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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this infection was specific because an antibody against CD81, as well as anti-E2 antibody, inhibited infection (17 and unpublished data).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 We found that JFH-1 RNA replication was not greater in these cell lines than in original Huh-7 cells (Wakita, unpublished data), but we observed a stable increase in the cell-surface expression of CD81 in these cell lines over that in original Huh-7 cells.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

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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

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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

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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.

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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 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.

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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).

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11. Megaprime^{TM} DNA labeling system (GE Healthcare).
12. S-300HR (GE Healthcare).
13. Rapid-Hyb^{TM} 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^{TM} 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**).

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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.

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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.

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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.

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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.

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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.

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4. Transfer 2 μL of 2 mg/mL RNA to a new tube and add 8 μL of sample buffer, then incubate at 65°C for 15 min and transfer onto ice.
5. Spin the tube down briefly, add 2 μL of gel-loading buffer, and apply the sample to a denaturing agarose gel.
6. After electrophoresis, rinse the agarose gel with DEPC-treated water once for 10 min with gentle shaking.
7. For a minigel, incubate in 150 mL of 50 mM NaOH/10 mM NaCl solution at room temperature for 20 min.
8. Discard the solution and rinse the gel with DEPC-treated water.
9. Incubate in 150 mL of 0.1 M Tris-HCl, pH 7.4, at room temperature for 20 min.
10. Discard the solution and rinse the gel with DEPC-treated water.
11. Incubate in 150 mL of 20× SSC at room temperature for 20 min.
12. Transfer the gel onto a Hybond-N+ membrane at 50–55 cm H_2O for 1 h (*see* **Note 4**).
13. Immobilize the membrane using a Stratalinker™ UV crosslinker.
14. Purify the DNA fragment containing the *NS3* to *5B* genes of JFH-1.
15. Transfer 25 ng of the purified DNA fragment into a new tube and add 5 μL of primer solution from the Megaprime™ DNA labeling system and distilled water to a total volume of 33 μL.
16. Boil the solution for 5 min and then transfer it onto ice.
17. Add 10 μL of labeling buffer and 5 μL of [$\alpha-^{32}$P]dCTP, followed by 2 μL of enzyme solution, and then incubate the mixture at 37°C for 10 min.
18. Purify the [$\alpha-^{32}$P]dCTP-labeled DNA probe using a S-300HR spin column.
19. Determine the specific activity with a scintillation counter.
20. Incubate the membrane with 0.125 mL/cm^2 Rapid-Hyb Buffer™ at 65°C for at least 15 min. During this incubation period, boil the probe for 5 min and then place it on ice for 2 min.
21. Add the boiled probe at 1×10^5 cpm/mL buffer and incubate at 65°C for 2 h with shaking.
22. Wash the hybridized membrane with 2 × SSC/0.1% SDS at room temperature for 10 min three times.
23. Wash the membrane with 1 × SSC/0.1% SDS at 65°C for 15 min once.
24. Wash the membrane with 0.1 × SSC/0.1% SDS at 65°C for 20 min three times.
25. Expose the membrane to X-ray film.

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3.12. Quantification of HCV Core Protein

For estimation of levels of HCV core protein in culture supernatant or cell lysate, concentrations of HCV core protein can be determined by HCV core protein immunoassay. Cell pellets can be lysed in lysis buffer.

1. Dilute the HCV core standard solution with standard diluent and prepare 3600, 1200, 400, 133, and 44.4 fmol/L solutions. Use standard diluent as a negative control (0 fmol/L).
2. Add 50 μL of pretreatment solution to 100 μL of sample and mix (*see* **Note 5**).
3. Incubate at 56–60°C for 30 min and then at room temperature for 5 min.
4. Determine the number of eight-well strips needed for the assay. Insert these into the frame for current use.
5. Add 100 μL of reaction buffer to each well.
6. Add 100 μL of each standard solution and pretreated samples to the appropriate wells. Mix them by pipetting.
7. Cover the plate with a plate cover and incubate it for 60 min at room temperature with shaking on a plate mixer.
8. Thoroughly aspirate or decant solution from the wells and discard the liquid. Take care not to scratch the inside of the well. After aspiration, fill the wells with wash solution, being careful not to overflow the wells. Soak for at least 20 s and then aspirate the liquid. Repeat this washing procedure six times.
9. Add 200 μL of labeled-antibody solution to each well.
10. Cover the plate with a plate cover and incubate it for 30 min at room temperature.
11. Thoroughly aspirate or decant solution from the wells and discard the liquid. Wash the wells six times as described above.
12. Add 200 μL of substrate solution to each well. Incubate for 30 min at room temperature in the dark.
13. Add 50 μL of stop solution to each well. Tap the side of the plate gently to mix.
14. Read the absorbance of each well at 492 nm against two negative control wells.
15. Plot the absorbance of each standard against the standard concentration. Draw the best smooth curve through these points to construct the standard curve.
16. Determine HCV core protein concentrations for unknown samples from the standard curve. Samples with concentrations exceeding the highest standard (3600 fmol/L) should be diluted and reanalyzed. Samples producing less than 100 fmol/L should also be reanalyzed for confirmation of results.
17. Determine the total lysate protein concentrations, for example using the Bradford method, and adjust them to 1 mg/mL with normal serum. HCV core protein concentrations within

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cell lysate can be expressed as fmol/g of total protein when divided by total protein concentrations (*see* **Note 6**).

