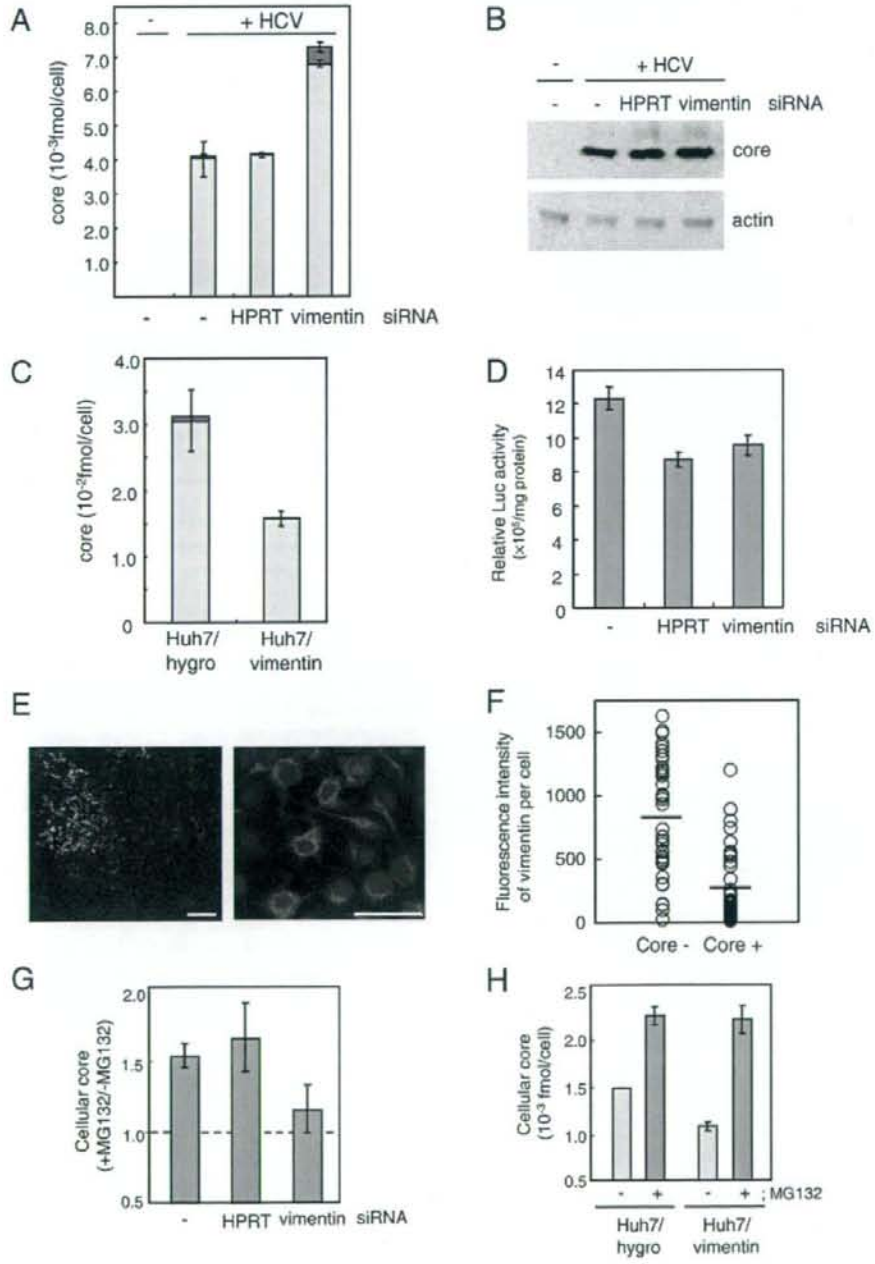
Kang et al. had reported previously (Kang et al., 2005). Co-staining of cellular vimentin and the core protein on immunofluorescence microscopy also supported the existence of a minor but definite association between them (data not shown). Based on these findings, we speculate that vimentin plays a role in the transport of the core protein to the nucleus, where it is then degraded, although further biochemical studies will be needed to demonstrate this.

HCV core protein is distributed mainly in the ER and lipid droplets in host cells (Barba et al., 1997), and the ER membrane associating the lipid droplets with core protein has been recognized as a site important for HCV production, particularly HCV RNA replication and virus particle assembly (Boulant et al., 2007; Miyanari et al., 2007). Vimentin is also closely associated with lipid droplets (Brasaemle et al., 2004; Lieber and Evans, 1996; Schweitzer and Evans, 1998). Thus, in addition to its degradative modulation of core protein, vimentin might also affect the function of lipid droplets and consequently inhibit HCV production. The effects of vimentin knock-down and overexpression on HCV production were actually stronger at the extracellular core protein level (secretion



of the virus) than at the intracellular core protein level (Figs. 5A, C), suggesting additional activity of vimentin in the processes of HCV particle release.

Since the level of expression of vimentin in carcinomas is correlated with parameters of malignant potential such as tumor grade and tumor invasion, vimentin has been used as a marker of malignant tumors (Bannasch et al., 1982). It has indeed been reported that some HCV-infected patients with hepatocellular carcinoma exhibited up-regulation of vimentin expression in tumor tissue (Kim et al., 2003) although further statistical studies are required to clearly demonstrate this. Tanaka et al. noted that in livers of HCV-infected patients with hepatocelllular carcinoma the virus existed predominantly in non-cancerous tissue, at levels 10- to 100-fold higher than in cancerous tissue (Tanaka et al., 2004). These observations in human liver samples suggest that the reduction in HCV levels in hepatic tumor can be explained by the increase of vimentin expression in tumor, consistent with our findings for cultured cells.

In this study we demonstrated that cellular vimentin expression enhanced the proteasomal degradation of core protein and eventually restricted HCV production. Vimentin itself and sites of vimentin/core interaction may thus be novel targets of treatment using anti-HCV strategies.

Materials and methods

Antibodies

Mouse monoclonal antibodies to annexin II, fatty acid synthase, calnexin, lamin A/C, and GFP were purchased from BD Transduction Laboratories. Mouse monoclonal antibodies to HCV core protein, prohibitin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Anogen, Lab Vision, and Abcam, respectively. Rabbit polyclonal antibodies to vimentin, lamin B1, p53, and HPRT were from Santa Cruz Biotechnology Inc., while those to actin were from Biomedical Technologies Inc.

Plasmids

The mammalian expression vector of HCV core protein, pcEF39neo (Ruggieri et al., 1997), and the empty vector pcEF321swxneo (Harada et al., 1995) were described previously. The mammalian expression vector of Flag-tagged HCV core protein, pCAG/Flag-core, and the empty vector, pCAG, were described previously (Moriishi et al., 2003). For construction of a mammalian expression vector of vimentin, pcDNA3.1/Hygro/vimentin, vimentin fragment was amplified by PCR using the reverse-transcribed cDNAs of Huh7 cells as a template. The PCR primer pairs used were 5′-GCCATGTCCACCAGGTCCGTGTCC-3′ and 5′-TTATTATTCAAGGTCATCGTGATG-3′. The PCR products were inserted into the EcoRV site of pBluescript SKII(+). pBluescript SK(+)/vimentin was digested with Hind III and Xba I, and the vimentin fragment was inserted into pcDNA3.1/Hygro (Invitrogen), which had been digested with Hind III and Xba I. For construction of pcDNA3.1/EGFP, EGFP fragment was prepared by digestion of pEGFP-N1 (Clontech Laboratories, Inc.) with Nhe I and Hind III and inserted into pcDNA3.1/Hygro, which had been digested with Nhe I and Hind III. The subgenomic replicon constructs, pSGR-JFH1/Luc (wild type) and pSGR-JFH1/Luc-GND (GND mutation in the NS5B sequence), with the firefly luciferase reporter gene were described previously (Kato et al., 2005).

