JOURNAL OF VIROLOGY, Mar. 2009, p. 2389–2392
0022-538X/09/$08.00+0 doi:10.1128/JVI.01690-08

Vol. 83, No. 5

Proteasomal Turnover of Hepatitis C Virus Core Protein Is Regulated by Two Distinct Mechanisms: a Ubiquitin-Dependent Mechanism and a Ubiquitin-Independent but PA28γ-Dependent Mechanism▽

Ryosuke Suzuki,[1] Kohji Moriishi,[2] Kouichirou Fukuda,[1] Masayuki Shirakura,[1] Koji Ishii,[1] Ikuo Shoji,[3] Takaji Wakita,[1] Tatsuo Miyamura,[1] Yoshiharu Matsuura,[2] and Tetsuro Suzuki[1]*

Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640,[1] Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871,[2] and Division of Microbiology, Kobe University Graduate School of Medicine, Hyogo 650-0017,[3] Japan

Received 8 August 2008/Accepted 5 December 2008

We have previously reported on the ubiquitylation and degradation of hepatitis C virus core protein. Here we demonstrate that proteasomal degradation of the core protein is mediated by two distinct mechanisms. One leads to polyubiquitylation, in which lysine residues in the N-terminal region are preferential ubiquitylation sites. The other is independent of the presence of ubiquitin. Gain- and loss-of-function analyses using lysineless mutants substantiate the hypothesis that the proteasome activator PA28γ, a binding partner of the core, is involved in the ubiquitin-independent degradation of the core protein. Our results suggest that turnover of this multifunctional viral protein can be tightly controlled via dual ubiquitin-dependent and -independent proteasomal pathways.

Hepatitis C virus (HCV) core protein, whose amino acid sequence is highly conserved among different HCV strains, not only is involved in the formation of the HCV virion but also has a number of regulatory functions, including modulation of signaling pathways, cellular and viral gene expression, cell transformation, apoptosis, and lipid metabolism (reviewed in references 9 and 15). We have previously reported that the E6AP E3 ubiquitin (Ub) ligase binds to the core protein and plays an important role in polyubiquitylation and proteasomal degradation of the core protein (22). Another study from our group identified the proteasome activator PA28γ/REG-γ as an HCV core-binding partner, demonstrating degradation of the core protein via a PA28γ-dependent pathway (16, 17). In this work, we further investigated the molecular mechanisms underlying proteasomal degradation of the core protein and found that in addition to regulation by the Ub-mediated pathway, the turnover of the core protein is also regulated by PA28γ in a Ub-independent manner.

Although ubiquitylation of substrates generally requires at least one Lys residue to serve as a Ub acceptor site (5), there is no consensus as to the specificity of the Lys targeted by Ub (4, 8). To determine the sites of Ub conjugation in the core protein, we used site-directed mutagenesis to replace individual Lys residues or clusters of Lys residues with Arg residues in the N-terminal 152 amino acids (aa) of the core (C152), within which is contained all seven Lys residues (Fig. 1A). Plasmids expressing a variety of mutated core proteins were generated by PCR and inserted into the pCAGGS (18). Each core-expressing construct was transfected into human embryonic kidney 293T cells along with the pMT107 (25) encoding a Ub moiety tagged with six His residues (His_6). Transfected cells were treated with the proteasome inhibitor MG132 for 14 h to maximize the level of Ub-conjugated core intermediates by blocking the proteasome pathway and were harvested 48 h posttransfection. His_6-tagged proteins were purified from the extracts by Ni^{2+}-chelation chromatography. Eluted protein and whole lysates of transfected cells before purification were analyzed by Western blotting using anticore antibodies (Fig. 1B). Mutations replacing one or two Lys residues with Arg in the core protein did not affect the efficiency of ubiquitylation: detection of multiple Ub-conjugated core intermediates was observed in the mutant core proteins comparable to the results seen with the wild-type core protein as previously reported (23). In contrast, a substitution of four N-terminal Lys residues (C152K6-23R) caused a significant reduction in ubiquitylation (Fig. 1B, lane 9). Multiple Ub-conjugated core intermediates were not detected in the Lys-less mutant (C152KR), in which all seven Lys residues were replaced with Arg (Fig. 1B, lane 11). These results suggest that there is not a particular Lys residue in the core protein to act as the Ub acceptor but that more than one Lys located in its N-terminal region can serve as the preferential ubiquitylation site. In rare cases, Ub is known to be conjugated to the N terminus of proteins; however, these results indicate that this does not occur within the core protein.

To investigate how polyubiquitylation correlates with proteasome degradation of the core protein, we performed kinetic analysis of the wild-type and mutated core proteins by use of the Ub protein reference (UPR) technique, which can compensate for data scatter of sample-to-sample variations such as levels of expression (10, 24). Fusion proteins expressed from UPR-based constructs (Fig. 2A) were cotranslationally cleaved by deubiquitylating enzymes, thereby generating equimolar quantities of the core proteins and the reference protein, dihydrofolate reductase-hemagglutinin (DHFR-HA) tag-modified Ub, in which the Lys at aa 48 was replaced by Arg to prevent its polyubiquitylation (Ub^{R48}). After 24 h of transfec-

* Corresponding author. Mailing address: Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1111. Fax: 81-3-5285-1161. E-mail: tesuzuki@nih.go.jp.

▽ Published ahead of print on 17 December 2008.

