicon RNA that was isolated from a selected cell clone in comparison to the original in vitro transcript (Lohmann et al., 2001). By using a functional screening approach, several adaptive mutations were found in replicons that were cloned from a single cell line. These mutations were scattered throughout the polyprotein coding sequence and increased the G418 transduction efficiency to various extents. The highest increase, which was $\sim$500-fold, was due to a single substitution in the NS5B RdRp at amino acid position 2884 of the polyprotein and this mutation was conserved among all replicon sequences cloned from this particular cell line (Lohmann et al., 2001; Fig. 3).

By determining amino acid substitutions that were conserved between replicons of a given cell line, Blight et al. (2000) observed a clustering of such mutations in the center of NS5A (Fig. 3(A)). From a total of 17 independent cell clones analyzed, nine different NS5A substitutions were found that localized to a region of $\sim$30 amino acids in the center of the molecule. Moreover, a deletion was identified in one replicon that spanned the region in NS5A which was shown to interact with the double stranded RNA-activated, interferon-induced protein kinase PKR (Gale et al., 1997, 1998). When transferred into the original replicon construct, each of these substitutions and the deletion was adaptive and increased the G418 transduction efficiency between 500 and up to $\sim$20 000-fold (Blight et al., 2000). Moreover, this increase in the number of G418-resistant colonies correlated well with the level of replication measured in a transient assay with quantitative RT-PCR.

Based on an analogous approach, we analyzed a total of 26 independent replicon-harboring cell lines for adaptive mutations. We also observed a clustering of such mutations in NS5A and some of them were very similar or even identical to the ones described by Blight and coworkers (Lohmann and Bartenschlager, unpublished results and Fig. 3). In addition, we developed a simple and convenient transient replication assay (Krieger et al., 2001). Instead of using cumbersome quantitative RT-PCR, this assay is based on the measurement of the luciferase activity expressed from the corresponding Photinus pyralis gene that was inserted into the replicon construct instead of the selectable marker neo (Fig. 2). With this approach we confirmed that the mutations in NS5A we and Blight et al. (2000) identified were adaptive and that the level of adaptation varied significantly between the various substitutions (Fig. 3(B)). However, the most efficient replicon that we designated rep5.1 was isolated from HCV RNAs that were passaged several times by electroporation into naive Huh-7 cells (Krieger et al., 2001). Upon amplification of the nearly complete replicon RNA from cells of the fourth passage and functional testing of $\sim$100 different clones, two were identified with a very high G418-transduction efficiency. Sequence analysis of these two RNAs and further functional studies revealed that two mutations in NS3 (E1202G and T1280I) and one mutation in NS5A (S2197P) were responsible for the adaptation. Moreover, the comparison of the replication efficiencies of rep5.1 with replicons harboring these mutations individually clearly demonstrated a synergistic effect when the three substitutions were combined (Fig. 3(B)). Replica-

tion of rep5.1 was high enough to allow detection of viral RNA and proteins by Northern-blot and immunofluorescence analyses in a transient assay, i.e. in cells analyzed 24–96 h post transfection (Krieger et al., 2001). Moreover, these cell culture-adaptive mutations permitted the development of cell lines carrying autonomously replicating and selectable full length HCV genomes (Pietschmann et al., 7th international meeting of hepatitis C virus and related viruses, Brisbane, Australia, 2000).

Currently we can only speculate about the mechanism of cell culture adaptation. It is possible that some HCV proteins are cytotoxic, for