Cell lines

All hepatic cells used in this study were plated on collagen-coated dishes (Asahi Techno Glass, Japan). Human hepatic Huh7 and Huh7.5.1 cells were grown in normal culture medium [Dulbecco's modified Eagle's medium (DMEM) (KOJIN BIO, Japan) supplemented with 10% fetal bovine serum (FBS), 100 U/ml Penicillin G, and 100 mg/ml streptomycin sulfate] containing 0.1 mM non-essential amino acids (GIBCO) under a 5% CO_2 atmosphere at 37 °C. We used human hepatic cell lines constitutively expressing HCV core protein, including Hep39 from HepG2 cells (Harada et al., 1995; Ruggieri et al., 1997) and Uc39-2 and Uc39-6 from Huh7 cells (Fukasawa et al., 2006; Sato et al., 2006). Huh7 and HepG2 cell lines carrying the empty vector, Hepswx and Uc321, respectively, were used as a mock control. All of these stable transfectants were maintained in normal culture medium containing 1 mg/ml G418 (Sigma). The human adrenal carcinoma cell line SW13, the subtypes 2CB5 and 1HF5 of which do or do not express vimentin, respectively (Sarria et al., 1990), was maintained in normal culture medium. When the pcDNA3.1/EGFP vector was transfected into 2CB5 and 1HF5 cells, the percentage of GFP-positive cells was 56.3% and 53.6%, respectively, 2 days after transfection ($n=3$), indicating that there was no difference in the transfection efficiency between these cells. To establish vimentin-overexpressing cells, pcDNA3.1/Hygro/vimentin was transfected into 1HF5 and Huh7 cells using FuGENE 6 transfection regent (Roche). The vimentin-overexpressing Huh7 and 1HF5 cells were selected under hygromycin for 2 weeks and cloned to obtain Huh7/vimentin cells and 1HF5/vimentin cells, respectively. Huh7 and 1HF5 cells carrying the empty vector pcDNA/Hygro were also established, as Huh7/hygro cells and 1HF5/hygro cells, respectively.

Preparation of DISFs

Confluent monolayers of Uc321 and Uc39 cells in four culture dishes (150 mm inner diameter) were harvested by trypsinization, and 1.5×10^7 cells of each were pelleted by centrifugation (218 ×g for 5 min at 4 °C). After washing with PBS three times, each cell pellet was resuspended in 1 ml of lysis buffer [10 mM HEPES-HCl, pH 7.5, 10 mM NaCl, 140 mM KCl, 0.5 mM DTT, 0.5% Triton X-100 (Pierce Biotechnology), 10 mM NaF, Complete™ EDTA-free (Roche)] (i.e. a 20% cell suspension). The cell suspension was lysed with a ball-bearing homogenizer (Hope et al., 2002). The soluble fraction (designated the detergent-soluble fraction, DSF) containing ~85% of the total cellular proteins was collected by centrifugation of the cell

Fig. 5. HCV production in vimentin-knockdown and vimentin-overexpressing Huh7 cells. (A) Huh7 cells (5×10^4 cells) in 48-well plates were incubated with or without HCV particles (including 8.0 fmol of core protein) for 6 h, and then treated twice with a 3-day interval without (−) or with siRNA duplexes of HPRT or vimentin. After 7-day culture, the amounts of HCV core protein per cell in cells (light gray bar) and culture medium (dark gray bar) were determined. $n=3$. (B) Culture medium was collected at day 6 in the infection experiment described above in (A). The concentration of HCV core protein in these samples of medium was adjusted to 2.7 fmol/ml with fresh medium. Cells were infected with these samples of medium containing 1.4 fmol of HCV core protein for 2 days, and harvested after 7-day incubation. Infectivity was analyzed by the immunoblotting of cell lysates with antibodies to HCV core protein and β-actin. (C) Vimentin-overexpressing Huh7/vimentin and control Huh7/hygro cells infected with HCV were harvested after 7-day incubation. The amounts of HCV core protein per cell in cells (light gray bar) and culture medium (dark gray bar) were determined. (D) Huh7 cells harboring the HCV subgenomic replicon containing a luciferase reporter gene were transfected without (−) or with siRNA duplexes of HPRT or vimentin. After 2.5-day culture, luciferase activity in cell extracts was determined. $n=3$. (E) Immunofluorescence microscopic analysis of HCV-infected Huh7 cells. After infection with HCV, Huh7 cells were cultured for 6 days. HCV core protein (green) and vimentin (red) were then detected with specific antibodies. Nuclei (blue) were stained with DAPI. Two views showing low and high magnifications are displayed. Bars, 100 μm in the left panel; 50 μm in the right panel. (F) Under the HCV-infected conditions in panel E, fluorescence intensity of vimentin in core-positive and core-negative Huh7 cells was determined by line profile analysis. $n=40$. Statistical significance of differences in fluorescence intensity of vimentin between core-positive and core-negative cells was evaluated using Student's t test, showing $p<10^{-8}$. (G) As in (A), Huh7 cells were incubated with HCV particles, and then treated twice with a 2-day interval without (−) or with siRNA duplexes of HPRT or vimentin. After 4.5-day culture, cells were treated with (+) or without (−) MG132 (50 μM) for 16 h. In each culture condition, the ratio of HCV core protein level in the MG132-treated cells to that in MG132-untreated cells was determined. $n=3$. (H) Huh7/vimentin and Huh7/hygro cells infected with HCV were cultured for 4 days and treated with (+) or without (−) MG132 (50 μM) for 16 h. The amounts of cellular core protein per cell were determined. $n=3$.

lysate performed twice at 218 ×g for 5 min at 4 °C. The insoluble pellet was suspended in 2 ml of lysis buffer containing 1.62 M sucrose and then centrifuged at 10,000 ×g for 1 h at 4 °C. The pellet was resuspended in 1 ml of lysis buffer containing 1.0 M sucrose and layered over 2 ml of lysis buffer containing 2.0 M sucrose. After centrifugation at 50,000 ×g for 2 h at 4 °C, the precipitated fraction containing ~15% of total cellular proteins was collected and resuspended in lysis buffer containing 0.25 M sucrose at a concentration of 3 mg protein/ml (designated the detergent-insoluble fraction, DISF). Each fraction was stored at −80 °C until use. The protein concentrations in these preparations were determined with BCA protein assay reagents (Pierce Biotechnology) using BSA as a standard.

2D-PAGE/MALDI-QJT-TOF MS analysis

The DISF (0.15 mg protein) of each cell line was cleaned using a PlusOne™ 2-D Clean Up kit (GE Healthcare) and resuspended in rehydration solution containing 9 M urea, 4% CHAPS, 65 mM dithioerythritol, and 0.5% ampholyte. The first-dimensional IEF was performed with an Immobiline Dry Strip pH 4–7 according to the manufacturer's instruction (GE Healthcare). The second-dimensional electrophoresis was carried out on 12% SDS-polyacrylamide gel, and the gel was stained with SYPRO-Ruby (Bio-Rad). Spot detection and comparison in 2D images were accomplished with PDQuest™ 2-D analysis software ver. 7.3 (Bio-Rad). The protein bands were excised from the gel and subjected to in-gel trypsin digestion. The tryptic peptide mixtures were analyzed by MALDI-QJT-TOF MS (AXIMA-QJT, Shimazu Biotech, Japan) as described previously (Sato et al., 2006; Shevchenko et al., 1996). Mascot software (Matrix Science) was used for protein identification.

Immunoblot analysis

The proteins were separated by electrophoresis in precast NuPAGE 10% or 12% Bis-Tris gels (Invitrogen), and then transferred to a polyvinylidene difluoride membrane. The membranes were blocked overnight at 4 °C or for 60 min at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 5% skim milk. The blots were probed with the first antibodies at 1:1000 dilution for 60 min at room temperature and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad) or HRP-conjugated goat anti-mouse IgG (GE Healthcare) at 1:2000 dilution for 45 min. Detection of immunoreactive proteins was performed using an ECL system (GE Healthcare).

Quantitative real-time PCR analysis

Cellular total RNAs were prepared with an RNeasy kit (Qiagen). The total RNA fraction (1 μg) was processed directly to cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche). Of the total 20 μl cDNA solution, an aliquot of 0.5–2 μl was used for each real-time PCR assay. The PCR primers used for HCV core protein were: forward, 5′-AGGAAGACTTCCGAGCG-3′, and reverse, 5′-GGGTGACAGGAGCCATC-3′. The PCR primers for actin were obtained from the LightCycler™-Primer Set (Roche). Quantitative real-time PCR was carried out in a LightCycler (Roche) using LightCycler-FastStart DNA Master SYBR Green I (Roche).