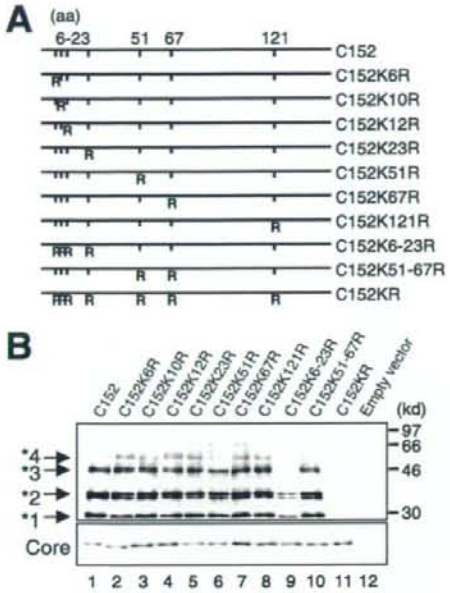

FIG. 1. In vivo ubiquitylation of HCV core protein. (A) The HCV core protein (N-terminal 152 aa) is represented on the top. The positions of the amino acid residues of the core protein are indicated above the bold lines. The positions of the seven Lys residues in the core are marked by vertical ticks. Substitution of Lys with Arg (R) is schematically depicted. (B) Detection of ubiquitylated forms of the core proteins. The transfected cells with core expression plasmids and pMT107 were treated with the proteasome inhibitor MG132 and harvested 48 h after transfection. His_6-tagged proteins were purified and subsequently analyzed by Western blot analysis using anticore antibody (upper panel). Core proteins conjugated to a number of His_6-Ub are denoted with asterisks. Whole lysates of transfected cells before purification were also analyzed (lower panel). Lanes 1 to 11, C152 to C152KR, as indicated for panel A. Lane 12; empty vector.

tion with UPR constructs, cells were treated with cycloheximide and the amounts of core proteins and DHFR-HA-Ub^{R48} at the indicated time points were determined by Western blot analysis using anticore and anti-HA antibodies. The mature form of the core protein, aa 1 to 173 (C173) (13, 20), and C152 were degraded with first-order kinetics (Fig. 2B and D). MG132 completely blocked the degradation of C173 and C152 (Fig. 2B), and C152K6-23R and C152KR were markedly stabilized (Fig. 2C). The half-lives of C173 and C152 were calculated to be 5 to 6 h, whereas those of C152K6-23R and C152KR were calculated to be 22 to 24 h (Fig. 2D), confirming that the Ub plays an important role in regulating degradation of the core protein. Nevertheless, these results also suggest possible involvement of the Ub-independent pathway in the turnover of the core protein, as C152KR is more destabilized than the reference protein (Fig. 2C and 2D).

We have shown that PA28γ specifically binds to the core protein and is involved in its degradation (16, 17). Recent studies demonstrated that PA28γ is responsible for Ub-independent degradation of the steroid receptor coactivator SRC-3 and cell cycle inhibitors such as p21 (3, 11, 12). Thus, we next investigated the possibility of PA28γ involvement in the degradation of either C152KR or C152. Since C152KR carries two amino acid substitutions in the PA28γ-binding region (aa 44 to 71) (17), we tested the influence of the mutations of C152KR on the interaction with PA28γ by use of a coimmunoprecipi-

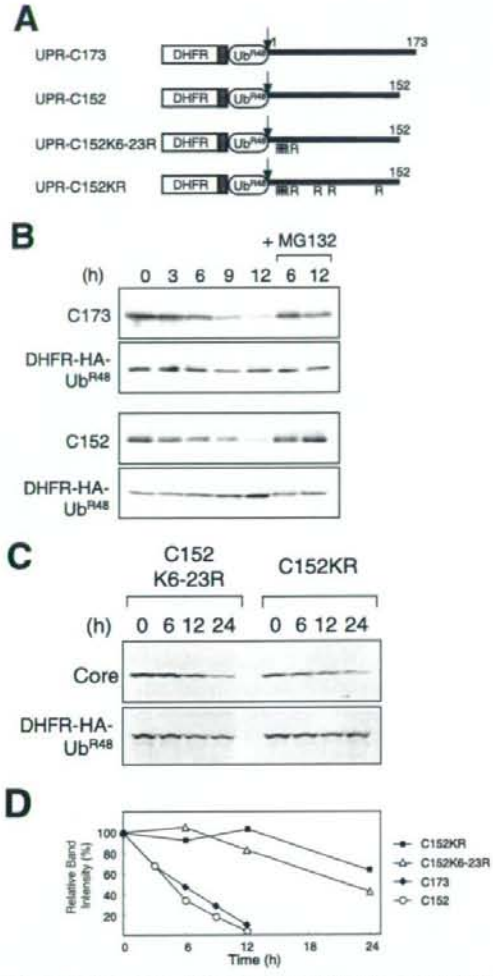

FIG. 2. Kinetic analysis of degradation of HCV core proteins. (A) The fusion constructs used in the UPR technique. Open boxes indicate the DHFR sequence, which is extended at the C terminus by a sequence containing the HA epitope (hatched boxes). Ub^{R48} moieties bearing the Lys-Arg substitution at aa 48 are represented by open ellipses. Bold lines indicate the regions of the core protein. The amino acid positions of the core protein are indicated above the bold lines. The arrows indicate the sites of in vivo cleavage by deubiquitylating enzymes. (B and C) Turnover of the core proteins. After a 24-h transfection with each UPR construct, cells were treated with 50 μg of cycloheximide/ml in the presence or absence of 10 μM MG132 for the different time periods indicated. Cells were lysed at the different time points indicated, followed by evaluation via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis using antibodies against the core protein and HA. (D) Quantitation of the data shown in panels B and C. At each time point, the ratio of band intensity of the core protein relative to the reference DHFR-HA-Ub^{R48} was determined by densitometry and is plotted as a percentage of the ratio at time zero.