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

Copy numbers of HCV RNA in culture supernatant or infected cells can be determined by real-time detection RT-PCR (RTD-PCR), using TaqMan EZ RT-PCR Core Reagents kit and the 7500 Real-Time PCR System.

1. To synthesize standard RNA for quantification, prepare plasmid DNA containing the HCV IRES sequence.
2. Synthesize an HCV RNA standard using an appropriate commercial kit, such as the MEGAscript™ T7kit. Check the integrity of the synthesized RNA by denaturing agarose gel electrophoresis as described above.
3. Determine RNA concentrations by measuring optical density at OD260 and OD280. Calculate the copy number of synthesized RNA from the concentration and length of RNA. Adjust the standard RNA concentration to 10^{10} copies/μL with nuclease-free water.
4. Prepare primers and the TaqMan probe listed in **Table 23.2**.
5. Dilute HCV RNA standard solution with RNA standard dilution buffer and prepare 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 copies/μLE solutions. Use nuclease-free water as a negative control.
6. Prepare the reaction mixture as follows:

Nuclease-free water	7.1 μL
5× TaqMan EZ buffer	5 μL
10 mM dATP	0.5 μL
10 mM dGTP	0.5 μL
10 mM dCTP	0.5 μL
20 mM dUTP	0.65 μL
10 μm 130S primer	0.5 μL
10 μm 290R primer	0.5 μL
3 μm TaqMan probe	2.5 μL
25 mM $Mn(OAc)_2$	3 μL
AmpErase UNG	0.25 μL
rTth DNA polymerase	1.5 μL
Total	22.5 μL

7. Add 22.5 μL of reaction mixture to each well of the PCR plate.

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Table 23.2
Primers and a probe used in RTD-PCR

Name	Sequence (5′ > 3′)
130S	5′- CGG GAG AGC CAT AGT GG-3′
290R	5′- AGT ACC ACA AGG CCT TTC G-3′
probe	5′- (6-Fam) CTG CGG AAC CGG TGA GTA CAC (Tamra) -3′

8. Add 2.5 μL of each RNA sample and standard to the appropriate wells. Use nuclease-free water as a negative control. All samples and controls should be evaluated in at least two wells.
9. Set the PCR plate in a 7500 Real-Time PCR System or an equivalent system.
10. Set the reaction conditions and incubate at 50°C for 2 min, 60°C for 30 min, 95°C for 5 min, followed by 50 cycles at 95°C for 20 s and 62°C for 1 min.
11. Confirm the absence of amplification in the negative control wells.
12. Determine RNA copy numbers in the samples using Sequence Detection Software.

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

1. Collect culture medium from RNA-transfected or infected cells.
2. Centrifuge the collected culture medium at 3000g for 20 min at 4°C, and pass the supernatant through a disk filter with a 0.45 μm pore size.
3. Concentrate the collected culture medium using an Amicon Ultra-15 device (100,000 MWCO) if necessary. Add up to 15 mL of culture medium to the upper chamber of the Amicon Ultra-15. Centrifuge at 3000g for 30 min at 4°C. The concentrated culture medium can be stored at −80°C until use.
4. Seed wells in poly-D-lysine-coated 96-well plates with Huh-7 cells at a density of 1×10^4 cells on the day before virus inoculation.
5. Dilute culture medium containing infectious JFH-1 virus.
6. Discard the culture medium from the plates seeded with Huh-7 cells.
7. Add 100 μL of serially diluted virus solution to at least six wells per dilution, and incubate for 4 h at 37°C.
8. Remove the inoculum and add 100 μL of fresh complete medium, then incubate the inoculated cells for 72 h at 37°C under 5% CO_2.
9. At the end of incubation, remove the culture medium.
10. Fix the cells by dipping the plate into ice-chilled 100% methanol.

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11. Incubate it at −20°C for 20 min.
12. Block the cells at room temperature for 1 h with 100 μL per well of blocking buffer, then wash with PBS(−) once.
13. Add 100 μL of anticore antibody solution to each well to make a concentration of 50 μg/mL in blocking buffer, and incubate at room temperature for 1 h.
14. Aspirate the antibody solution and wash the wells with PBS(−) three times.
15. Add 100 μL per well of AlexaFluor 488-conjugated anti-mouse IgG in blocking buffer, and incubate at room temperature for 1 h.
16. Aspirate the antibody solution and wash the wells with PBS(−) three times.
17. Count the stained cells using fluorescence microscopy.
18. Calculate the infectivity of the inoculum form the focus number and inoculum dilution, which can be expressed as focus-forming units per milliliter (ffu/mL). Alternatively, determine the TCID50 according to the method of Reed and Muench *(44)*.