Transfection of siRNA

Subconfluent cells cultured in a 48-well plate were transfected twice at a 2- or 3-day interval with 30 nM of vimentin-specific, HPRT-specific, or negative control (Invitrogen) siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions. The siRNA target sequences were as follows: vimentin (sense), 5′-ACCTTGAACGCAAAGTGGAATCTTT-3′; HPRT-S1 (sense), 5′-AAGCCAGACUUUGUUGGAUUUGAAA-3′.

Infection of Huh7 cells with HCV

Infectious HCV (JFH1 strain) particles were produced in Huh7.5.1 cells as described previously (Wakita et al., 2005). Culture supernatant containing infectious HCV particles was collected and stored at −80 °C until use. Subconfluent naïve Huh7, Huh7/hygro, or Huh7/vimentin cells in 24-well or 48-well plates were exposed to normal culture medium containing HCV particles (1.4–8 fmol core protein/well, corresponding to moi=0.0175–0.1) for 6 h at 37 °C. Cells were then washed and maintained in 500 μl (24-well) or 250 μl (48-well) of normal culture medium for 6–7 days at 37 °C. To determine HCV production activity, the amounts of HCV core protein in the culture medium and cell lysates were quantified with an enzyme-linked immunosorbent assay (ELISA) (Ortho® HCV antigen ELISA test, Ortho-Clinical Diagnostics, Japan).

Assay for activity of HCV genomic RNA replication

The RNAs (30 μg) transcribed from pSGR-JFH1/Luc and pSGR-JFH1/Luc-GND (Kato et al., 2005) were transfected into Huh7 cells (1.6×10^6 cells) by electroporation. Transfected cells in normal culture medium were immediately seeded into 48-well plates at 9.0×10^4 cells/well. Four hours after transfection, siRNAs were also transfected into these cells. After incubation for 2.5 days, cells were harvested and the luciferase activity in cell lysates was determined with the Luciferase Assay System (Promega). Since the luciferase activities of the JFH1/Luc replicon were ~400-fold higher than those of the JFH1/Luc-GND mutant replicon, background luciferase activity, which is independent of replication activity, was very low in our experimental conditions.

Immunofluorescence microscopy

Cells cultured on glass cover slips (in 24-well plates) were fixed in 1% formaldehyde-PBS for 1 h at 4 °C, permeabilized in PBS containing 0.1% Triton X-100 for 5 min, and washed twice with PBS. The cell monolayers were incubated with rabbit anti-vimentin antibodies (1:100) and mouse anti-HCV core protein antibodies (1:100) for 60 min at room temperature. After washing with PBS, the cells were incubated with Alexa488-conjugated anti-mouse IgG, Alexa594-conjugated anti-rabbit IgG, and DAPI (4′, 6′-diamidino-2-phenylindole) (Invitrogen) for 60 min at 4 °C. Coverslips were washed with PBS and mounted on glass slides. Immunofluorescence was visualized and quantitated with a confocal laser-scanning microscope (Axiovert 100M, Carl Zeiss) equipped with a LSM510 system (Carl Zeiss).

Acknowledgments

Huh-7.5.1 cells and Huh-7 cells were kindly provided by F. V. Chisari (Scripps Research Institute).

This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan, and by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

Aizaki, H., Lee, K.J., Sung, V.M., Ishiko, H., Lai, M.M., 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology 324 (2), 450–461.

Ariumi, Y., Kuroki, M., Abe, K., Dansako, H., Ikeda, M., Wakita, T., Kato, N., 2007. DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication. J. Virol. 81 (24), 13922–13926.

Bannasch, P., Zerban, H., Mayer, D., 1982. The cytoskeleton in tumor cells. Pathol. Res. Pract. 175 (2–3), 196–211.

Barba, G., Harper, F., Harada, T., Kohara, M., Goulinet, S., Matsuura, Y., Eder, G., Schaff, Z., Chapman, M.J., Miyamura, T., Brechot, C., 1997. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc. Natl. Acad. Sci. U. S. A. 94 (4), 1200–1205.

Bartenschlager, R., Lohmann, V., 2000. Replication of hepatitis C virus. J. Gen. Virol. 81 (Pt 7), 1631–1648.
Boulant, S., Targett-Adams, P., McLauchlan, J., 2007. Disrupting the association of hepatitis C virus core protein with lipid droplets correlates with a loss in production of infectious virus. J. Gen. Virol. 88 (Pt 8), 2204–2213.
Brasaemle, D.L., Dolios, G., Shapiro, L., Wang, R., 2004. Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. J. Biol. Chem. 279 (45), 46835–46842.
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 (4902), 359–362.
Fukasawa, M., Tanaka, Y., Sato, S., Ono, Y., Nitahara-Kasahara, Y., Suzuki, T., Miyamura, T., Hanada, K., Nishijima, M., 2006. Enhancement of de novo fatty acid biosynthesis in hepatic cell line Huh7 expressing hepatitis C virus core protein. Biol. Pharm. Bull. 29 (9), 1958–1961.
Harada, T., Kim, D.W., Sagawa, K., Suzuki, T., Takahashi, K., Saito, I., Matsuura, Y., Miyamura, T., 1995. Characterization of an established human hepatoma cell line constitutively expressing non-structural proteins of hepatitis C virus by transfection of viral cDNA. J. Gen. Virol. 76 (Pt 5), 1215–1221.
Hope, R.G., McLauchlan, J., 2000. Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. J. Gen. Virol. 81 (Pt 8), 1913–1925.
Hope, R.G., Murphy, D.J., McLauchlan, J., 2002. The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins. J. Biol. Chem. 277 (6), 4261–4270.
Johnston, J.A., Ward, C.L., Kopito, R.R., 1998. Aggresomes: a cellular response to misfolded proteins. J. Cell Biol. 143 (7), 1883–1898.
Kang, S.M., Shin, M.J., Kim, J.H., Oh, J.W., 2005. Proteomic profiling of cellular proteins interacting with the hepatitis C virus core protein. Proteomics 5 (8), 2227–2237.
Kato, T., Date, T., Miyamoto, M., Sugiyama, M., Tanaka, Y., Orito, E., Ohno, T., Sugihara, K., Hasegawa, I., Fujiwara, K., Ito, K., Ozasa, A., Mizokami, M., Wakita, T., 2005. Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. J. Clin. Microbiol. 43 (11), 5679–5684.
Kim, W., Oe Lim, S., Kim, J.S., Ryu, Y.H., Byeon, J.Y., Kim, H.J., Kim, Y.I., Heo, J.S., Park, Y.M., Jung, G., 2003. Comparison of proteome between hepatitis B virus- and hepatitis C virus-associated hepatocellular carcinoma. Clin. Cancer Res. 9 (15), 5493–5500.
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., et al., 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244 (4902), 362–364.
Lieber, J.G., Evans, R.M., 1996. Disruption of the vimentin intermediate filament system during adipose conversion of 3T3-L1 cells inhibits lipid droplet accumulation. J. Cell. Sci. 109 (Pt 13), 3047–3058.
Matto, M., Rice, C.M., Aroeti, B., Glenn, J.S., 2004. Hepatitis C virus core protein associates with detergent-resistant membranes distinct from classical plasma membrane rafts. J. Virol. 78 (21), 12047–12053.
McLauchlan, J., Lemberg, M.K., Hope, G., Martoglio, B., 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J. 21 (15), 3980–3988.
Miyanari, Y., Atsuzawa, K., Usuda, N., Watashi, K., Hishiki, T., Zayas, M., Bartenschlager, R., Wakita, T., Hijikata, M., Shimotohno, K., 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. 9 (9), 1089–1097.
Moriishi, K., Okabayashi, T., Nakai, K., Moriya, K., Koike, K., Murata, S., Chiba, T., Tanaka, K., Suzuki, R., Suzuki, T., Miyamura, T., Matsuura, Y., 2003. Proteasome activator PA28gamma-dependent nuclear retention and degradation of hepatitis C virus core protein. J. Virol. 77 (19), 10237–10249.
Morimoto, T., Fujita, M., Kawamura, T., Sunagawa, Y., Takaya, T., Wada, H., Shimatsu, A., Kita, T., Hasegawa, K., 2008. Myocardial regulation of p300 and p53 by doxorubicin involves ubiquitin pathways. Circ. J. 72 (9), 1506–1511.
Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Matsuura, Y., Miyamura, T., Koike, K., 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J. Gen. Virol. 78 (Pt 7), 1527–1531.
Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T., Koike, K., 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat. Med. 4 (9), 1065–1067.
Randall, G., Panis, M., Cooper, J.D., Tellinghuisen, T.L., Sukhodolets, K.E., Pfeffer, S., Landthaler, M., Landgraf, P., Kan, S., Lindenbach, B.D., Chien, M., Weir, D.B., Russo, J.J., Ju, J., Brownstein, M.J., Sheridan, R., Sander, C., Zavolan, M., Tuschl, T., Rice, C.M., 2007. Cellular cofactors affecting hepatitis C virus infection and replication. Proc. Natl. Acad. Sci. U. S. A. 104 (31), 12884–12889.
Reed, K.E., Rice, C.M., 2000. Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. Curr. Top. Microbiol. Immunol. 242, 55–84.
Ruggieri, A., Harada, T., Matsuura, Y., Miyamura, T., 1997. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. Virology 229 (1), 68–76.
Sarria, A.J., Nordeen, S.K., Evans, R.M., 1990. Regulated expression of vimentin cDNA in cells in the presence and absence of a preexisting vimentin filament network. J. Cell Biol. 111 (2), 553–565.
Sato, S., Fukasawa, M., Yamakawa, Y., Natsume, T., Suzuki, T., Shoji, I., Aizaki, H., Miyamura, T., Nishijima, M., 2006. Proteomic profiling of lipid droplet proteins in hepatoma cell lines expressing hepatitis C virus core protein. J. Biochem. (Tokyo) 139 (5), 921–930.
Schweitzer, S.C., Evans, R.M., 1998. Vimentin and lipid metabolism. Sub-cell. Biochem. 31, 437–462.
Shevchenko, A., Wilm, M., Vorm, O., Jensen, O.N., Podtelejnikov, A.V., Neubauer, G., Shevchenko, A., Mortensen, P., Mann, M., 1996. A strategy for identifying gel-separated proteins in sequence databases by MS alone. Biochem. Soc. Trans. 24 (3), 893–896.
Shi, S.T., Lee, K.J., Aizaki, H., Hwang, S.B., Lai, M.M., 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J. Virol. 77 (7), 4160–4168.
Shirakura, M., Murakami, K., Ichimura, T., Suzuki, R., Shimoji, T., Fukuda, K., Abe, K., Sato, S., Fukasawa, M., Yamakawa, Y., Nishijima, M., Moriishi, K., Matsuura, Y., Wakita, T., Suzuki, T., Howley, P.M., Miyamura, T., Shoji, I., 2007. E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. J. Virol. 81 (3), 1174–1185.
Suzuki, R., Tamura, K., Li, J., Ishii, K., Matsuura, Y., Miyamura, T., Suzuki, T., 2001. Ubiquitin-mediated degradation of hepatitis C virus core protein is regulated by processing at its carboxyl terminus. Virology 280 (2), 301–309.
Suzuki, T., Ishii, K., Aizaki, H., Wakita, T., 2007. Hepatitis C viral life cycle. Adv. Drug Deliv. Rev. 59 (12), 1200–1212.
Tanaka, T., Inoue, K., Hayashi, Y., Abe, A., Tsukiyama-Kohara, K., Nuriya, H., Aoki, Y., Kawaguchi, R., Kubota, K., Yoshiba, M., Koike, M., Tanaka, S., Kohara, M., 2004. Virological significance of low-level hepatitis B virus infection in patients with hepatitis C virus associated liver disease. J. Med. Virol. 72 (2), 223–229.
Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11 (7), 791–796.