tation assay. When Flag-tagged PA28γ (F-PA28γ) was expressed in cells along with C152 or C152KR, F-PA28γ precipitated along with both C152 and C152KR, indicating that PA28γ interacts with both core proteins (Fig. 3A). Figure 3B reveals the effect of exogenous expression of F-PA28γ on the steady-state levels of C152 and C152KR. Consistent with previous data (17), the expression level of C152 was decreased to a nearly undetectable level in the presence of PA28γ (Fig. 3B, lanes 1 and 3). Interestingly, exogenous expression of PA28γ led to a marked reduction in the amount of C152KR expressed (Fig. 3B, lanes 5 and 7). Treatment with MG132 increased the steady-state level of the C152KR in the presence of F-PA28γ as well as the level of C152 (Fig. 3B, lanes 4 and 8).

We further investigated whether PA28γ affects the turnover of Lys-less core protein through time course experiments. C152KR was rapidly destabilized and almost completely degraded in a 3-h chase experiment using cells overexpressing F-PA28γ (Fig. 3C, left panels). A similar result was obtained using an analogous Lys-less mutant of the full-length core protein C191KR (Fig. 3C, right panels), thus demonstrating that the Lys-less core protein undergoes proteasomal degradation in a PA28γ-dependent manner. These results suggest that PA28γ may play a role in accelerating the turnover of the HCV core protein that is independent of ubiquitylation.

Finally, we examined gain- and loss-of-function of PA28γ with respect to degradation of full-length wild-type (C191) and mutated (C191KR) core proteins in human hepatoma Huh-7 cells. As expected, exogenous expression of PA28γ or E6AP caused a decrease in the C191 steady-state levels (Fig. 4A). In contrast, the C191KR level was decreased with expression of PA28γ but not of E6AP. We further used RNA interference to inhibit expression of PA28γ or E6AP. An increase in the abundance of C191KR was observed with PA28γ small interfering RNA (siRNA) but not with E6AP siRNA (Fig. 4B). An increase in the C191 level caused by the activity of siRNA against PA28γ or E6AP was confirmed as well.

Taking these results together, we conclude that turnover of the core protein is regulated by both Ub-dependent and Ub-independent pathways and that PA28γ is possibly involved in Ub-independent proteasomal degradation of the core protein. PA28 is known to specifically bind and activate the 20S proteasome (19). Thus, PA28γ may function by facilitating the delivery of the core protein to the proteasome in a Ub-independent manner.

Accumulating evidence suggests the existence of proteasome-dependent but Ub-independent pathways for protein degradation, and several important molecules, such as p53, p73, Rb, SRC-3, and the hepatitis B virus X protein, have two distinct degradation pathways that function in a Ub-dependent and Ub-independent manner (1, 2, 6, 7, 14, 21, 27). Recently, critical roles for PA28γ in the Ub-independent pathway have been demonstrated; SRC-3 and p21 can be recognized by the 20S proteasome independently of ubiquitylation through their interaction with PA28γ (3, 11, 12). It has also been reported that phosphorylation-dependent ubiquitylation mediated by GSK3 and SCF is important for SRC-3 turnover (26). Nevertheless, the precise mechanisms underlying turnover of most of the proteasome substrates that are regulated in both Ub-dependent and Ub-independent manners are not well understood. To our knowledge, the HCV core protein is the first

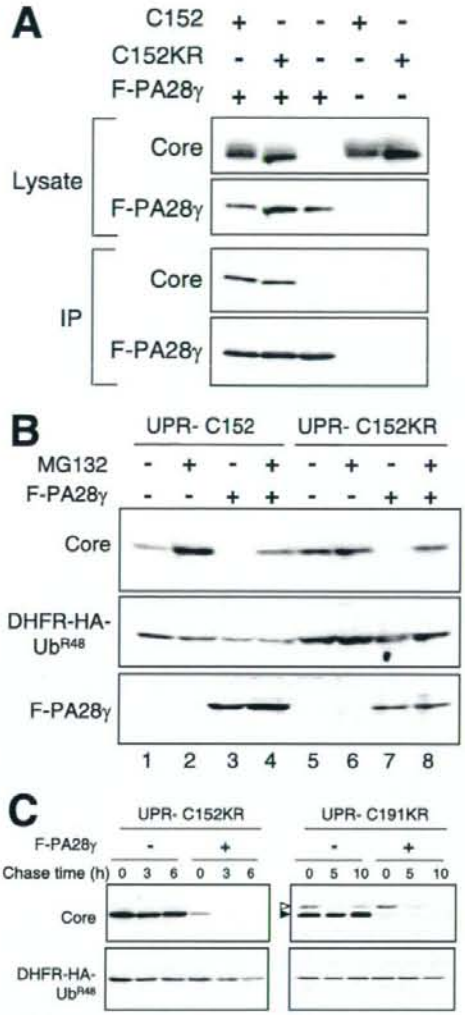

FIG. 3. PA28γ-dependent degradation of the core protein. (A) Interaction of the core protein with PA28γ. Cells were cotransfected with the wild-type (C152) or Lys-less (C152KR) core expression plasmid in the presence of a Flag-PA28γ (F-PA28γ) expression plasmid or an empty vector. The transfected cells were treated with MG132. After 48 h, the cell lysates were immunoprecipitated with anti-Flag antibody and visualized by Western blotting with anticore antibodies. Western blot analysis of whole cell lysates was also performed. (B) Degradation of the wild-type and Lys-less core proteins via the PA28γ-dependent pathway. Cells were transfected with the UPR construct with or without F-PA28γ. In some cases, cells were treated with 10 μM MG132 for 14 h before harvesting. Western blot analysis was performed using anticore, anti-HA, and anti-Flag antibodies. (C) After 24 h of transfection with UPR-C152KR and UPR-C191KR with or without F-PA28γ (an empty vector), cells were treated with 50 μg of cycloheximide/ml for different time periods as indicated (chase time). Western blot analysis was performed using anticore and anti-HA antibodies. The precursor core protein and the core that was processed, presumably by signal peptide peptidase, are denoted by open and closed triangles, respectively.

