ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

VIROLOGY

Virology 314 (2003) 16–25

www.elsevier.com/locate/yviro

Production and release of infectious hepatitis C virus from human liver cell cultures in the three-dimensional radial-flow bioreactor

Hideki Aizaki,[a] Seishi Nagamori,[b,*] Mami Matsuda,[a] Hayato Kawakami,[c] Osamu Hashimoto,[d] Hiroaki Ishiko,[d] Masaaki Kawada,[e] Tomokazu Matsuura,[f] Satoshi Hasumura,[e] Yoshiharu Matsuura,[g] Tetsuro Suzuki,[a] and Tatsuo Miyamura[a]

[a] *Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan*
[b] *Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611, Japan*
[c] *Department of Anatomy, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611, Japan*
[d] *Mitsubishi Kagaku Bio-clinical Laboratories, Inc., Itabashi-ku, Tokyo 174-0056, Japan*
[e] *Kiba Hospital, Koutou-ku, Tokyo 135-0042, Japan*
[f] *Department of Laboratory Medicine, The Jikei University School of Medicine, Minato-ku, Tokyo 105-8461, Japan*
[g] *Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan*

Received 5 February 2003; returned to author for revision 19 March 2003; accepted 22 April 2003

Abstract

Lack of efficient culture systems for hepatitis C virus (HCV) has been a major obstacle in HCV research. Human liver cells grown in a three-dimensional radial-flow bioreactor were successfully infected following inoculation with plasma from an HCV carrier. Subsequent detection of increased HCV RNA suggested viral replication. Furthermore, transfection of HCV RNA transcribed from full-length cDNA also resulted in the production and release of HCV virions into supernatant. Infectivity was shown by successful secondary passage to a new culture. Introduction of mutations in RNA helicase and polymerase regions of HCV cDNA abolished virus replication, indicating that reverse genetics of this system is possible. The ability to replicate and detect the extracellular release of HCV might provide clues with regard to the persistent nature of HCV infection. It will also accelerate research into the pathogenicity of HCV, as well as the development of prophylactic agents and new therapy.

Keywords: HCV; Three-dimensional radial-flow bioreactor; Replication; Full-length cDNA; Infectious clone; Particles; Reverse genetics; Infection; In vitro culture model; Artificial liver

Introduction

More than 100 million people are infected with hepatitis C virus (HCV) worldwide, and many are prone to developing chronic hepatitis with subsequent cirrhosis and hepatocellular carcinoma (HCC) (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990). Measures to eliminate HCV from carriers before progressive and degenerative liver diseases develop are needed. However, studies aimed at elucidating the mechanism behind persistent HCV infection have been hampered by lack of an efficient in vitro culture system capable of supporting viral replication.

Many attempts have been made to culture HCV in vitro. However, to date, only limited HCV replication was suggested by reverse transcription-polymerase chain reaction (RT-PCR) techniques, and HCV-specific proteins were not generally detected (Ito et al., 2001; Kato and Shimotohno, 1999; Thomson and Liang, 2000). Infectious cDNA clones have been successfully developed for positive-sense RNA viruses (Boyer and Haenni, 1994). Full-length HCV cDNA clones have also been successfully used to infect chimpanzees in in vivo transfection experiments (Kolykhalov et al., 1997, 2000; Yanagi et al., 1997). Furthermore, replication of genome-length dicistronic HCV RNA was shown in a

* Corresponding author. Fax: +81-3-5285-1161.
E-mail address: nagamori@ma.rosenet.ne.jp

0042-6822/03/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0042-6822(03)00383-0

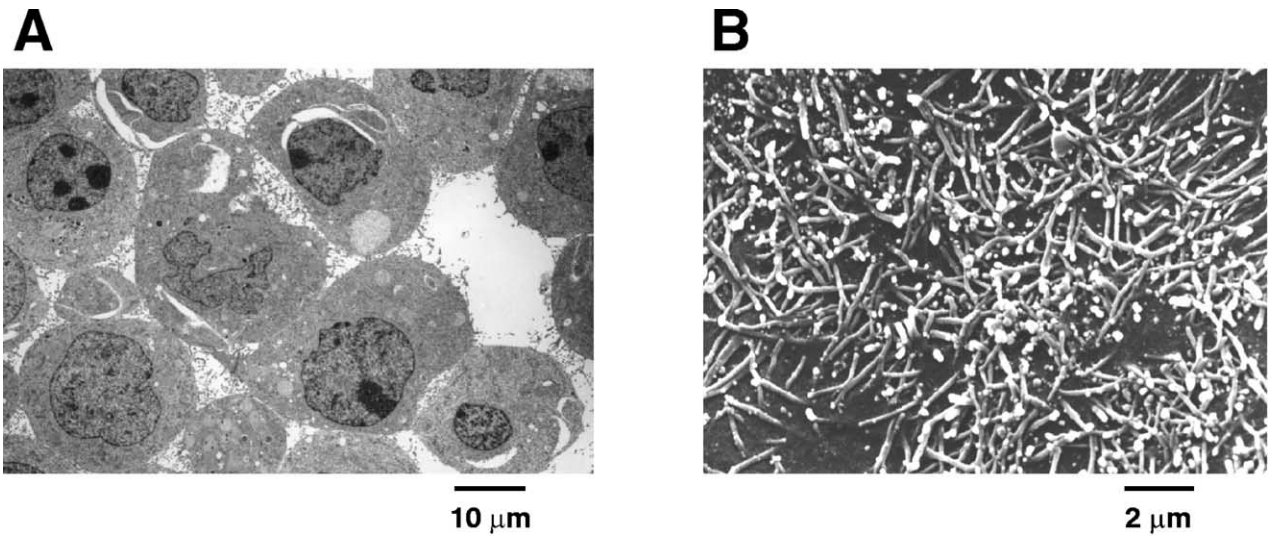

Fig. 1. FLC4 cells in the RFB. (A) Transmission electron micrograph image of FLC4 cells cultured in the RFB. Scale bar shows 10 μm. (B) Scanning electron micrographs showing FLC4 cells attached with the RFB matrix. Scale bar shows (B).

human liver cell line when HCV RNA was dicistronically transfected together with selective markers (Ikeda et al., 2002; Pietschmann et al., 2002). Although replication of the entire HCV genome was also observed, any infectious HCV virions could not be detected (Pietschmann et al., 2002). Thus, viral RNA replication and synthesis of fully processed viral proteins may not be sufficient to produce infectious virus. One or more host factor(s) provided only by permissive cells may be required for the assembly of infectious virions. Alternatively, monolayer cultures of mammalian cells, typically human liver-derived cell lines, may not permit the assembly and release of HCV from cells.