Chapter 23

Isolation of JFH-1 Strain and Development of an HCV Infection System

Takaji Wakita

Abstract

Detailed analysis of hepatitis C virus (HCV) has been hampered by the lack of an appropriate viral culture system and small animal models of infection. My group and others have recently reported the production of infectious virus after full-length HCV RNA transfection into Huh-7 cells. This system depends primarily on isolation of a JFH-1 strain from a patient with fulminant hepatitis. The JFH-1 strain belongs to genotype 2a and has high colony-formation efficiency when tested with a subgenomic replicon system. Here, I describe various protocols for isolation of the JFH-1 strain and construction of the HCV infection system. The HCV infection system contributes to our understanding of HCV virology and may permit development of novel antiviral strategies.

Key words: Fulminant hepatitis, JFH-1, patient sera, hepatocytes, nested RT-PCR, virus particles.

1. Introduction

To date, propagation of HCV in cultured cells has been difficult *(1)* for a number of reason, including low replication capacity of the virus and its tropism for highly differentiated hepatocytes. Inoculation of patient sera or plasma into cultured cells results in only a limited level of HCV replication, as determined by nested RT-PCR. This problem hindered the efforts of a number of HCV researchers, but in 1999, Lohmann et al. *(2)* were the first to report efficient replication of an HCV subgenomic replicon, in which an HCV structural region was replaced with a neomycin-resistance gene. After transfection of replicon RNA into Huh-7 hepatocellular carcinoma cells, followed by several weeks of G418 selection culture, replicons were established, and robust replicon

Hengli Tang (ed.), *Hepatitis C: Methods and Protocols, Second Edition, vol. 510*

DOI 10.1007/978-1-59745-394-3_23 Springerprotocols.com

RNA replication was observed in these cells. Adaptive mutations were found in most replicon genomes that increased virus replication at different levels, and some combinations of these adaptive mutations were observed to increase replication strongly *(3–5)*. Genomic replicons containing a structural region with adaptive mutations in a nonstructural region demonstrated efficient replication in transfected Huh-7 cells *(6–8)*, but viral particles were not produced from these genomic replicons. Furthermore, a full-length viral RNA genome with adaptive mutations synthesized in vitro was not infectious in chimpanzees, unlike the wild-type genome *(9)*. These results suggest that adaptive mutations enhance the replication capacity of the HCV RNA genome in cultured cells at the expense of efficient viral particle formation in cultured cells and in vivo.

The JFH-1 strain was isolated from a 32-year-old male patient *(10)*. He was admitted with acute liver failure and had serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations of 9160 IU/L and 6970 IU/L, respectively. The minimum prothrombin time was 16%. Stage II encephalopathy developed 5 days after admission, after which he was diagnosed with fulminant hepatitis. HCV RNA was detected by reverse transcription polymerase chain reaction (RT-PCR) with sera obtained during the acute phase. Anti-HCV antibody was also tested for but not detected on admission (by second-generation enzyme-linked immunosorbent assay, Ortho Diagnostics, Tokyo, Japan). All viral markers indicating exposure to other hepatitis viruses were negative. After admission, the patient's liver function and clinical condition improved with conservative treatment. Anti-HCV antibody became positive 6 weeks after admission. These findings suggest that his fulminant hepatitis was in fact due to HCV infection. The infectious strain of HCV was analyzed in 12 sets of nested RT-PCR, as well as 5′ RACE and 3′ RACE RT-PCR, which covered the entire HCV genome. All of the PCR products were cloned and sequenced. Five clones of each PCR fragment were sequenced, and the consensus sequence was determined. According to sequence analysis, the JFH-1 strain belongs to genotype 2a, and its sequence deviates slightly from other genotype 2a clones isolated from patients with chronic hepatitis (10).

Subgenomic replicon and full-length constructs were assembled with cloned PCR fragments *(11–13)*. The colony-formation efficiency of the JFH-1 replicon was much greater than that of the Con1 replicon with adaptive mutations. Furthermore, transient transfection of replicon RNA into Huh-7 cells resulted in autonomous RNA replication, as determined by northern-blot analysis *(11, 14)*. Importantly, adaptive mutations were not necessary for efficient JFH-1 replicon replication in Huh-7 cells. In addition, the JFH-1 replicon produced colonies in several

other cell lines, including HepG2, IMY-N9, HeLa, and 293 human cells, as well as mouse NIH3T3 fibroblast cells and mouse AML12, MMHD3, and MMH1-1 hepatocytes *(12, 15, 16)*. On the basis of this analysis, JFH-1 demonstrates markedly greater replication efficiency than other reported HCV clones. So that full advantage could be taken of this characteristic of the JFH-1 clone, full-length JFH-1 was examined with regard to replication in Huh-7 cells after transfection of synthesized RNA *(17)*. Transfected full-length RNA replicated efficiently in Huh-7 cells, and surprisingly, infectious virus particles were secreted into the culture medium. Culture supernatant was harvested from the transfected cells, cleared by centrifugation and filtration, and used to inoculate naïve Huh-7 cells. After inoculation, several infected cells were identified by immunostaining with HCV-specific antibodies. In standard Huh-7 cells, the infection efficiency was less than 0.5%, but this infection was specific because an antibody against CD81, as well as anti-E2 antibody, inhibited infection (17 and unpublished data). Furthermore, the virus particles secreted into the culture supernatant were infectious in chimpanzees *(17)*. These results strongly suggest that the secreted virus particles were authentic HCV.