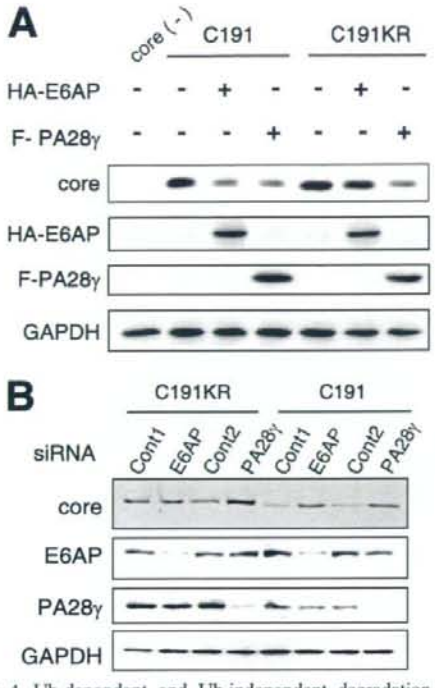

FIG. 4. Ub-dependent and Ub-independent degradation of the full-length core protein in hepatic cells. (A) Huh-7 cells were cotransfected with plasmids for the full-length core protein (C191) or its Lys-less mutant (C191KR) in the presence of F-PA28γ or HA-tagged-E6AP expression plasmid (HA-E6AP). After 48 h, cells were lysed and Western blot analysis was performed using anticore, anti-HA, anti-Flag, or anti-GAPDH. (B) Huh-7 cells were cotransfected with core expression plasmids along with siRNA against PA28γ or E6AP or with negative control siRNA. Cells were harvested 72 h after transfection and subjected to Western blot analysis.

viral protein studied that has led to identification of key cellular factors responsible for proteasomal degradation via dual distinct mechanisms. Although the question remains whether there is a physiological significance of the Ub-dependent and Ub-independent degradation of the core protein, it is reasonable to consider that tight control over cellular levels of the core protein, which is multifunctional and essential for viral replication, maturation, and pathogenesis, may play an important role in representing the potential for its functional activity.

This work was supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labor and Welfare of Japan, and from the Ministry of Education, Culture, Sports, Science and Technology, by Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation, Japan, and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan.