Nagamori et al. established a three-dimensional (3D) radial-flow bioreactor (RFB) system, in which human liver cells retained their differentiated hepatocyte functions and morphological appearance for an extended period of time (Kawada et al., 1998). This system was originally designed to develop artificial liver tissue (Kawada et al., 1998; Matsuura et al., 1998). However, in this study, we utilized the system to examine cells transfected with full-length HCV RNA or inoculated with infectious serum. Although the titer was not particularly high, propagation of HCV in vitro was clearly shown. Furthermore, infectious virus was released into the supernatant in the absence of obvious cell lysis.

Results

FLC4 cell culture in RFB system

We found that cells from a human hepatocellular carcinoma-derived cell line, FLC4 (Functional Liver Cell 4) (Aoki et al., 1998), produced albumin at a rate of 18 $\mu g/10^5$ cells/day, when cultured in the RFB system. In conventional monolayer cultures, FLC4 cells produced albumin at a rate of 2.5 $\mu g/10^5$ cells/day.

α-Fetoprotein production by FLC4 cells also differed among the two culture systems (7 $\mu g/10^5$ cells/day were produced by cells in the RFB system versus an undetectable amount by cells in the monolayer culture). Production of albumin and α-fetoprotein by FLC4 cells in the RFB culture continued for more than 100 days without cell passage, during which time the temperature was reduced from 37 to 32°C, causing a gradual increase in oxygen consumption.

Transmission electron microscopy (TEM) has shown maintenance of tight junctions among RFB-cultured FLC4 cells and normal intercellular spaces (Fig.1A). Scanning electron microscopy (SEM) has shown that, unlike FLC4 cells in monolayer cultures, FLC4 cells cultured in the RFB system retain structurally intact microvilli on their surface (Fig. 1B). RFB culture is therefore thought to provide an environment in which the natural architecture and function of cells are maintained. We tested the replication of HCV under these conditions as a model of HCV replication in vivo.

Infection experiments

First, we inoculated FLC4 cells with infectious human plasma in RFB culture. The infectious plasma (no. 6) was derived from a healthy HCV carrier and its infectivity was eventually proven after posttransfusion hepatitis C was observed in a patient that had received the carrier's blood (Takeuchi et al., 1990; Aizaki et al., 1998). Afterward, the infectivity of the carrier's plasma was tested by inoculation of chimpanzees, and its titer was determined to be $10^{5.5}$ chimpanzee infectious doses per milliliter (Sugitani and Shikata, 1998).

Table 1
HCV infection of FLC4 cells cultured in the RFB following inoculation with the plasma

Days	HCV RNA[a] (log_{10} copies/ml)	ALT (IU/l)
−2	−	7
0[b]	5	2
1	+	NT[c]
2	−	NT
3	−	2
4	−	NT
5	−	2
7	−	NT
9	−	2
11	−	NT
12	−	2
14	−	NT
16	+	2
19	4–5	2
23	4	2
26	+	2

[a] HCV RNA in the culture fluids was determined by qualitative and quantitative RT-PCR. Log of copy numbers identified by the quantitative assay are shown. +, positive by the qualitative assay but below the detection limit (10^3 copies/ml) by the quantitative one. −, negative by both of assays.
[b] On the day 0, plasma no. 6 was inoculated.
[c] NT, not tested.

After cells (1×10^9) were inoculated with 1 ml of the plasma in RFB culture by infusion, HCV RNA was detected by PCR in the culture fluid for 2 days. This was thought to be due to residual virus following the infusion. RNA was not detected in the culture fluid for the next 2 weeks (less than 50 copies/ml). HCV RNA was then detected on day 16 postinfection (p.i.) and reached $10^4 – 10^5$ copies/ml by days 19 and 23 p.i. (Table 1). After this, HCV RNA was detected in the culture fluid for more than 80 days by the qualitative assay (data not shown). Subsequent experimentation using the same cells and inoculum reproduced this profile of HCV RNA detection in the culture fluid (data not shown). In this experiment, the cells were harvested on day 14, and further culture was discontinued. Intracellular HCV core protein levels were as high as 75 pg/mg of total cellular protein in the cells. Although the precise number of cells supporting virus replication could not be determined, this level of core protein expression is almost equivalent to that observed in the liver tissue of chronic hepatitis C patients (Koike et al., 2002).

The hypervariable regions (HVR) of HCV RNA detected on day 23 p.i. were compared to those of plasma no. 6 (Table 2). Plasma no. 6 (donor plasma) was found to contain a mixture of clones with different HVR sequences, but more than half of these contained A1 sequences (Sugitani and Shikata, 1998). After transmission, these mixed clones converged until one clone was primarily detected (clone A1) in infected chimpanzees (Chimp.) (Sugitani and Shikata, 1998), and in the human hepatitis patient, who received no. 6 plasma for transfusion (Recip.) (Aizaki et al., 1998). Interestingly, of the 20 clones tested in this RFB culture study, 19 had A1 clone sequences. These data suggest that the HCV RNA detected was not that of the infused virus but, rather, it appears that the RFB culture selectively supported the replication of certain clones. Clones having A1 sequences may have a replication advantage in RFB culture.