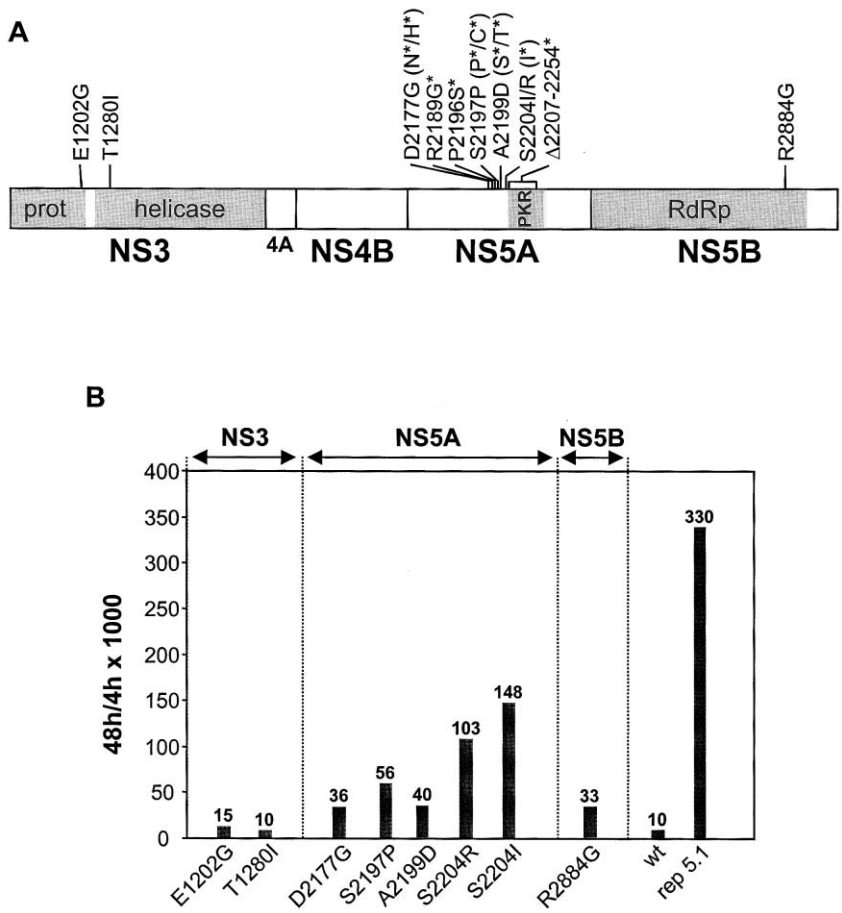

Fig. 3. Cell culture-adaptive mutations and their effect on HCV RNA replication. (A) Location of adaptive mutations within the NS3-5B coding region. The two domains within NS3 carrying the proteinase (prot) and helicase activities as well as the NS5B domain required for RdRp activity are indicated by shading. Mutations described by Blight et al. (2000) are labeled with a star and they are shown together with mutations identified in the authors' lab (Krieger et al., 2001; Lohmann et al., 2001; Lohmann and Bartenschlager, unpublished results). Some of the mutations found by both groups were identical (S2197P, S2204I) whereas in some cases different substitutions were observed at the same positions. (B) Efficiency of transient RNA replication of replicons carrying cell culture-adaptive mutations. The amino acid substitutions indicated at the bottom were introduced into a non-adapted replicon construct harboring the firefly luciferase gene. Forty eight hours post transfection of the corresponding cRNA, cells were lysed and reporter activities were determined. Values correspond to the ratio of the luciferase activity measured 48 and 4 h after transfection multiplied by 1000. The 4 h value was used to correct for different transfection efficiencies because at this time only luciferase translated from the input RNA was measurable (Krieger et al., 2001). The results obtained with the non-adapted and the highly adapted replicons (wt and rep5.1, respectively) are shown in the right.

instance via induction of apoptosis as suggested for the pestivirus bovine viral diarrhea virus or the HCV core protein (Zhang et al., 1996; Hoff and Donis, 1997; Ruggieri et al., 1997). Cell culture-adaptive mutations might lead to a loss of cytotoxicity, thus allowing continued RNA replication. However, in a series of cotransfection experiments with adapted and parental replicons we found no evidence for this assumption (Lohmann et al., 2001). Alternatively, at least in case of the NS3 NTPase/helicase and the NS5B RdRp, cell culture-adaptive mutations may directly modulate the activities of the enzymes, but modeling studies revealed that these substitutions affect residues that are exposed on the surface of the molecules and reside far away from the active sites (Lohmann et al., 2001). This result implies that cell culture-adaptive mutations alter the interaction between viral and cellular factors, e.g. via an increased binding of an activating molecule or the loss of an interaction with a cellular inhibitor. The observed clustering of the mutations in NS5A indicates that this is a region potentially involved in such a (loss of) interaction.

3.4.4. Usefulness of the HCV replicon system for drug development

The availability of cell lines carrying high levels of self-replicating HCV RNAs for the first time enables detailed molecular studies of virus replication in cell culture. The system is also very useful for the development and the evaluation of antiviral drugs for several reasons. First, all known viral enzymes (NS2-3 and NS3/4A proteinases, NS3 NTPase/helicase and NS5B RdRp) that are prime targets for antiviral therapy are encoded in the replicon and they are essential for replication; second, the replicons can be propagated stably in the cells for years; third, the cells can be adapted to various culture formats like 96-well plates, and fourth, by using a highly cell culture adapted replicon, we were able to establish cell lines that carry a selectable subgenomic HCV RNA with an easily measurable reporter gene (Lohmann et al., unpublished).