REFERENCES

1. **Asher, G., J. Lotem, L. Sachs, C. Kahana, and Y. Shaul.** 2002. Mdm-2 and ubiquitin-independent p53 proteasomal degradation regulated by NQO1. Proc. Natl. Acad. Sci. USA **99:**13125–13130.
2. **Asher, G., P. Tsvetkov, C. Kahana, and Y. Shaul.** 2005. A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73. Genes Dev. **19:**316–321.
3. **Chen, X., L. F. Barton, Y. Chi, B. E. Clurman, and J. M. Roberts.** 2007. Ubiquitin-independent degradation of cell-cycle inhibitors by the REGγ proteasome. Mol. Cell **26:**843–852.
4. **Ciechanover, A.** 1998. The ubiquitin-proteasome pathway: on protein death and cell life. EMBO J. **17:**7151–7160.
5. **Hershko, A., A. Ciechanover, and A. Varshavsky.** 2000. The ubiquitin system. Nat. Med. **6:**1073–1081.
6. **Jariel-Encontre, I., M. Pariat, F. Martin, S. Carillo, C. Salvat, and M. Piechaczyk.** 1995. Ubiquitinylation is not an absolute requirement for degradation of c-Jun protein by the 26 S proteasome. J. Biol. Chem. **270:**11623–11627.
7. **Jin, Y., H. Lee, S. X. Zeng, M. S. Dai, and H. Lu.** 2003. MDM2 promotes p21waf1/cip1 proteasomal turnover independently of ubiquitylation. EMBO J. **22:**6365–6377.
8. **Ju, D., and Y. Xie.** 2006. Identification of the preferential ubiquitination site and ubiquitin-dependent degradation signal of Rpn4. J. Biol. Chem. **281:**10657–10662.
9. **Lai, M. M. C., and C. F. Ware.** 1999. Hepatitis C virus core protein: possible roles in viral pathogenesis. Springer, Berlin, Germany.
10. **Lévy, F., N. Johnsson, T. Rumenapf, and A. Varshavsky.** 1996. Using ubiquitin to follow the metabolic fate of a protein. Proc. Natl. Acad. Sci. USA **93:**4907–4912.
11. **Li, X., L. Amazit, W. Long, D. M. Lonard, J. J. Monaco, and B. W. O'Malley.** 2007. Ubiquitin- and ATP-independent proteolytic turnover of p21 by the REGγ-proteasome pathway. Mol. Cell **26:**831–842.
12. **Li, X., D. M. Lonard, S. Y. Jung, A. Malovannaya, Q. Feng, J. Qin, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley.** 2006. The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REGγ proteasome. Cell **124:**381–392.
13. **Liu, Q., C. Tackney, R. A. Bhat, A. M. Prince, and P. Zhang.** 1997. Regulated processing of hepatitis C virus core protein is linked to subcellular localization. J. Virol. **71:**657–662.
14. **Lonard, D. M., Z. Nawaz, C. L. Smith, and B. W. O'Malley.** 2000. The 26S proteasome is required for estrogen receptor-α and coactivator turnover and for efficient estrogen receptor-α transactivation. Mol. Cell **5:**939–948.
15. **Moradpour, D., F. Penin, and C. M. Rice.** 2007. Replication of hepatitis C virus. Nat. Rev. Microbiol. **5:**453–463.
16. **Moriishi, K., R. Mochizuki, K. Moriya, H. Miyamoto, Y. Mori, T. Abe, S. Murata, K. Tanaka, T. Miyamura, T. Suzuki, K. Koike, and Y. Matsuura.** 2007. Critical role of PA28γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. Proc. Natl. Acad. Sci. USA **104:**1661–1666.
17. **Moriishi, K., T. Okabayashi, K. Nakai, K. Moriya, K. Koike, S. Murata, T. Chiba, K. Tanaka, R. Suzuki, T. Suzuki, T. Miyamura, and Y. Matsuura.** 2003. Proteasome activator PA28γ-dependent nuclear retention and degradation of hepatitis C virus core protein. J. Virol. **77:**10237–10249.
18. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108:**193–199.
19. **Realini, C., C. C. Jensen, Z. Zhang, S. C. Johnston, J. R. Knowlton, C. P. Hill, and M. Rechsteiner.** 1997. Characterization of recombinant REGα, REGβ, and REGγ proteasome activators. J. Biol. Chem. **272:**25483–25492.
20. **Santolini, E., G. Migliaccio, and N. La Monica.** 1994. Biosynthesis and biochemical properties of the hepatitis C virus core protein. J. Virol. **68:**3631–3641.
21. **Sheaff, R. J., J. D. Singer, J. Swanger, M. Smitherman, J. M. Roberts, and B. E. Clurman.** 2000. Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination. Mol. Cell **5:**403–410.
22. **Shirakura, M., K. Murakami, T. Ichimura, R. Suzuki, T. Shimoji, K. Fukuda, K. Abe, S. Sato, M. Fukasawa, Y. Yamakawa, M. Nishijima, K. Moriishi, Y. Matsuura, T. Wakita, T. Suzuki, P. M. Howley, T. Miyamura, and I. Shoji.** 2007. E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. J. Virol. **81:**1174–1185.
23. **Suzuki, R., K. Tamura, J. Li, K. Ishii, Y. Matsuura, T. Miyamura, and T. Suzuki.** 2001. Ubiquitin-mediated degradation of hepatitis C virus core protein is regulated by processing at its carboxyl terminus. Virology **280:**301–309.
24. **Suzuki, T., and A. Varshavsky.** 1999. Degradation signals in the lysine-asparagine sequence space. EMBO J. **18:**6017–6026.
25. **Treier, M., L. M. Staszewski, and D. Bohmann.** 1994. Ubiquitin-dependent c-Jun degradation in vivo is mediated by the δ domain. Cell **78:**787–798.
26. **Wu, R. C., Q. Feng, D. M. Lonard, and B. W. O'Malley.** 2007. SRC-3 coactivator functional lifetime is regulated by a phospho-dependent ubiquitin time clock. Cell **129:**1125–1140.
27. **Zhang, Z., and R. Zhang.** 2008. Proteasome activator PA28γ regulates p53 by enhancing its MDM2-mediated degradation. EMBO J. **27:**852–864.

Biochemical and Biophysical Research Communications 377 (2008) 747–751

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Characterization of infectious hepatitis C virus from liver-derived cell lines

Daisuke Akazawa [a,b], Tomoko Date [b], Kenichi Morikawa [b], Asako Murayama [b], Noriaki Omi [a,b], Hitoshi Takahashi [a,b], Noriko Nakamura [a], Koji Ishii [b], Tetsuro Suzuki [b], Masashi Mizokami [c], Hidenori Mochizuki [a], Takaji Wakita [b,*]

[a] Pharmaceutical Research Laboratories, Toray Industries, Inc., Kanagawa, Japan
[b] Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[c] Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

ARTICLE INFO

Article history:
Received 20 September 2008
Available online 23 October 2008

Keywords:
Cell culture
Hepatitis C virus
Infectivity
Particle
Replicon

ABSTRACT

The efficient production of infectious HCV from the JFH-1 strain is restricted to the Huh7 cell line and its derivatives. However, the factors involved in this restriction are unknown. In this study, we examined the production of infectious HCV from other liver-derived cell lines, and characterized the produced viruses. Clones of the Huh7, HepG2, and IMY-N9, harboring the JFH-1 full-genomic replicon, were obtained. The supernatant of each cell clone exhibited infectivity for naïve Huh7. Each infectious supernatant was then characterized by sucrose density gradient. For all of the cell lines, the main peak of the HCV-core protein and RNA exhibited at approximately 1.15 g/mL of buoyant density. However, the supernatant from the IMY-N9 differed from that of Huh7 in the ratio of core:RNA at 1.15 g/mL and significant peaks were also observed at lower density. The virus particles produced from the different cell lines may have different characteristics.