Table 2
Comparison of amino acid sequences of HVR among HCV clones derived from plasma no. 6

Species	Sequence of HVR[a]	Number of clones			
		Donor[b]	Chimp.	Recip.	RFB
	384 410				
A1	H Y R V T R G V Q G H V T S T L T S L F R P G A S Q K	11	20	2	19
A2	S -[c]	1	0	0	0[c]
A3	- L - - -	1	0	0	0
A4	- F - -	0	0	0	1
B1	N - H -	1	0	0	0
B2	N - H - - - A - - - - G A F G - - - - - Q - - - - - -	3	0	0	0
B3	N - H - - - A - - - - G A F - - - - - - T S - - - - -	0	0	1	0
B4	D - H - - - A - - - - G A F - - - - - - T S - - - - -	3	0	0	0
B5	D - H - - - A - - - - G A F Q - - - - - T S - - - - -	0	0	1	0
B6	D - H - - - A - - - - G A F Q - - - - - T S - - - - R	0	0	1	0
B7	D - H - - - A - - - - G A F H - - - - - T S - - - - -	0	0	2	0
B8	D - H - - - A - - C - G A F H - - - - - T S - - - - -	0	0	1	0
C1	N - H - - - - - - - R - A - K - - - F - T - - P - - -	0	0	1	0
D1	- - H - - - - - - - - - - - - - - - F K - S - - - - -	0	0	1	0
		20	20	10	20

[a] Sequences of HVR in HCV clones recovered from the culture fluid of the RFB (RFB), the plasmas of a donor (Donor), a chimpanzee (Chimp.), and a recipient (Recip.) are shown.
[b] The sequences and diversity in Donor, Chimp., and Recip. are from Sugitani and Shikata (1998), and Aizaki et al. (1998).
[c] Dashes represent amino acid residues identical to the clone A-1.

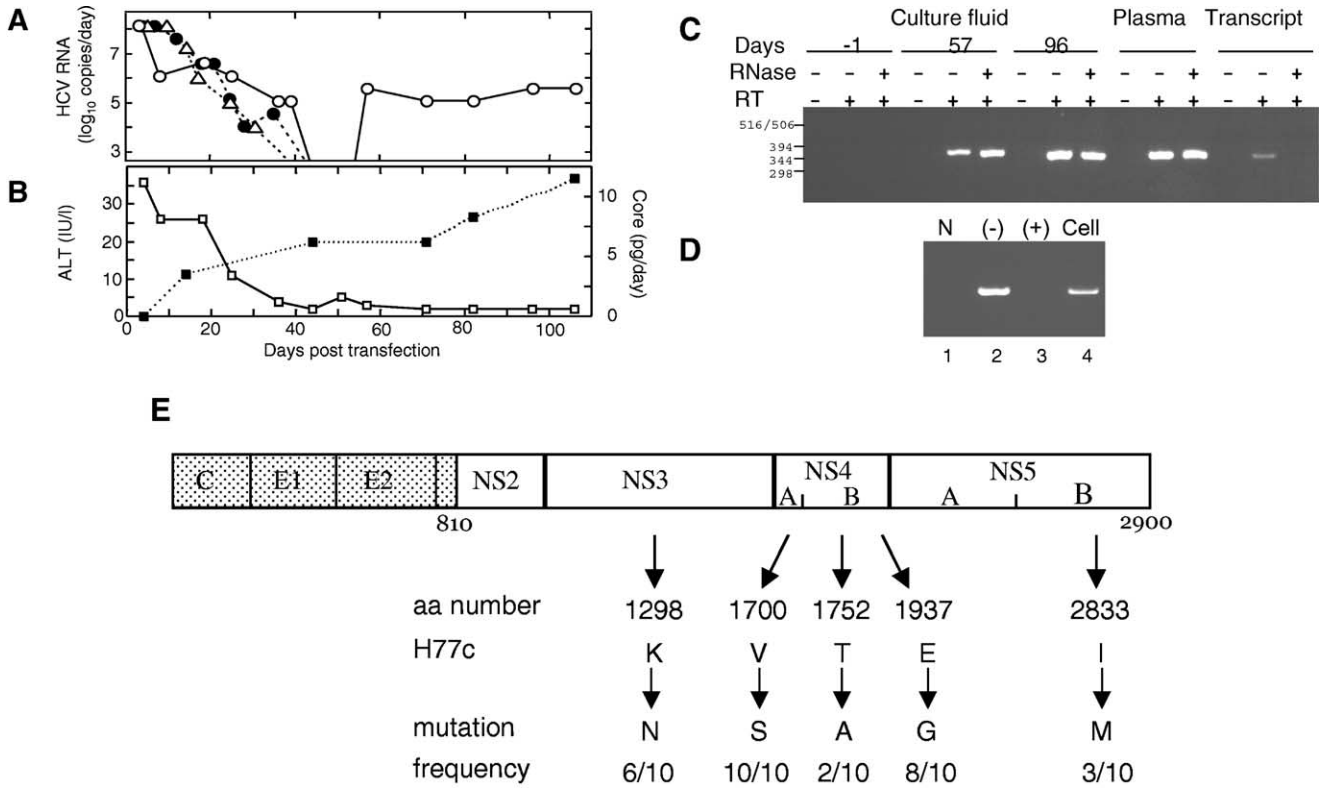

Fig. 2. HCV propagation in FLC4 cells in RFB culture following transfection with the RNA derived from the infectious cDNA clone. (A) HCV RNA titers in the culture fluid after transfection of the infectious HCV RNA H77c (○), helicase inactive mutant RNA H77cHel- (△), and RdRp inactive mutant RNA H77cRdRp- (●). On the day 0, the full-length HCV RNA H77c was transfected. HCV RNAs at days 44, 47, 51, and 54 were positive, but their RNA titers were $<10^3$ copies/ml. (B) ALT levels (□) and the amount of HCV core protein (■) in the culture fluid after transfection of H77c, respectively. (C) Culture fluids of the H77c transfectant were treated with (+) or without (−) RNase A. Samples were then amplified by RT-PCR with (+) or without (−) enzyme in the RT step. The culture fluids collected on day −1, 57, and 96 after inoculation, patient plasma, and the cell-culture medium added with 6×10^4 molecules of HCV RNA transcript were analyzed. (D) Negative-strand-specific RT-PCR, Lane 1, cellular RNA without added synthetic RNA. Lanes 2 and 3, with added synthetic negative (−) and positive (+)-strand RNA, respectively. Lane 4, cellular RNA from the cells harvested on the day 110 p.t. (E) HCV clones recovered from the culture fluids of the RFB. (Top) Schematic presentation of the complete HCV genome. The ORF with the structural proteins (shaded box) located in the amino-terminal portion of polyprotein, and the remainder encodes the nonstructural protein (NS2 to NS5B). (Middle) The positions and frequencies. Overlapping RT-PCR products were sequenced and the mutations observed in nonstructural protein regions are illustrated, along with the frequency with each arose. The amino acid number is indicated above the H77c sequence.