One example that illustrates the applicability of the replicon system for drug development is given by the dose response curve shown in Fig. 4. When

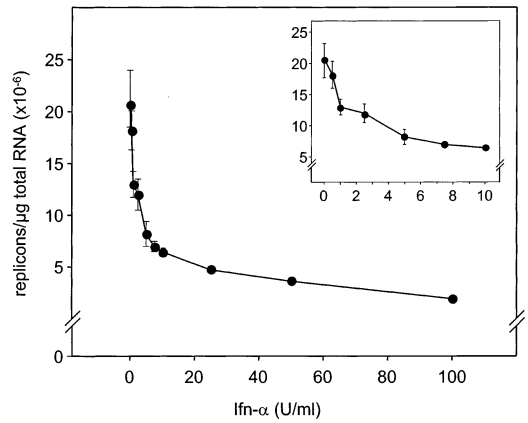

Fig. 4. Inhibition of HCV RNA replication by Ifn-α. A cell line harboring a bicistronic selectable replicon was incubated with increasing concentrations of Ifn-α for 72 h and HCV RNA was determined by quantitative RT-PCR using the TaqMan technique. Values (means of quadruplicates and error ranges) are normalized for total RNA by using a multiplex assay that allows the co-amplification of both the HCV 5′ NTR and a house keeping gene (Kaul and Bartenschlager, unpublished). The insert in the upper right is a detail of the same dose response curve in the range between 0 and 10 U/ml Ifn-α.

cells were incubated with increasing concentrations of Ifn-α, the amount of replicon RNA as measured by quantitative RT-PCR dropped with an IC_{50} of ~ 5 U/ml. A similar inhibition (IC_{50} 1–5 U/ml) was found with several other cell lines harboring a selectable subgenomic RNA and it was independent from the adaptive mutation in NS3, NS5A or NS5B (Blight et al., 2000; Frese et al., 2001). More recently, NS5B RdRp inhibitors effective in the micromolar range have been presented (R. DeFrancesco, 7th International Meeting of Hepatitis C virus and related viruses, Brisbane, Australia, 2000). The compounds that presumably act at the pyrophosphate binding site of the enzyme have been shown to block replication of subgenomic replicons. These results illustrate that cell lines with a subgenomic replicon are a suitable tool for the development of HCV-specific antiviral therapies. However, several properties of the system must be considered when it is used for drug development:

1. Since HCV RNA replication is tightly coupled to host cell proliferation (Pietschmann et al.,

2001), compounds inhibiting host cell growth will also reduce the level of replicon RNA. Therefore, conclusions about the specific inhibition of HCV replication can only be drawn when cytotoxicity or cytostatic effects exerted by a substance have been excluded. However, the availability of simple viability tests (e.g. MTT assay) should make it possible to easily differentiate between these two effects.

2. It can not be excluded that the structural proteins influence the antiviral activity of a compound or contribute to antiviral resistance. For instance, it has been suggested that E2 is involved in the resistance against the antiviral cellular activity induced by Ifn-α (Taylor et al., 1999). Therefore, inhibitory compounds identified by drug screenings that were performed with a subgenomic replicon should be validated with cell lines harbouring a self-replicating full length HCV genome. Although a primary screen with such a cell line is also possible, it might be limited by the higher biosafety level required when working with full length HCV RNAs compared to the subgenomic replicons. Moreover, it seems unlikely that for instance nucleoside analogues identified by screenings with a subgenomic RNA are not active against the NS5B RdRp expressed from a complete genome.

3. Cell culture-adaptive mutations may affect the biochemical properties of a protein altering its sensitivity towards an antiviral drug. However, as alluded to in a previous section, we and others have characterized >20 different cell lines each harbouring a replicon that carries a different cell culture adaptive mutation(s). Therefore, when screening for instance for inhibitors of the NS5B RdRp, cell lines with replicons can be used that carry cell culture adaptive mutations in regions other than NS5B.

4. Summary

In the last few years, a number of cell culture systems have been developed that support HCV replication after infection ex vivo. Although these systems are useful to study some aspects of HCV replication, the level of virus multiplication is not satisfactory because in all cases the detection of viral RNA had to rely on RT-PCR. In this respect, the development of the replicon system can be considered as a breakthrough that may have a great impact both on basic research and for the development of antiviral drugs. The availability of cell lines that support the replication of subgenomic HCV RNAs as well as full length genomes to a level that is easily detectable by Northern-blot permits studies that have not been possible with other systems. Moreover, the identification of cell culture-adaptive mutations allowed the development of systems that permit the analysis of HCV RNA replication in transient assays. In spite of this progress, much more work will be needed to further optimize the cell culture system. For instance, thus far only Huh-7 cells support replication of the HCV RNAs whereas all attempts with other cell lines, including those permissive for HCV (e.g. HepG2 or PH5CH, Table 1) failed. This may be due to the composition of host cell factors that differs between Huh-7 and all the other cell lines. Another enigma is the observation that thus far only the HCV isolate we cloned replicates in cell culture whereas functional genomes with proven infectivity failed (Blight et al., 2000). This is another restriction that might be overcome by the generation of chimeras between our and other HCV isolates in order to map the mutation(s) causing the replication defect. Finally, what we still need is a system that efficiently produces infectious HCV as well as a permissive cell line. Although we can not predict how long it will take until these systems become available, the progress made in the last few years will keep us optimistic that this will happen in the not too distant future.

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