Although the infection efficiency with original Huh-7 cells maintained in our laboratory was quite low, efficient viral infection was achieved with cured cells, such as Huh-7.5 and Huh-7.5.1 *(18, 19)*. These cell lines were produced by interferon treatment of subgenomic replicons. Huh-7.5 cells have a defective point mutation in RIG-I, resulting in defective intracellular interferon signaling against HCV RNA replication *(20)*. Transfection of the JFH-1 genome or inoculation of Huh-7.5 and Huh-7.5.1 cells with infectious JFH-1 virus thus produces robust replication and HCV virus infection *(18, 21)*. We therefore tested the permissiveness of JFH-1 replication in these cell lines by transient transfection of a subgenomic JFH-1 replicon. We found that JFH-1 RNA replication was not greater in these cell lines than in original Huh-7 cells (Wakita, unpublished data), but we observed a stable increase in the cell-surface expression of CD81 in these cell lines over that in original Huh-7 cells. Of note, Koutsoudakis et al. *(22)* have reported that the level of CD81 cell-surface expression is a key determinant of HCV infection. In fact, we have observed that the Huh-7 cells used in the initial infection assay were in fact a mixture of cell clones with varying levels of CD81 expression and infectivity *(23)*. Interestingly, we have isolated several Huh-7 subclones without cell-surface CD81 expression. Among these subclones, some support highly efficient subgenomic replicon replication. We therefore transfected a CD81 expression vector into these cell clones to produce stable cells with a high level of ectopic CD81 expression. These cells supported a greater degree of infectivity of the JFH-1 virus than

did original Huh-7 cells or the cured cell lines *(23)*. Therefore achievement of a high level of infectivity depends on cell-surface expression of CD81, as well as the replicon replication efficiency of Huh-7 cells. Huh-7 cells were first isolated more than 20 y ago *(24)* and were distributed worldwide. The phenotype of Huh-7 cells, including permissiveness for JFH-1 virus infectivity, may differ in subclones maintained in different laboratories.

Interestingly, CD81 may play an important role after virus binding to the cell surface *(25,26)*. Furthermore, SR-BI and SR-BII are thought to play roles in early infection, but the role of SR-BI/II receptors in HCV infection remains unclear *(27,28)*. Heparan sulfate and heparinase reduce cell-surface HCV binding, and anti-E2 and E1 antibodies also block HCV infection in cultured cells. These results suggest that HCV first binds to the cell surface by means of a heparan sulfate proteoglycan at low affinity, after which it may be transferred to high-affinity receptors, such as CD81, which may facilitate virus internalization *(25,26)* but CD81 may also be involved in initial virus attachment to the cell surface. HCV has also been reported to enter target cells by clathrin-mediated endocytosis, followed by fusion within an acidic endosomal compartment *(29)*. Recently, Evans et al. *(30)* reported that claudin-1, a tight-junction component, is a coreceptor for HCV infection. 293T cells are both CD81 and SR-B1 positive but not permissive for HCV infection. 293T gained permissiveness for HCV infection when claudin-1 was ectopically expressed *(30)*. Further studies will be necessary to elucidate how these molecules are cooperatively involved in the process of virus entry. These observations may also explain the tissue tropism of HCV.

Human hepatocytes (immortalized by HCV core protein [IHH]) were also used to develop a permissive cell line for HCV infection. A similar degree of virus particle production was observed upon transfection of RNA from genotype 1a (H77) and genotype 2a (JFH-1) into IHH, so IHH may support HCV genome replication and virus assembly *(31)*. These results suggest that a number of host factors are involved in the virus-host interaction and thus determine the permissiveness of a host cell for HCV infection. Furthermore, a regulatory link may exist between innate antiviral and inflammatory cellular responses to viral infection. HCV infection triggers dsRNA signaling pathways that induce CXCL-8 (IL-8) through transcriptional activation and mRNA stabilization *(32)*. Proinflammatory cytokines induce indoleamine-2,3-dioxygenase (IDO), which is an important mediator of peripheral immune tolerance. Huh-7 cells supporting HCV replication express higher levels of IDO mRNA than do noninfected cells when stimulated with IFN-gamma or when cocultured with activated T cells *(33)*. Proinflammatory cytokines induced by HCV infection may therefore play an

important role in the pathogenesis of HCV infection and escape from host immune responses.

Development of an efficient therapy that eliminates infected HCV from chronic carriers is important. To date, interferon and ribavirin have been used in clinics, with limited efficacy. NS3 protease inhibitors, as well as NS5B polymerase inhibitors and other drugs, are undergoing clinical trials but have not yet been approved for therapeutic use [reviewed in **ref.** *(34)*]. The HCV infection system described here, using a JFH-1 clone, may provide a good method for screening new antiviral agents. Furthermore, stable JFH-1 cDNA-transfected cell lines capable of producing infectious virus may be suitable for screening antiviral agents *(35, 36)*. Further understanding of the HCV life cycle remains important because each step provides a potential target for control of HCV infection and replication. HCV-infected cell systems enable us to characterize the subcellular localization of HCV structural proteins in the context of an infectious cycle. Interestingly, Rouillé et al. *(37)* have reported colocalization of core and NS3 proteins in infected cells, which may suggest that interaction of structural and nonstructural proteins is important for infectious virus formation. On the other hand, Shirakura et al. *(38)* have identified ubiquitin ligase E6AP as an HCV core–binding protein. E6AP has been observed to bind to the core protein and promote its degradation. Exogenous expression of E6AP has also been found to decrease intracellular core protein levels, as well as supernatant HCV infectivity titers, in HCV JFH-1–infected Huh-7 cells. Furthermore, knockdown of endogenous E6AP by RNA interference has been observed to increase intracellular core protein levels and supernatant HCV infectivity titers in HCV JFH-1–infected cells. These studies suggest several novel targets for control of HCV infection.

A great deal of research has also been focused on the development of an HCV vaccine [reviewed in **ref.** *(39)*]. Efforts to produce an HCV vaccine have been met with skepticism because the presence of neutralizing antibody after recovery from HCV infection is difficult to demonstrate, but the JFH-1 infection system, as well as a pseudotype retrovirus carrying HCV envelope proteins, have been used to demonstrate that most chronic HCV carriers develop neutralizing antibodies in coexistence with virus particles within their circulation *(17, 40)*. Determining how HCV evades host immune surveillance remains important, as does determining how protective immunity against HCV infection develops; both will contribute to development of efficient vaccines and immunotherapies.

The development of an HCV infection system using the JFH-1 strain will aid our virological understanding of this important virus. A genotype 1a strain, H77S containing 5 adaptive mutations, has been reported to produce infectious virus after

synthesized RNA transfection into Huh-7 cells, albeit with limited efficiency *(41)*. Clearly, therefore, JFH-1 is not the only HCV strain that can be propagated in cultured cells. Further study will be necessary to develop other genotypic infectious HCV in cell culture. Understanding why JFH-1 is the only strain that replicates efficiently without adaptive mutations in cultured cells will also be important. Further mechanistic analysis of evaluation of the mechanics of JFH-1 genomic replication will help further these goals.

2. Materials

2.1. Cell Culture

1. Huh-7 cells (24), which can be purchased from Cell Bank, RIKEN BioResource Center (Cat. no. RCB1366).
2. Dulbecco's modified Eagle's medium (DMEM) (high glucose; Sigma-Aldrich Japan K.K., Tokyo, Japan) supplemented with 10% fetal bovine serum (an appropriate lot for Huh-7 cells and derivatives), 0.1 mM MEM nonessential amino acids solution (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 10 mM HEPES, pH 7.4, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin.