Transfection experiments

The above results suggest that RFB culture supports the replication and release of HCV in culture fluid. To confirm this, we transfected cells in RFB culture with HCV RNA. RNA derived from an infectious full-length HCV cDNA clone (H77c) (Yanagi et al., 1997) was used. This clone was derived from HCV genotype 1a and was proven infectious by direct inoculation into chimpanzee livers.

Cells were transfected with HCV RNA and cultured using the RFB system. HCV RNA was assayed by RT-PCR using samples of culture fluid. As in the infection experiments described above, the amount of viral RNA in the culture fluid declined immediately after transfection. At days 44, 47, 51, and 54 posttransfection (p.t.), HCV RNA was positive by the qualitative assay but below the detection limit (10^3 copies/day) by the quantitative one (Aizaki et al., 1998). However, the sample collected on day 57 p.t. indicated HCV RNA levels as $10^5 – 10^6$ copies/day. This level remained here for 100 days of culture (Fig. 2A, open circles and solid line). In addition, HCV core protein gradually increased in the culture fluid until a maximum level of approximately 13.2 pg/day was reached on day 106 p.t. (Fig. 2B, filled squares and dotted line). The relative amounts of HCV core protein (13.2 pg/day) and HCV RNA ($10^5 – 10^6$ copies/day) detected on day 106 p.t. are consistent with previously reported data on native HCV virions (Kashiwakuma et al., 1996). Since ALT levels did not increase within the culture fluid (Fig. 2B, open squares and solid line), direct hepatocyte injury did not appear to result from HCV replication.

To determine whether the HCV RNA detected was packaged within virions, the culture fluid collected on days 57 and 96 p.t. was treated with RNase and examined by quantitative RT-PCR. As shown in Fig. 2C, the HCV nucleic acid was first confirmed to be RNA. However, the RNA was RNase resistant, suggesting that it is contained within virions. The RNase resistance might also indicate HCV RNA of

the double-stranded replicative form. Nevertheless, such RNA, if any, trapped in membranous structures of the cells may not be conferred resistance to nucleases. In the RFB system, RNase-resistant HCV RNA was not detected after transfection of mutant clones (described later). Furthermore, in cells harvested on day 110 p.t., negative-strand HCV RNA was detected by tagged RT-PCR (Fig. 2D).

To verify that this system supports the replication of transfected HCV RNA, we constructed two mutated full-length HCV RNAs, termed H77cHel− and H77cRdRp−, containing lethal mutations of helicase and polymerase, respectively. After transfection, the residual RNA of these mutant clones gradually declined in the culture fluid, and no RNA was detected by RT-PCR after day 38 and 40 p.t., for H77cHel− and H77cRdRp−, respectively (Fig. 2A, H77cHel−: open triangles; H77cRdRp−: filled circles). Thus the detection of HCV RNA 44 days after transfection of wild-type HCV RNA must have been due to actual viral replication.

To confirm HCV replication and to determine whether mutations arose during the course of the study, we determined the nucleotide sequences of entire nonstructural regions of the clone detected on day 110 p.t. and compared them with the original clone (Fig. 2E). We observed 43 substitution mutations, all of which resulted in amino acid changes. This is not derived from RT errors during RT-PCR amplification because similar sequence changes were seen in the independent RT-PCR reaction. In particular, mutations of V1700S in the NS4A, E1937G in the NS4B, and K1298N in the NS3 regions were frequently observed (in 60% or more of the sequenced clones), which suggests that these mutations might enhance RNA replication within the FLC4-RFB system. We are now analyzing whether or not transcripts with these mutations enhance infectivity or have a replication advantage in RFB culture.

Late-stage culture fluid (80 to 110 days posttransfection) was collected, concentrated, and examined by TEM. A number of spherical particles, between 30 and 80 nm in diameter, were observed, and probably represent two entities of approximately 30 and 60 nm in size (Fig. 3A). The suspension was then fractionated by spinning within a continuous 10–60% (w/w) sucrose gradient. The fractions were then treated with Triton X-100/NaOH/polyethylene glycol and assayed for HCV core protein. The core protein was detected in both 1.07 and 1.20 g/ml fractions, while HCV RNA was predominantly detected in fractions of 1.03–1.09 g/ml (Fig. 3B). Although quantitative assays to detect HCV envelope proteins have not been developed, the lower density fraction observed in this study (1.03–1.09 g/ml) might reflect the presence of HCV particles (1.03–1.12 g/ml), as previously observed in the serum of hepatitis C patients (Kanto et al., 1994; Kaito et al., 1994). The higher density fraction, in contrast, may represent empty particles without RNA. To distinguish between the particles observed in these fractions, an indirect immunogold electron microscopic study was carried out (Figs. 3C and D). Spherical virus-like particles, 55 to 60 nm in diameter, in the 1.07 g/ml fraction reacted with monoclonal antibody to HCV E1 protein. We did not observe any specific reactions of concentrated supernatant from transfected cells with anti-Histidine antibody (Fig. 3E).

The secondary passage

The virus particle-containing supernatant collected at days 93, 94, and 95 p.t. was pooled and transferred to a fresh RFB culture of FLC4 cells. No HCV RNA was detected in supernatant collected at days 1–16 p.i. However, HCV RNA was detected on day 18, 24, and 28 p.i. The HVR sequences of this HCV RNA were mostly (90%) A1 clone sequences. New clones with a single base change were detected after another 14 days. In addition, HCV core protein was detected in the culture fluid on day 24 and 28 p.i. (Table 3). These results further suggest that HCV is produced in RFB culture and that released HCV is infectious.

Discussion

Despite numerous efforts to grow HCV, full replication of HCV has not been achieved in conventional monolayer cultures using any type of cell (Kato and Shimotohno, 1999; Thomson and Liang, 2000). Even when HCV RNA is detected by PCR in monolayer cultures, synthesis of newly synthesized HCV-specific proteins is not observed (Bartenschlager and Lohmann, 2001). A robust and reliable cell-culture system by which to grow HCV is urgently needed (Randall and Rice, 2001).