Hepatitis C virus (HCV) is an enveloped virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family. HCV is a human pathogen and HCV infection is a major cause of chronic hepatitis, liver cirrhosis and hepatic carcinoma. The main therapy for HCV is treatment with pegylated-interferon and rivabirin. However, these agents show little effect for patients that have a high titer of HCV-RNA, genotype 1. Thus, it is necessary to develop new, more effective therapies and preventive treatments to counteract HCV infection. It was discovered that a genotype 2a strain of HCV, JFH-1, can efficiently replicate in the Huh7 cell line [1], and an *in vitro* culture model of infectious HCV has also been successfully developed using the JFH-1 genome [2–4]. Recently, it has become possible to produce various chimeric HCV by replacement of the JFH-1 structural protein region with that of other strains. The HCV particles produced from such chimera are expected to lead to the development of a HCV vaccine, and new anti-HCV pharmaceuticals.

The infectious HCV-derived JFH-1 genome was developed using the human hepatoma Huh7 cell line [5]. Although the sub-genomic replicon RNA of JFH-1 can autonomously replicate, not only in Huh7 cells, but in other human liver [6], non-hepatic [7], and mouse [8] cells, infectious HCV production has been restricted to Huh7-derived cells. In this study, we undertook a comparative study of infectious HCV particles produced from different cell lines including Huh7. Infectious HCV particles were successfully produced into the culture media and characterized.

* Corresponding author. Fax: +81 3 5285 1161.
E-mail address: wakita@nih.go.jp (T. Wakita).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.10.063

Materials and methods

Cell culture. Huh7, Huh7.5.1 ([3], a generous gift from Dr. Francis V. Chisari), HepG2, and IMY-N9 cells were cultured at 37 °C in 5% CO_2. The HepG2 cells were cultured in modified Eagle's medium containing 10% fetal bovine serum. All of the other cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, as described previously [6].

Plasmids. The pFGR-JFH1 and pFGR-JFH1/deltaE12 plasmids, encoding the full-genomic replicon, and envelope-deleted replicons, respectively, were generated as previously described [9].

RNA synthesis. RNA synthesis was performed as described previously [2]. Briefly, the pFGR-JFH1 plasmid was digested with XbaI and then treated with Mung Bean nuclease (New England Biolabs, Beverly, MA). The digested plasmid DNA fragment was then purified and used as a template for RNA synthesis. HCV-RNA was synthesized *in vitro* using a MEGAscript™ T7 kit (Ambion, Austin, TX). The synthesized RNA was treated with DNaseI, followed by acid phenol extraction to remove any remaining template DNA.

Establishment of replicon cells. Cell lines harboring FGR-JFH1 replicons were produced as described previously [9]. Briefly, trypsinized cells were washed with Opti-MEM I™ reduced-serum medium (Invitrogen, Carlsbad, CA) and resuspended at 7.5×10^6 cells/mL with Cytomix buffer [1]. RNA (10 μg), synthesized from pFGR-JFH1, was mixed with 400 μL of cell suspension and transferred to an electroporation cuvette (Precision Universal Cuvettes, Thermo Hybrid, Middlesex, UK). The cells were then pulsed at 260 V and 950 μF with the Gene Pulser II™ apparatus (Bio-Rad,

Hercules, CA). Transfected cells were immediately transferred to 10-cm culture dishes, each containing 8 mL of culture medium. G418 (0.8–1.0 mg/mL) (Nacalai Tesque, Kyoto, Japan) was added to the culture medium at 16–24 h after transfection. Culture medium, supplemented with G418, was replaced twice per week. Three weeks after transfection, sparsely grown G418-resistant colonies were independently isolated using a cloning cylinder (Asahi Techno Glass Co., Tokyo, Japan), and were expanded.

Preparation of supernatants from FGR-JFH1 replicon cells. Culture media was collected from Huh7, IMY-N9, and HepG2 cell lines harboring the FGR-JFH1 replicon and was passed through a 0.45-μm filter. Filtrated culture media was then pooled and concentrated 50-fold using Amicon Ultra-15 (100,000 Molecular weight cut off; Millipore, Bedford, MA), and stored at −80 °C until use.

Assay of infection of naïve Huh7 cells. Infection of naïve Huh7 cells were assayed by immunofluorescence and colony formation assays. For the immunofluorescence assay naïve Huh7.5.1 cells were seeded at 1×10^4 cells/well in an 8-well chamber slide (Becton Dickinson, Franklin Lakes, NJ), cultured overnight and then inoculated with diluted culture media containing infectious HCV particles (1×10^6 HCV-RNA copies). At 72 h after inoculation, the cells were fixed in acetone/methanol (1:1) for 10 min at −20 °C, and the infected foci were visualized by immunofluorescence as follows.

An anti-core HCV protein monoclonal antibody 2H9 [2] was added to the cells at 50 μg/mL in BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan). After incubation for 1 h at room temperature, the cells were washed and incubated with a 1:400 dilution of AlexaFluor 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) diluted in BlockAce. The cells were then washed, treated with DAPI solution (Sigma, Saint Louis, MO) at 0.1 μg/mL and examined by Biozero fluorescence microscopy (Keyence, Osaka, Japan).

Colony formation assays were performed as described previously [9]. Briefly, naïve Huh7 cells were inoculated with culture supernatants from replicon-expressing cell lines for 2 h, and then cultured with complete medium. Inoculated cells were cultured for 3 weeks in medium supplemented with G418 (0.3 mg/mL). Cell survival was assessed by staining with crystal violet.