4. Notes

1. The final concentration of formaldehyde is shown.
2. If the expected amount of recovered RNA is low, transfer RNA or glycogen should be added before isopropanol precipitation to enhance the precipitation efficiency.
3. If the 3′ residue might be A, other 3′ RACE methods should also be used, such as the linker ligation method.
4. The membrane should be rinsed with DEPC-treated water, then 20× SSC.
5. Multiple dilutions of samples may be necessary to produce core protein concentrations within a standard range. Fetal bovine serum can be used as the diluent.
6. Adjusting the total protein concentration may reduce background contamination.

Acknowledgments

This work was partially supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labour and Welfare of Japan, and from the Ministry of Education, Culture, Sports, Science and Technology and by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation.

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References

1. Bartenschlager, R. and Lohmann, V. (2001) Novel cell culture systems for the hepatitis C virus. *Antiviral Res.* **52,** 1–17.
2. Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285,** 110–113.
3. Blight, K. J., Kolykhalov, A. A., and Rice, C. M. (2000) Efficient initiation of HCV RNA replication in cell culture. *Science* **290,** 1972–1974.
4. Lohmann, V., Korner, F., Dobierzewska, A., and Bartenschlager, R. (2001) Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J. Virol.* **75,** 1437–1449.
5. Krieger, N., Lohmann, V., and Bartenschlager, R. (2001) Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. *J. Virol.* **75,** 4614–4624.
6. Ikeda, M., Yi, M., Li, K., and 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.
7. Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., et al. (2002) Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J. Virol.* **76,** 4008–4021.
8. Blight, K. J., McKeating, J. A., Marcotrigiano, J., and Rice, C. M. (2003) Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. *J. Virol.* **77,** 3181–3190.
9. Bukh, J., Pietschmann, T., Lohmann, V., Krieger, N., Faulk, K., Engle, R. E., et al. (2002) Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. *Proc. Natl. Acad. Sci. USA* 99, 14416–14421.
10. Kato, T., Furusaka, A., Miyamoto, M., Date, T., Yasui, K., Hiramoto, J., et al. (2001) Analysis of hepatitis C virus isolated from a fulminant hepatitis patient. *J. Med. Virol.* **64,** 334–339.
11. Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M., et al. (2003) Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* **125,** 1808–1817.
12. Uprichard, S. L., Chung, J., Chisari, F. V., and Wakita, T. (2006) Replication of a hepatitis C virus replicon clone in mouse cells. *Virol. J.* **3,** 89.
13. Date, T., Miyamoto, M., Kato, T., Morikawa, K., Murayama, A., Akazawa, D., et al. (2007) An infectious and selectable full-length replicon system with hepatitis C virus JFH-1 strain. *Hepatol. Res.* in press.
14. Kato, T., Date, T., Miyamoto, M., Sugiyama, M., Tanaka, Y., Orito, E., et al. Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. *J. Clin. Microbiol.* **43,** 5679–5684.
15. Date, T., Kato, T., Miyamoto, M., Zhao, Z., Yasui, K., Mizokami, M., et al. (2004) Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells. *J. Biol. Chem.* **279,** 22371–22376.
16. Kato, T., Date, T., Miyamoto, M., Zhao, Z., Mizokami, M., and Wakita, T. (2005) Nonhepatic cell lines HeLa and 293 cells support efficient replication of hepatitis C virus genotype 2a subgenomic replicon. *J. Virol.* **79,** 592–596.
17. Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., et al. (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* **11,** 791–796.
18. Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., et al. (2005) Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. USA* **102,** 9294–9299.
19. Blight, K. J., McKeating, J. A., and Rice, C. M. (2002) Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J. Virol.* **76,** 13001–13014.
20. Sumpter, R., Jr., Loo, Y. M., Foy, E., Li, K., Yoneyama, M., Fujita, T., et al. (2005) Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J. Virol.* **79,** 2689–2699.
21. Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., et al. (2005) Complete replication of hepatitis C virus in cell culture. *Science* **309,** 623–626.
22. Koutsoudakis, G., Herrmann, E., Kallis, S., Bartenschlager, R., and Pietschmann, T. (2007) The level of CD81 cell surface expression is a key determinant for productive entry of hepatitis C virus into host cells. *J. Virol.* 81, 588–598.
23. Akazawa, D., Date, T., Morikawa, K., Murayama, A., Miyamoto, M., Kaga, M., et al. (2007) CD81 expression is important for heterogeneous HCV permissiveness of Huh7 cell clones. *J. Virol.* [Epub ahead of print].

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