Recent reports have demonstrated HCV replication in human hepatocytes following transplantation into mice (Ilan et al., 2002; Mercer et al., 2001). This model provided a useful system to evaluate anti-HCV agents. However, researchers have yet to isolate infectious HCV virions.

In this connotation, the establishment of a HCV replication system (engineered HCV minigenomes) has been a real breakthrough in recent HCV research (Lohmann et al., 1999; Blight et al., 2000). This has also made reverse genetic HCV research possible. Furthermore, the ability to passage these cells has revealed that clones with “adaptive” mutations eventually become dominant (Blight et al., 2000; Lohmann et al., 2001; Krieger et al., 2001). However, the consensus sequences responsible for enhancement of HCV RNA replication have not been identified, even after examinations of the same clone (type 1b) and hepatocyte cell line (Huh-7 cells). This system was first developed to replicate only the nonstructural region of HCV genome, but further developed to enable replication of the full-length HCV genome. Although HCV RNA was synthesized, along with all properly processed HCV proteins, infectious virions were not produced (Ikeda et al., 2002; Pietschmann et al., 2002). Host cell factor(s) essential for virus assembly and release might not be provided, even by Huh-7 cells, which

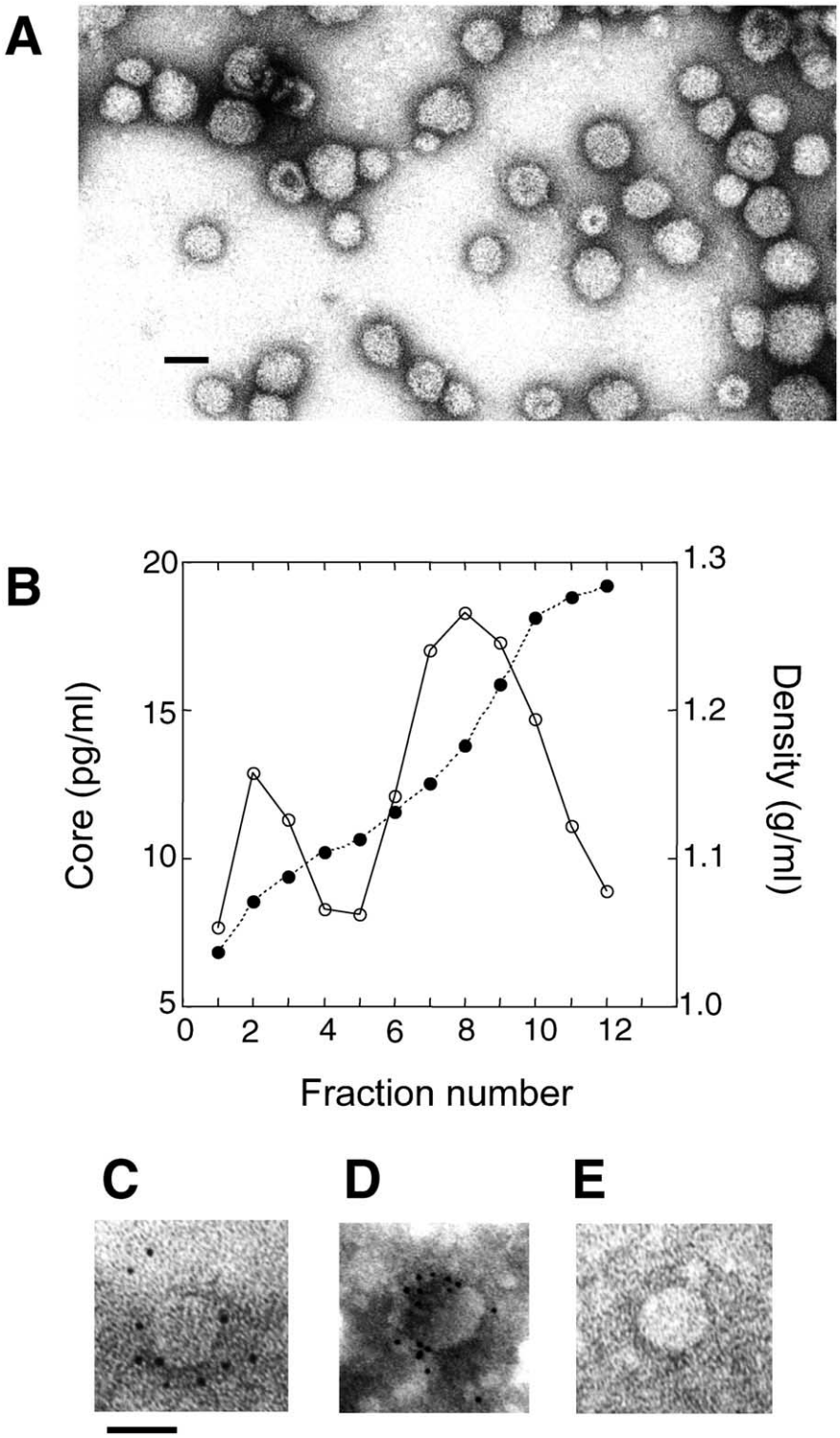

Fig. 3. Analysis of HCV-like particles in culture medium. (A) Culture fluid of the H77c transfectant was concentrated, stained with uranyl acetate, and examined by TEM. (B) HCV core protein in fractions from sucrose density gradient centrifugation. Amounts of HCV core protein (○) and buoyant densities (●) are shown. (C, D, E) Immunoelectron microscopy of the particles purified by sucrose gradient centrifugation. (C, D) Virus-like particles reacted with mouse monoclonal antibody against HCV E1 protein at a dilution of 1:10, and their antibody haloes were identified by binding to goat anti-mouse IgG-conjugated colloidal gold particles (6 nm) at a dilution of 1:20. (E) Control reaction using mouse monoclonal antibody against histidine residues antigen. The scale bars represent 50 nm.

Table 3
Secondary passage of HCV produced from the viral RNA transfection in the RFB culture

Days	HCV RNA[a]	Core[b] (pg/day)
−2	−	0
0[c]	+	5.3
1	+	2.1
3	−	0
5	−	0
7	−	0
9	−	0
11	−	0
13	−	0
16	−	0
18	+	0
20	−	0
22	−	0
24	+	6.9
28	+	14.5

[a] HCV RNA was detected qualitatively by RT-PCR (positive, +; negative, −).
[b] Core antigen was measured by the EIA method.
[c] Supernatant fluid of the RFB culture transfected with HCV RNA was transferred to new FLC4 cells in the RFB on day 0.

are capable of hosting viral RNA replication and HCV protein synthesis.