2.2. RNA Extraction and cDNA Synthesis

1. TRIzol and TRIzol LS (Invitrogen).
2. Nuclease-free water (Ambion, Austin, TX).
3. Random hexamer (TAKARA Bio, Kyoto, Japan).
4. RNase inhibitor (TAKARA Bio).
5. Moloney murine leukemia virus reverse transcriptase (Superscript II, Invitrogen).

2.3. RT-PCR for Isolation of HCV cDNA

1. TaKaRa LA Taq (TAKARA Bio).
2. Primers (**Table 23.1**).

2.4. 5′ RACE RT-PCR

1. 5′ RACE System, Version 2.0 (Invitrogen, cat no. 18374-058).
2. TaKaRa Ex Taq (TAKARA Bio).
3. Primers (**Table 23.1** and included in the 5′ RACE System).

2.5. 3′ RACE RT-PCR

1. Poly(A) Polymerase (TaKaRa).
2. 5× Poly(A) Polymerase buffer (200 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$, 12.5 mM $MnCl_2$, 1.25 M NaCl, 2.5 mg/mL bovine serum albumin).
3. Nuclease-free water
4. RNase inhibitor.
5. TRIzol.
6. Superscript II.

Table 23.1
Primers used in cloning the JFH-1 strain

Name		Sequence (5′ > 3′)
1st PCR	44S	5′-CTG TGA GGA ACT ACT GTC TT-3′
	1323R	5′-GGC GAC CAG TTC ATC ATC AT-3′
2nd PCR	44S	
	486R	5′-GTC GTG CGC ACA CCC AAC CT-3′
2nd PCR	317S	5′-GGG AGG TCT CGT AGA CCG TG-3′
	849R	5′-GGT AGG TTC CCT GTT GCA TA-3′
2nd PCR	617S	5′-TGG GCA GGA TGG CTC CTG TC-3′
	1323R	
1st PCR	1050S	5′-GGT GTT GGG TGC CAG TCT C-3′
	2445R	5′-TCC ACG ATG TTC TGG TGA AG-3′
2nd PCR	1141S	5′-TGT CCG CCA CCT TCT GCT-3′
	2367R	5′-CAT TCC GTG GTA GAG TGC A-3′
1st PCR	2099S	5′-ACG GAC TGT TTT AGG AAG CA-3′
	3568R	5′-TGT TCC GAG GAA GGA CTG AG-3′
2nd PCR	2285S	5′-AAC TTC ACT CGT GGG GAT CG-3′
	3509R	5′-TCC TGT CAC GCC CCG TCA-3′
1st PCR	3425S	5′-CTT CTC GCC CCC ATC ACT G-3′
	4706R	5′-TTG CAG TCG ATC ACG GAG TC-3′
2nd PCR	3471S	5′-TGG GCG CCA TAG TGG TGA G-3′
	4665R	5′-TCG GTG GCG ACG ACC AC-3′
1st PCR	4547S	5′-AAG TGT GAC GAG CTC GCG G-3′
	5970R	5′-TTC TCG CCA GAC ATG ATC TT-3′
2nd PCR	4547S	5′-AAG TGT GAC GAG CTC GCG G-3′
	5970R	5′-TTC TCG CCA GAC ATG ATC TT-3′
1st PCR	5714S	5′-GCT TCC ATG ATG GCA TTC AG-3′
	7220R	5′-TGT AAT CAG GCC GTG CCC A-3′
2nd PCR	5883S	5′-TGG GTA AGG TGC TGG TGG A-3′
	7003R	5′-GTG GTG CAG GTG GCT CGC A-3′
1st PCR	6537S	5′-TCA ATT GTT ACA CGG AGG GC-3′
	8091R	5′-TTT TTG GCC ATG ATG GTT GTA-3′
2nd PCR	6950S	5′-GAG CTC CTC AGT GAG CCA G-3′

(Continued)

Table 23.1 (Continued)

	8035R	5′-CCA CAC GGA CTT GAT GTG GT-3′
1st PCR	7848S	5′-ACG CCC ATT ATG ACT CAG TC-3′
	8892R	5′-AGC CAT GAA TTG ATA GGG GA-3′
2nd PCR	7952S	5′-TCT GCA AGA TCC AAG TAT GG-3′
	8892R	5′-AGC CAT GAA TTG ATA GGG GA-3′
1st PCR	8337S	5′-TTT CGT ATG ATA CCC GAT GCT T-3′
	9330R	5′-GCG CCG ACG GTG AAC CAA CT-3′
2nd PCR	8680S	5′-CTT CAC GGA GGC CAT GAC CA-3′
	9283R	5′-CAA TGG AGT GAG TTT GAG CTT-3′
1st PCR	9095S	5′-TAC TCT CAC CAC GAA CTG AC-3′
	3X-75R	5′-TAC GGC ACT CTC TGC AGT CA-3′
2nd PCR	9231S	5′-GCC GAT ATC TCT TCA ATT GG-3′
	3X-54R	5′-GCG GCT CAC GGA CCT TTC AC-3′
5′RACE		
cDNA synthesis	444R	5′-TAT ACT CCG CCA ACG ATC TG-3′
1st PCR	408R	5′-TTA ACG TCT TCT GGG CGA CG-3′
2nd PCR	258R	5′-ACT CGG CTA GCA GTC TTG CG-3′
3′RACE		
	3X-10S	5′-ATC TTA GCC CTA GTC ACG GC-3′
	CACT35	5′-CAC TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT-3′

7. Primers (**Table 23.1**).
8. Ribonuclease H (TaKaRa).
9. AmpliTaq Gold DNA polymerase (Applied Biosystems Japan, Tokyo, Japan).

2.6. Cloning of PCR Products

1. QIAquick Gel Extraction Kit (QIAGEN K.K., Tokyo, Japan).
2. pGEM-T EASY vector (Promega Corp., Madison, WI).
3. Big Dye Terminator Mix and an automated DNA sequencer (Applied Biosystems Japan).

2.7. HCV Clone and Plasmid Construction

1. pGEM-T easy vector.
2. Restriction enzymes.

2.8. Plasmid DNA Preparation

1. DH5α competent cells.
2. Luria-Bertani medium.
3. Solution 1: 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose; solution 2: 0.2 N NaOH, 1% sodium dodecyl sulfate (SDS); solution 3: 3 M KOAc, 11.5% glacial acetic acid, for plasmid DNA preparation
4. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0.
5. 10 mg/mL ethidium bromide solution (Nippon Gene, Tokyo, Japan).
6. Cesium chloride (Iwai Chemicals, Tokyo, Japan, molecular biology grade).
7. Opti-Seal polyallomer centrifuge tube (Beckmann, Palo Alto, CA).
8. TLN100 rotor (Beckmann).
9. Optima TLX Ultracentrifuge (Beckmann).
10. Isopropanol saturated with NaCl.

2.9. RNA Synthesis

1. *Xba*I
2. Mung bean nuclease (New England Biolabs, Beverly, MA).
3. MEGAscript™ T7 kit (Ambion, Austin, TX).

2.10. RNA Transfection

1. Opti-MEM I™ reduced-serum medium (Invitrogen).
2. Cytomix buffer *(42)*.
3. Electroporation cuvette (Precision Universal Cuvettes, Thermo Hybrid, Middlesex, UK).
4. Gene Pulser II™ apparatus (Bio-Rad, Hercules, CA).

2.11. Northern Blot Analysis

1. To prepare DEPC-treated water, add 0.1% of diethylpyrocarbonate (DEPC) to distilled water and shake well. Incubate the solution at 37°C for 2 h, and then autoclave it before use.
2. TRIzol.
3. 37% formaldehyde.
4. Sample buffer: 0.4× 3-(*N*-morpholino) propanesulfonic acid (MOPS), 6.7% formaldehyde (*see* **Note 1**), 50% formamide.
5. 0.4× MOPS, prepared from 10× MOPS buffer stock: 0.2 M MOPS, pH 7.0, 50 mM sodium acetate, 10 mM EDTA, pH 8.0.
6. Gel-loading buffer: 1% SDS, 50% glycerol, 0.05% bromophenol blue).
7. To prepare 100 mL of 1% denaturing agarose gel, melt 1 g of SeaKem GTG Agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME) completely in 50 mL of DEPC-treated water, then add 10 mL of prewarmed 10× MOPS buffer and 18 mL of prewarmed 37% formaldehyde in a fume hood.
8. 20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
9. Hybond-N+(GE Healthcare, Piscataway, NJ).
10. Stratalinker™ UV crosslinker (Stratagene, La Jolla, CA).