Titration of infectivity. The infectivity titer of the culture supernatants was determined on Huh7.5.1 cells by end point dilution and immunofluorescence as described above. Briefly, each sample was serially diluted 10-fold in DMEM-10% FBS and 100 μL was used to inoculate Huh7.5.1 cells. Infection was examined 72 h post-inoculation by immunofluorescence using a mouse monoclonal anti-core antibody and secondary anti-mouse IgG-Alexa 488 conjugated antibodies. Infectious foci were counted and the titer was calculated and expressed as focus forming units per mL (FFU/mL).

Sucrose density gradient analysis. Concentrated cell supernatants were layered on top of a preformed continuous 10–60% sucrose gradient in TNE buffer containing 10 mM Tris, pH7.5, 150 mM NaCl, and 0.1 mM EDTA. Gradients were centrifuged in an SW41 rotor (Beckman Coulter, Fullerton, CA) at 35,000 rpm for 16 h at 4 °C, and fractions (400 μL each) were collected from the bottom of the tube. The density of each fraction was estimated by weighing a 100 μL drop from each fraction following a gradient run.

Quantification of HCV-core protein and RNA. The level of the HCV-core protein in culture supernatants or sucrose density gradient fractions, was assayed using an immunoassay as described elsewhere [10]. Viral RNA was isolated from harvested culture media, or sucrose density gradient fractions, using the QiaAmp Viral RNA Extraction kit (Qiagen, Tokyo, Japan). The copy number of HCV RNA was determined by real-time detection reverse transcription-polymerase chain reaction (RTD-PCR), using an ABI Prism 7500fast sequence detector system (Applied Biosystems, Tokyo, Japan) [11].

Results

Production of infectious HCV from human liver-derived cell lines

We first determined if it was possible to produce infectious HCV from cell lines other than Huh7. We selected the HepG2 and IMY-N9 cell lines to establish human liver-derived cell lines that enable replication of the JFH-1 genome [6]. Since full-genomic JFH-1 did not transiently replicate in these cells (data not shown), we established FGR-JFH1 replicon cells that stably replicate the JFH-1 genome. In the culture media obtained from these full-genomic replicon cells, HCV-RNA titers were detected by RTD-PCR. The titer of HCV-RNA was highest in the supernatant from an IMY-N9 cell clone and lowest from a HepG2 cell clone (Table 1). When naïve Huh7.5.1 cells were inoculated with culture supernatants from the replicon cells, infected cells could be detected by immunofluorescence using an anti-HCV-core protein antibody (Fig. 1A). These data suggested that HepG2 and IMY-N9 cells are able to produce infectious HCV.

We then compared the specific infectivity of the replicon containing culture supernatants from the different cells. Specific infectivity was calculated by dividing the infectious titer, calculated by immunofluorescence of infectious foci, of the culture media by the titer obtained for HCV-RNA. Using these calculations the culture media from Huh7 and HepG2 cells showed almost the same specific infectivity whereas that from IMY-N9 cell was relatively higher (Table 1). Thus the infectious HCV in the culture media might differ according to the cell line from which it was obtained.

To clarify the differences observed in specific infectivity, we next examined the ability of the various cellular supernatants to induce colony formation. For this assay naïve Huh7 cells were inoculated with culture media of the same HCV-RNA titer as that of the FGR-JFH1 virus and were cultured in G418-containing medium. Cell survival was assayed by staining with crystal violet, and the number of colonies formed was counted. Consistent with the specific infectivity results, the supernatant of the IMY-N9 replicon cell showed higher colony formation compared with that of Huh7 and HepG2 replicon cells (Fig. 1B and C). Thus IMY-N9 cells produce infectious HCV with a relatively higher infectivity than the other cell lines suggesting that the supernatant derived from the different replicon producing cells may differ.

Characterization of the FGR-JFH1 virus from different liver-derived cells

To further characterize potential differences between the viruses produced by the different cell lines we next characterized the FGR-JFH1 virus in the media of the different cell lines by sucrose density gradient analysis. Concentrated cell supernatants were layered on top of a preformed continuous 10–60% sucrose gradient and centrifuged. Twenty-four fractions were collected and the HCV-core protein and RNA was assayed in each fraction. The peak fraction of the HCV-core protein and that of the RNA coincided at a density of 1.15 g/mL in all supernatants. However, the supernatant of the IMY-N9 cells showed different profiles for both the HCV-core protein and RNA compared to those of Huh7. Thus the IMY-N9 cells had a different ratio of

Table 1
Infectivity of the supernatant of replicon cell lines.

Producing cell	HCV-RNA (copies/mL)	Infectious titer (FFU/mL)	Specific infectivity (FFU/RNA copy)
Huh7	$1.36 \pm 0.02 \times 10^8$	$1.30 \pm 0.32 \times 10^4$	9.56×10^{-5}
IMY-N9	$2.80 \pm 0.04 \times 10^8$	$3.75 \pm 0.38 \times 10^4$	1.34×10^{-4}
HepG2	$8.80 \pm 0.75 \times 10^7$	$7.70 \pm 1.41 \times 10^3$	7.96×10^{-5}