The results presented in this article clearly indicate that HCV replicates in FLC4 cells in the RFB system. In this study, we repeated the infection, transfection, and secondary passage experiments three times and obtained reproducible results. We also performed a mock-infected RFB culture for 4 months, during which time culture supernatant was harvested every week and examined for HCV RNA and HCV core protein. Negative results were obtained for both (data not shown).

In the culture medium of FLC4 cells in RFB culture, 10^6 – 10^7 copies/day of HCV were detected 2–3 weeks after infection, and 10^5 – 10^6 copies/day were detected 2–4 months after transfection. Although the viral growth of HCV in culture remains inefficient compared to that of other known viruses, this is the first report to unequivocally demonstrate HCV replication in tissue culture, as well as the production of infectious HCV derived from full-length HCV RNA.

The reason for different profiles of detection of HCV RNA and core protein is unclear (Fig. 2B). It is possible that the two assays have different sensitivities. Differences in the half-lives of viral RNA and HCV core protein may be also explain the difference. Nonetheless, the slow but gradual increase in HCV RNA and core protein observed in this study excludes the possibility that the results are due to HCV contamination or detection of the originally infused HCV RNA. These results also suggest that selection of clones capable of viral replication occurs during FLC4/RFB culture. Furthermore, it was shown that the viral quasispecies have changed before and after passages. Efforts to select clones with adaptive mutations enhancing both RNA replication and the production of infectious progeny are ongoing.

The key point of this work is the RFB culture, which allows liver cells to maintain their physiological function for a long time in culture. The RFB is a vertically extended cylindrical matrix with porous bead microcarriers having a honeycomb-like structure. This structure enables contact between cells and circulating components, including oxygen and nutrients, without excessive shear stress. The RFB has an effective air space ratio of 50%; thus, the microcarriers have much of their surface area exposed, which supports the long-term viability of the cells in culture. Cells cultured in the RFB system maintain their polarity within a well-defined 3D structure, having tight intercellular junctions and close connectivity with other epithelial cell membranes. These characteristics are important for the sustained function of liver cells in culture, including secretory and endocytic/transcytotic hepatocyte pathways.

A number of enveloped viruses have been reported to mature at distinct membrane domains within monolayers of polarized epithelial cells. For example, paramyxoviruses and orthomyxoviruses bud from the apical surface, while rhabdoviruses, retroviruses, and baculoviruses bud from a basolateral domain (Boulan and Sabatini, 1978). Similarly, FLC4 cells in the RFB are thought to maintain their polarity in a well-defined 3D structure, which is necessary for the replication and secretion of HCV.

The use of FLC4 cells may contribute to the success of this system. We have previously shown that this cell line supports efficient HCV structural gene expression by a recombinant adenovirus vector (Aoki et al., 1998). The results of this report also suggest that some host factors which increase the efficiency of translation of HCV minigene RNA are present in FLC4, but not other cells, including several commonly used human hepatoma cell lines. Furthermore, using a cell fusion assay, we recently found that FLC4 cells exhibit a high affinity for cell fusion with CHO cells expressing HCV envelope proteins on their surface (Takikawa et al., 2000). However, FLC4 cells in conventional monolayer cultures were not observed to support HCV replication following infection with no. 6 plasma. Although a small amount of virus was detected by PCR on day 19 p.i., less than 1 to 10–100th of the amount of virus per cell obtained with RFB culture was observed (data not shown). HCV RNA was not detected beyond day 19 p.i.

We are now attempting to simplify the method of RFB culture, by culturing fewer cells to better monitor HCV replication and intracellular events. In the immediate future, we will attempt to confirm the infectivity of HCV isolated from RFB culture by inoculation of chimpanzees. In addition, we would like to study the intracellular localization of processed HCV proteins, as well as virus assembly and the effects of specific antibodies and inhibitors. All of these things need to be done. Nevertheless, the evidence of infectivity of HCV RNA shown in the present work illustrates the potential of reverse genetics to delineate the mechanism

of HCV replication at a molecular level. This system will no doubt prove quite useful for studying the mechanism of persistent HCV infection and to evaluate the efficacy of vaccines, as well as various HCV specific inhibitors.

Materials and methods

Inoculum

We used plasma (no. 6) from a donor known to carry infectious HCV. Competitive RT-PCR detected 10^5 genome/ml of HCV RNA in the plasma (Sugitani and Shikata, 1998). pCV-H77C, which contains a full-length cDNA clone of strain H77 of HCV, was kindly provided by Dr. J. Bukh, National Institutes of Health, USA (Yanagi et al., 1997). We also constructed two full-length mutant clones (H77cHel- and H77cRdRp-) of HCV, containing D1316A (4286 to 4289), and G2737A (8551 to 8552), D2738A (8554), and D2739G (8557 to 8559) substitutions, respectively (Kolykhalov et al., 2000).

RFB culture of FLC4 cells

We developed a RFB culture system (Able, Japan) (Kawada et al., 1998; Matsuura et al., 1998) of FLC4 cells, produced from the cloning of JHH4 cells, in an attempt to maintain hepatocyte function in culture for an extended period of time. The RFB system, having an area of 2.7 m^2, was seeded with 1×10^9 FLC4 cells. Culture medium containing 2% FCS was added at a flow rate of 50 ml/day. To monitor the viability of cells, oxygen and glucose consumption, as well as albumin secretion, were continuously monitored throughout the duration of the study. The culture temperature was reduced from 37 to 35°C immediately following inoculation with plasma, and again to 32°C on day 51 p.i. to maintain continual slow growth of cells. As a consequence, oxygen consumption increased steadily, reaching 250 mg/day by day 30 p.i. and 350 mg/day by day 70 p.i. Despite the effects of low temperature on the culture, albumin secretion remained above 75 μg/ml throughout the culture period of 100 days.