11. Megaprime™ DNA labeling system (GE Healthcare).
12. S-300HR (GE Healthcare).
13. Rapid-Hyb™ buffer (GE Healthcare).

2.12. Quantification of HCV Core Protein

1. HCV core protein immunoassay (Ortho-Clinical Diagnostic K. K., Tokyo, Japan).
2. Lysis buffer: 10 mM Tris-HCl, pH 7.4, 0.5% NP40, 0.15 M NaCl, 1 mM EDTA, pH 8.0, 0.1% SDS.

2.13. Quantification of HCV RNA by Real-Time RT-PCR

1. TaqMan EZ RT-PCR Core Reagents kit (Applied Biosystems Japan).
2. 7500 Real-Time PCR System (Applied Biosystems Japan).
3. MEGAscript™ T7 kit (Ambion, Austin, TX).
4. RNA standard dilution buffer (10 mM DTT, 2000 U/mL RNase inhibitor, 0.2 mg/mL transfer RNA).

2.14. Infection of Cells with Secreted HCV and Determination of Infectivity

1. A disk filter with a 0.45 μm pore size (Millipore, Bedford, MA).
2. Amicon Ultra-15 (100,000 MWCO; Millipore).
3. poly-D-lysine-coated 96-well plates (Corning, New York, NY).
4. Blocking buffer: PBS(-) containing 1% bovine serum albumin and 2.5 mM EDTA.
5. Anticore antibody (e.g., C7-50) solution.
6. AlexaFluor 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR).

3. Methods

3.1. Patients

A 32-year-old man was admitted with acute liver failure to Jikei University Hospital (Daisan). He was diagnosed with fulminant hepatitis, and HCV RNA was detected by RT-PCR in sera during the acute phase of his illness. Serum HCV RNA was quantified with an Amplicor Monitor HCV test (Roche Diagnostic Systems, NJ). The titer was 10^5 copies/mL at admission. Anti-HCV antibody was negative at admission. All viral markers of the other hepatitis viruses, anti-HAV antibodies (IgG and IgM), and HBV markers (HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc, and HBV DNA), as well as GB virus-C/hepatitis G virus RNA, were negative. Analysis of antibodies to Epstein Barr virus and cytomegalovirus revealed a past history of infection.

3.2. RNA Extraction and cDNA Synthesis

1. Extract total RNA from 250 μL of patient serum using the acid-guanidinium-isothiocyanate-phenol-chloroform method (TRIzol LS), in accordance with the manufacturer's instructions (*see* **Note 2**).

2. Resuspend the RNA pellet in 20 μL of nuclease-free water.
3. Transfer 10 μL of the RNA sample to a new tube, and add 8 μL of 5× 1st strand buffer: 10 μL of 2 mM dNTP mixture, 2 μL of 0.1 M DTT, 2 μL of 25 μm random hexamer, 5 μL of nuclease-free water.
4. Mix it well and incubate it at 90°C for 3 min, then transfer the tube onto ice.
5. Spin it down briefly, and add 1 μL of RNase inhibitor and 2 μL of Superscript II reverse transcriptase.
6. Spin it down briefly and incubate at 42°C for 1 h, and then at 70°C for 15 min to terminate the reaction.
7. The cDNA sample can be stored at −70°C until use.

3.3. RT-PCR for Isolation of HCV cDNA

1. Prepare primer sets for the 1st and 2nd PCR to amplify the entire HCV genome as shown in **Table 23.1**.
2. Transfer 1 μL of the cDNA to a PCR tube and add 5 μL of 10× LA Taq buffer, 50 pmol of appropriate sense and antisense primers, 4 μL of 2.5 mM dNTP mix, 2.5 U of TaKaRa LA Taq, and distilled water to bring total volume to 50 μL.
3. Conduct 40 cycles of PCR, each of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min.
4. Amplified products can be separated by agarose gel electrophoresis

3.4. 5′ RACE RT-PCR

For determination of the terminal 5′ end sequence, cDNA can be synthesized with a 5′UTR primer (antisense), tailed with terminal deoxynucleotidyl transferase and a dCTP homopolymer, and then amplified by PCR (5′ RACE System for Rapid Amplification of cDNA Ends Version 2.0)

1. Transfer 5 μL of the RNA sample to a new tube, add 2.5 μL of 1 μm antisense primer (444R, **Table 23.1**), and mix.
2. Incubate the mixture at 70°C for 10 min and then transfer onto ice.
3. Add 2.5 μL of 10× Ex Taq buffer, 2.5 μL of 25 mM $MgCl_2$, 1 μL of 10 mM dNTP mix, 1.2 μL of 0.1 M DTT, 9.3 μL of nuclease-free water, and 1 μL of Superscript II.
4. Mix gently and incubate the mixture at 42°C for 1 h, then at 70°C for 15 min.
5. Spin it down briefly and maintain it at 37°C.
6. Add 1 μL of RNase Mix and incubate at 37°C for 30 min.
7. Purify cDNA using a RACE DNA Purification Spin Cartridge according to the manufacturer's instructions.
8. Transfer 10 μL of purified cDNA into a new tube and add 6.5 μL of nuclease-free water, 5 μL of 5× tailing buffer, and 2 mM dCTP.
9. Incubate the mixture at 94°C for 3 min, and then transfer onto ice.

10. Add 1 μL of recombinant Terminal deoxynucleotidyl Transferase (TdT), and mix gently.
11. Incubate the mixture at 37°C for 10 min, and then at 65°C for 10 min.
12. Perform the 1st PCR of dC-tailed cDNA using the Abridged Anchor Primer and 408 R primer (**Table 23.1**). Prepare the PCR mixture as follows:

dC-tailed cDNA		5 μL
primer	10 pM	2 μL
Abridged Anchor Primer		2 μL
dNTP mix	10 mM	1 μL
10× Ex Taq buffer		5 μL
$MgCl_2$	25 mM	3 μL
Distilled water		31.5 μL
TaKaRa Ex Taq	5 U/μL	0.5 μL

13. Conduct 35 cycles of PCR, each of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min.
14. Prepare the 2nd PCR mixture as follows:

1st PCR product		1 μL
primer	10 pM	2 μL
Abridged Universal Anchor Primer		1 μL
dNTP mix	10 mM	1 μL
10× Ex Taq buffer		5 μL
$MgCl_2$	25 mM	3 μL
Distilled water		33.5 μL
TaKaRa Ex Taq	5 U/μL	0.5 μL

15. Conduct 35 cycles of PCR, each of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min.
16. Separate amplified products by 3% agarose gel electrophoresis.

3.5. 3′ RACE RT-PCR

For determination of the terminal 3′ end sequence, extracted RNA can be polyadenylated with Poly(A) Polymerase (Takara Biochemicals), converted to cDNA with a 38-mer oligonucleotide containing $(T)_{35}$, and amplified with a 3′UTR primer and a primer used for reverse transcription (*see* **Note 3**).

1. Transfer 5 μL of the RNA sample to a new tube and incubate it at 90°C for 3 min and then transfer it onto ice.
2. Spin it down briefly and add 10 μL of 5× Poly(A) Polymerase buffer, 5 μL of 10 mM DTT, 1 μL of RNase inhibitor, 5 μL of 10 mM ATP, and 23 μL of nuclease-free water, followed by 1 μL of Poly(A) Polymerase, and mix gently.
3. Incubate the mixture at 37°C for 60 min.
4. Extract poly(A)-tailed RNA using TRIzol reagent according to the manufacturer's instructions.
5. Resuspend the RNA pellet in 10 μL of nuclease-free water.
6. Add 8 μL of 5× 1st strand buffer, 10 μL of 2 mM dNTP mixture, 2 μL of 0.1 M DTT, 5 μL of 10 μm CACT35, and 2 μL of nuclease-free water.
7. Mix well and incubate the mixture at 90°C for 3 min, then transfer the tube onto ice.
8. Spin it down briefly and add 1 μL of RNase inhibitor and 2 μL of Superscript II.
9. Mix well and incubate the tube at 50°C for 1 h, then at 72°C for 15 min to terminate the reaction.
10. Add 12 U of Ribonuclease H and incubate at 37°C for 20 min, then at 95°C for 3 min to terminate the reaction.
11. The cDNA sample can be stored at −70°C until use.
12. Conduct PCR of poly(A)-tailed cDNA using 3X-10S and CACT35 primers. Prepare the PCR mixture as follows:

poly(A)-tailed cDNA		2 μL
3X-10S primer	10 pM	1 μL
CACT35 Primer	10 pM	1 μL
dNTP mix	10 mM	2 μL
10× PCR Gold Buffer		2 μL
Distilled water		11.75 μL
AmpliTaq Gold DNA polymerase	0.05 U/μL	0.25 μL

13. Incubate the PCR mixture at 95°C for 7 min before PCR cycling, and perform PCR using 70 cycles, each of denaturing at 95°C for 30 s and annealing and extension at 60°C for 1 min.
14. Amplified products can be separated by 3% agarose gel electrophoresis

3.6. Cloning of PCR Products

1. Amplified products can be separated by agarose gel electrophoresis.
2. Excise the DNA fragment containing the PCR product from the agarose gel under UV light.