Infection and transfection

In the infection experiments, FLC4 cells were seeded in the RFB and cultured in ASF medium (Ajinomoto, Tokyo, Japan), containing 2% fetal calf serum, at a flow rate of 100 ml/day. One milliliter of plasma no. 6 was added, after which HCV RNA and ALT levels were measured in the culture fluid. To maintain an oxygen concentration of less than 1.0 ppm at the RFB outlet during culture, the culture temperature was gradually reduced from 37 to 32°C.

In the transfection experiments, serum-free ASF medium was added to FLC4 cells in RFB culture at a rate of 50 ml/day. pCV-H77C, pCV-H77cHel-, or pCV-H77cHel- was linealized with *Xba*I, and 10 μg of each RNA transcript was mixed with lipofectin (Invitrogen, Carlsbad, CA) and diluted with 10 ml of Opti-MEM (Invitrogen). The mixture was then used to inoculate cells in the RFB.

For secondary passage, supernatant was collected on days 93, 94, and 95 p.t. (pooled to approximately 150 ml) and frozen at −80°C, until which time it was thawed and centrifuged at 8000 *g* for 15 min. After this, the supernatant, containing approximately 1.5×10^6 copies of HCV, was transferred to new FLC4 cells in RFB culture. Fifty milliliters of culture fluid was collected every day and replaced with 50 ml of new, fresh medium.

Analysis and quantitation of HCV RNA

HCV RNA in the medium was qualitatively detected by RT-PCR as described previously (Aizaki et al., 1998). Quantitative determination of HCV RNA was performed according to the competitive PCR method described by Kaneko et al. (1992). The limit of detection for HCV RNA by quantitative PCR was 10^3 copies/ml (Aizaki et al., 1998). RT-PCR was performed independently in two different laboratories (National Institute of Infectious Diseases and Mitsubishi Kagaku Bio-clinical Laboratories, Japan), and the same results were obtained. The nucleotide and amino acid sequences of HVRs of HCV envelope glycoprotein E2 were compared. Treatment with RNase was done as described previously by Kolykhalov et al. (1997). Negative-stranded HCV RNA was detected by strand-specific PCR as described previously by Lanford et al. (1994). Synthetic positive-and negative-strand RNAs encompassing the 5′ untranslated region of HCV were prepared by in vitro transcription using T7 RNA polymerase, followed by extensive purification with DNase to remove DNA. The purified RNAs were mixed with cellular RNA from FLC4 to mimic the conditions of transfected cells and to check for the generation of false positives by PCR. PCR products from one round of amplification were analyzed by agarose gel electrophoresis.

Enzyme immunoassay for HCV core antigen

The culture medium was centrifuged at 8000 *g* for 90 min, after which the supernatant was centrifuged at 100,000 *g* for 3 h. The precipitates were suspended in lysis buffer (Promega, Madison, WI) and HCV core antigen was measured using the highly sensitive enzyme immunoassay (EIA) method (Kashiwakuma et al., 1996).

Electron microscopy study

Cell samples for TEM and SEM were prepared as described previously (Kawada et al., 1998). Briefly, FLC4 cells in RFB culture were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After treatment with 0.1% osmium tetroxide in the same buffer, specimens were de-

hydrated in ethanol, after which they were embedded in a mixture of propyloxide and EPOK812 epoxy resin. Thin sections were examined with a transmission electron microscope (JEOL DEM1200-EX, Nihon Denshi Co., Japan). FLC4 cells attached to microcarriers were fixed to the observation table and dehydrated, after which they were submerged in 100% isoamine acetate. Samples coated with carbon and gold were observed with a scanning electron microscope (JEOL-35CF, Nihon Denshi Co.).

To analyze the virus-like particles, culture fluid of cells transfected with HCV RNA was collected and concentrated by ultracentrifugation. The pellet obtained by centrifugation was suspended in 1.5 ml of ASF medium and one-third of the suspension was reconcentrated. After this, the resultant pellet was suspended in 3 μl of ASF medium and applied to a formvar-carbon grid for negative staining. For IEM study, mouse monoclonal antibody against HCV E1 protein (1:10 dilution) and goat anti-mouse IgG-conjugated colloidal gold particles (6 nm; 1:20 dilution) were used as first and second antibodies, respectively.

Acknowledgments

We thank Drs. J. Bukh and R. Purcell for providing a full-length HCV cDNA. This work was supported by Grants-in-Aid from the Second-Term Comprehensive 10-Year Strategy for Cancer Control, Research on Advanced Medical Technology, Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health and Welfare, the Organization for Pharmaceutical Safety and Research, Japan, and the Promotion and Mutual Aid Corporation for Private Schools of Japan.

References

Aizaki, H., Aoki, Y., Harada, T., Ishii, K., Suzuki, T., Nagamori, S., Toda, G., Matsuura, Y., Miyamura, T., 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. Hepatology 27, 621–627.

Aoki, Y., Aizaki, H., Shimoike, T., Tani, H., Ishii, K., Saito, I., Matsuura, Y., Miyamura, T., 1998. A human liver cell line exhibits efficient translation of HCV RNAs produced by a recombinant adenovirus expressing T7 RNA polymerase. Virology 250, 140–150.

Bartenschlager, R., Lohmann, V., 2001. Novel cell culture systems for the hepatitis C virus. Antiviral Res. 52, 1–17.

Blight, K.J., Kolykhalov, A.A., Rice, C.M., 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290, 1972–1974.

Boulan, E.R., Sabatini, D.D., 1978. Asymmetric budding of viruses in epithelial monolayers: a model system for study of epithelial polarity. Proc. Natl. Acad. Sci. USA 75, 5071–5075.

Boyer, J.C., Haenni, A.L., 1994. Infectious transcripts and cDNA clones of RNA viruses. Virology 198, 415–426.

Choo, Q.-L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., Houghton, M., 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244, 359–362.

Ikeda, M., Yi, M.-K., Li, K., Lemon, S.M., 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J. Virol. 76, 2997–3006.

Ilan, E., Arazi, J., Nussbaum, O., Zauberman, A., Eren, R., Lubin, I., Neville, L., Ben-Moshe, O., Kischitzky, A., Litchi, A., Margalit, I., Gopher, J., Mounir, S., Cai, W., Daudi, N., Eid, A., Jurim, O., Czerniak, A., Galun, E., Dagan, S., 2002. The hepatitis C virus (HCV)-trimera mouse: a model for evaluation of agents against HCV. J. Infect. Dis. 185, 153–161.

Ito, T., Yasui, K., Mukaigawa, J., Katsume, A., Kohara, M., Mitamura, K., 2001. Acquisition of susceptibility to hepatitis C virus replication in HepG2 cells by fusion with primary human hepatocytes: establishment of a quantitative assay for hepatitis C virus infectivity in a cell culture system. Hepatology 34, 566–72.

Kaito, M., Wastanabe, S., Tsukiyama-Kohara, K., Yamaguchi, K., Kobayashi, Y., Konishi, M., Yokoi, M., Ishida, S., Suzuki, S., Kohara, M., 1994. Hepatitis C virus particle detected by immunoelectron microscopic study. J. Gen. Virol. 75, 1755–1760.

Kaneko, S., Murakami, S., Unoura, M., Kobayashi, K., 1992. Quantitation of hepatitis C virus RNA by competitive polymerase chain reaction. J. Med. Virol. 37, 278–282.

Kanto, T., Hayashi, N., Takehara, T., Hagiwara, H., Mita, E., Naito, M., Kasahara, A., Fusamoto, H., Kamada, T., 1994. Buoyant density of hepatitis C virus recovered from infected hosts: two different features in sucrose equilibrium density-gradient centrifugation related to degree of liver inflammation. Hepatology 19, 296–302.

Kashiwakuma, T., Hasegawa, A., Kajita, T., Takata, A., Mori, H., Ohta, Y., Tanaka, E., Kiyosawa, K., Tanaka, T., Tanaka, S., Hattori, N., Kohara, M., 1996. Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA). J. Immunol. Methods 190, 79–89.

Kato, N., Shimotohno, K., 1999. Systems to culture hepatitis C virus, in: Hagedorn, C.H., Rice, C.M. (Eds.), The Hepatitis C Viruses, Springer, Berlin, pp. 261–278.

Kawada, M., Nagamori, S., Aizaki, H., Fukaya, K., Niiya, M., Matsuura, T., Sujino, H., Hasumura, S., Yashida, H., Mizutani, S., Ikenaga, H., 1998. Massive culture of human liver cancer cells in a newly developed radial flow bioreactor system: ultrafine structure of functionally enhanced hepatocarcinoma cell lines. In Vitro Cell. Dev. Biol. Anim. 34, 109–115.

Koike, K., Tsutsumi, T., Fujie, H., Shintani, Y., Kyoji, M., 2002. Molecular mechanism of viral hepatocarcinogenesis. Oncology 62, 29–37.

Kolykhalov, A.A., Agapov, E.V., Blight, K.J., Mihalik, K., Feinstone, S.M., Rice, C.M., 1997. Transmission of hepatitis C by intrahepatic inoculation with transcibed RNA. Science 277, 570–574.

Kolykhalov, A.A., Mihalik, K., Feinstone, S.M., Rice, C.M., 2000. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3′ nontranslated region are essential for virus replication in vivo. J. Virol. 74, 2046–2051.

Krieger, N., Lohmann, V., Bartenschlager, R., 2001. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J. Virol. 75, 4614–4624.

Kuo, G., Choo, Q.-L., Alter, H.J., Gitnick, G.L., Redeker, A.G., Purcell, R.H., Miyamura, T., Dienstag, J.L., Alter, M.J., Stevens, C.E., Tegtmeier, G.E., Bonino, F., Colombo, M., Lee, W.-S., Kuo, C., Berger, K., Shuster, F.R., Overby, L.R., Bradley, D.W., Houghton, M., 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244, 362–364.

Lanford, R.E., Sureau, C., Jacob, J.R., White, R., Fuerst, T.R., 1994. Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. Virology 202, 606–614.

Lohmann, V., Komer, F., Dobierzewska, A., Bartenschlager, R., 2001. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J. Virol. 75, 1437–1449.

Lohmann, V., Komer, F., Koch, J., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110–113.

Matsuura, T., Kawada, M., Hasumura, S., Nagamori, S., Obata, T., Yamaguchi, M., Hataba, Y., Tanaka, H., Shimizu, H., Unemura, Y., Nonaka, K., Iwaki, T., Kojima, S., Aizaki, H., Mizutani, S., Ikenaga, H., 1998. High density culture of immortalized liver endothelial cells in the radial-flow bioreactor in the development of an artificial liver. Int. J. Artif. Organs 21, 229–234.

Mercer, D.F., Schiller, D.E., Elliott, J., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R.T., Tyrrell, D.L.J., Kneteman, N.M., 2001. Hepatitis C virus replication in mice with chimeric human livers. Nat. Med. 7, 927–933.

Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, C., Strand, D., Bartenschlager, R., 2002. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J. Virol. 76, 4008–4021.

Randall, G., Rice, C.M., 2001. Hepatitis C virus cell culture replication systems: their potential use for the development of antiviral therapies. Curr. Opin. Infect. Dis. 14, 743–747.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q.-L., Houghton, M., Kuo, G., 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA 87, 6547–6549.

Sugitani, M., Shikata, T., 1998. Comparison of amino acid sequences in hypervariable region-1 of hepatitis C virus clones between human inocula and the infected chimpanzee sera. Virus Res. 56, 177–182.

Takeuchi, K., Boonmar, S., Kubo, Y., Katayama, T., Harada, H., Ohbayashi, A., Choo, Q.-L., Kuo, G., Houghton, M., Saito, I., Miyamura, T., 1990. Hepatitis C virus cDNA clones isolated from a healthy carrier donor implicated in post-transfusion non-A, non-B hepatitis. Gene 91, 287–291.

Takikawa, S., Ishii, K., Aizaki, H., Suzuki, T., Asakura, H., Matsuura, Y., Miyamura, T., 2000. Cell fusion activity of hepatitis C virus envelope proteins. J. Virol. 74, 5066–5074.

Thomson, M., Liang, T.J., 2000. Molecular biology of hepatitis C virus, in: Liang, T.J., Hoofnagle, J.H. (Eds.), Hepatitis C. Academic Press, San Diego, pp. 1–24.

Yanagi, M., Purcell, R.H., Emerson, S.U., Bukh, J., 1997. Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. Proc. Natl. Acad. Sci. USA 94, 8738–8743.