3. Purify the DNA from the excised agarose gel using the QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions.
4. Purified PCR products can be cloned into a pGEM-T EASY vector (Promega).
5. Sequence the cloned DNA using Big Dye Terminator Mix and an automated DNA sequencer (Applied Biosystems Japan). In our laboratory, at least five clones for each RT-PCR fragment were sequenced and a consensus sequence for JFH-1 determined (accession number: AB047639).

3.7. HCV Clone and Plasmid Construction

1. To isolate the JFH-1 strain as it was isolated in our laboratory, amplify 14 fragments of HCV cDNA, covering the entire genome, by RT-PCR, then purify and clone all amplified products into a number of pGEM-T easy™ vectors: pGEM1-258, pGEM44-486, pGEM317-849, pGEM617-1323, pGEM1141-2367, pGEM2285-3509, pGEM3471-4665, pGEM4547-5970, pGEM5883-7003, pGEM6950-8035, pGEM7984-8892, pGEM8680-9283, pGEM9231-9634, and pGEM9594-9678 (the number assigned to each clone indicates its position within JFH-1).
2. Assemble a subgenomic replicon construct of JFH-1 [ours is pSGR-JFH1; accession number AB114136; see **ref.** *(11)*] Based on the consensus sequence of JFH-1. Then assemble the 5′ half of the JFH-1 cDNA (nt 1–5970) into plasmid pGEM1-5970 using nine plasmids containing overlapping cDNA. Cut out an *Age*I and *Eco*T22I fragment (nt 154-5293) from pGEM1-5970 and insert it into pSGR-JFH1; the resulting plasmid (pJFH-1) will contain full-length JFH-1 cDNA downstream of the T7 RNA promoter sequence [pJFH-1; **ref.** *(17)*].
3. Then derive two mutant constructs from pJFH-1: pJFH-1/GND, with a point mutation of GDD to GND, abolishing the RNA polymerase activity of NS5B, and pJFH-1/ΔE1-E2, with an in-frame deletion of 351 amino acids spanning most of the E1-to-E2 region *(17)*.

3.8. Plasmid DNA Preparation

1. Transform DH5α competent cells with pJFH-1 and other plasmid DNA.
2. Inoculate 200 mL Luria-Bertani medium containing 100 μg/mL ampicillin with a single colony.
3. Incubate the culture at 37°C overnight with vigorous shaking.
4. Transfer the culture to a centrifuge bottle, and centrifuge it at 4000g for 15 min.
5. Discard the supernatant, and resuspend the pellet with 12 mL of solution 1.

6. Add 24 mL of solution 2, mix gently by inverting the bottles five to six times, and then incubate them at room temperature for 10 min.
7. Add 18 mL of solution 3, and mix gently by inverting the bottles five to six times, then incubate them on ice for 10 min.
8. Centrifuge the solution at 12,000g for 15 min at 4°C.
9. Transfer the supernatant to a new centrifuge bottle.
10. Add 32 mL isopropanol, mix vigorously, and incubate at room temperature for 10 min.
11. Centrifuge the mixture at 12,000g for 15 min at 4°C.
12. Discard the supernatant and resuspend the pellet with 5.2 mL of TE buffer, then add 6 g of cesium chloride and mix until the CsCl is dissolved. Add 0.14 mL of 10 mg/mL ethidium bromide solution and incubate at room temperature for 10 min with gentle shaking.
13. Centrifuge the mixture at 3000g for 10 min at room temperature. Transfer the supernatant to two Opti-Seal polyallomer centrifuge tubes.
14. Place caps on the tubes and insert the tubes into a Beckmann TLN100 rotor. Centrifuge them at 360,000× g for 3 h, or at 140,000 × g overnight, in a Beckmann Optima TLX Ultracentrifuge at 25°C.
15. After centrifugation, remove the caps from the tubes and insert a 2.5 mL syringe attached to a 21-gauge needle into each tube a few millimeters below the band of DNA and then bring it in line with the bottom edge of the band. Draw the plunger slowly until most of the DNA is collected. Remove the needle from the tube and transfer the DNA solution into a new Opti-Seal polyallomer centrifuge tube.
16. Fill up the tube with CsCl/TE solution (1.1 g/mL, wt/vol) and centrifuge again at 360,000 × g for 3 h or at 140,000 × g overnight in a Beckmann TLN100 rotor at 25°C.
17. After this second round of ultracentrifugation, draw up the DNA band again as above and transfer the DNA solution into a new centrifuge tube.
18. Extract the DNA solution with isopropanol saturated with NaCl to remove ethidium bromide. Extraction should be repeated one more time after the orange color of the ethidium bromide disappears.
19. Add three volumes of TE buffer and eight volumes of 99.5% ethanol to the DNA solution. Mix well and let it stand at −70°C for 20 min.
20. Centrifuge at 9,100 × g for 20 min at 4°C.
21. After centrifugation, discard the supernatant and add 70% ethanol.
22. Centrifuge at 9,100 × g for 20 min at 4°C.
23. Discard the supernatant and dry the pellet completely.

24. Resuspend the pellet in TE buffer. Determine the DNA concentration by measuring optical density at OD260 and OD280. Also, double check the concentration of DNA, purity of the supercoiled DNA, and RNA contamination by agarose gel electrophoresis. Adjust the DNA concentration to 2 mg/mL with TE buffer.
25. Sequence the purified pJFH-1 plasmid at each preparation. Some unintended mutations are incorporated through the procedures.

3.9. RNA Synthesis

This protocol of RNA synthesis has also been described elsewhere *(43)*.

1. Digest pJFH-1 and other plasmids with *Xba*I.
2. Treat the *Xba*I-digested plasmid with mung bean nuclease to remove four nucleotides, leaving the correct 3′ end of the HCV cDNA.
3. Purify the digested plasmid DNA using phenol/chloroform extraction.
4. Synthesize HCV RNA using purified plasmid as templates for RNA with a MEGAscript™ T7 kit in accordance with the manufacturer's instructions.
5. Treat synthesized RNA with DNaseI then by acid phenol extraction to remove any remaining template DNA.
6. Determine the RNA concentration by measuring optical density at OD260 and OD280. Check the purity of synthesized RNA by agarose gel electrophoresis. Adjust the RNA concentration to 10 mg/mL with nuclease-free water.

3.10. RNA Transfection

A detailed protocol of RNA transfection has been described previously (44).

1. Trypsinize Huh-7 cells, wash them with Opti-MEM I™ Reduced-Serum Medium, and resuspend them at 7.5×10^6 cells/mL with Cytomix buffer *(42)*.
2. Add 10 μg RNA to 400 μL of cell suspension (3×10^6 cells) and transfer the mixture into an electroporation cuvette.
3. Pulse the cells at 260 V and 950 μF using a Gene Pulser II™ apparatus.
4. Transfer the transfected cells immediately into two 10 cm culture dishes, each containing 8 mL of culture medium.

3.11. Northern Blot Analysis

RNA replication in the transfected or infected Huh-7 cells can be analyzed by northern-blot analysis.

1. Extract cellular RNA from RNA-transfected cells using TRIzol solution according to the manufacturer's instructions.
2. Determine the concentration of the isolated RNA by measuring optical density. Adjust the concentration to 1 mg/mL with nuclease-free water.
3. Prepare a denaturing 1% agarose gel.