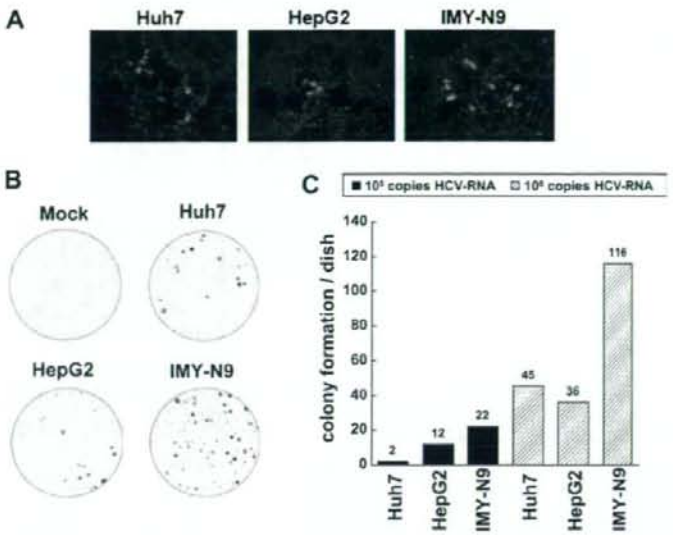

Fig. 1. Naïve Huh7 cell infection assay of JFH-1 full-genomic replicon cell culture supernatants. (A) JFH-1 full-genomic replicon (FGR-JFH1) cells were established in Huh7, HepG2, and IMY-N9 cell lines. Supernatants derived from Huh7 (*left*), HepG2 (*middle*), and IMY-N9 (*right*) cells (1×10^6 HCV-RNA copies) were inoculated into naïve Huh7.5.1 cells (1×10^4) for 48 h, and infected cells were then detected by immunofluorescence using an anti-core antibody (clone 2H9) (green). (B) Naïve Huh7 cells (5×10^5) were inoculated with mock, Huh7, HepG2, and IMY-N9-derived supernatants (10^6 HCV-RNA copies per 10-cm dish) of FGR-JFH1 cells for 2 h. Inoculated cells were cultured for 3 weeks in complete medium supplemented with G418 (0.3 mg/mL), and G418-resistant cells were stained using crystal violet. (C) The number of G418-resistant colonies obtained in (B) was calculated when 10^5 or 10^6 copies of HCV-RNA were tested. Mean values of colony number were indicated in duplicate experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HCV-core protein and RNA at a density of 1.15 g/mL (RNA/Core ratio; Huh7: 511, IMY-N9: 133 copies/fmol) and also showed a secondary peak at lower density (approximately 1.05 g/mL). For all supernatants the peak of infectivity exhibited at a density of 1.10 g/mL that was slightly lower than that of the HCV-core protein and RNA peaks. Furthermore infectivity was barely detectable in the lower density fractions (Fig. 2) suggesting that the HCV-core protein and RNA that was detected at lower density was irrelevant for infectivity of the different supernatants.

We considered the possibility that the core protein and RNA in the lighter fractions may be due to cellular debris containing a replication complex. To determine if this might be the case we therefore analyzed the supernatants from Huh7 and IMY-N9 envelope-deleted replicon cells (FGR-JFH1/deltaE12). The HCV-core protein and RNA were detected in the supernatants of these cells although the titers were very low. These supernatants were not infective for naïve Huh7 cells (data not shown). Furthermore, analysis of the concentrated supernatants of these cell lines by sucrose density gradient analysis detected both the HCV-core protein and RNA, and the major peaks of HCV-RNA were detected in the lower density (approximately 1.10 g/mL) fractions (Fig. 3). However, the profiles of HCV-core protein and RNA did not coincide for either cell line.

Discussion

Infectious HCV can be produced in cell culture by using the JFH-1 genome. This system permits investigation of various aspects of the HCV life cycle such as the steps of entry into cells, replication, and secretion. Infectious HCV derived from JFH-1 is robustly produced in Huh7 cell lines [2,3], and the infectious particles have been characterized. However the difficulty in robustly producing infectious HCV from other cell lines prevents a comparative study of HCV production among different cell lines. In this study, we compared infectious HCV production in Huh7 with that of other cell lines, and characterized the viruses produced.

First, we established Huh7, IMY-N9, and HepG2 FGR-JFH1 replicon cells. These cell lines were able to replicate the JFH-1 subgenomic replicon [6]. The HCV-core protein and RNA were detected in all of the supernatants and all of these supernatants showed infectivity for naïve Huh7. Infectivity was evaluated by transient infection and colony formation assays. These assays indicated that the infectious supernatant from IMY-N9 cell had higher infectivity than the other cell lines for naïve Huh7 cells.

Next, we characterized each supernatant by sucrose density gradient analysis, which revealed both similarities and differences among the infectious supernatants. All samples showed typical peaks at 1.15 g/mL buoyant density for HCV-core protein and RNA, and infectious fractions showed an almost identical buoyant density of 1.10 g/mL. However, the supernatant from the IMY-N9 cells showed a difference in the core/RNA ratio at a density of 1.15 g/mL and higher secondary peak of HCV-core protein and RNA at a lower density (approximately 1.05 g/mL). Since the fractions at lower density did not correlate with infectivity, it is believed that the component at lower density does not contain infectious HCV particles but rather cellular debris that contains HCV proteins, RNA, and lipids [12]. HCV can associate with lipoprotein [13,14], and is secreted with VLDL [15]. Thus, the observed differences in the HCV-producing cells may derive from differences in lipoprotein synthesis. However, it is also possible that the components migrating at lower density contain virus particles. The deletion mutant of FGR-JFH1 (FGR-JFH1/deltaE12) did replicate in Huh7 and IMY-N9 cells, and these replicon cells secreted the HCV-core protein into the culture media, although at low levels. HCV-RNA was also detected in the same culture medium, and the profile of this HCV-RNA differed from that of the HCV-core protein in sucrose density gradient analysis. Thus, the

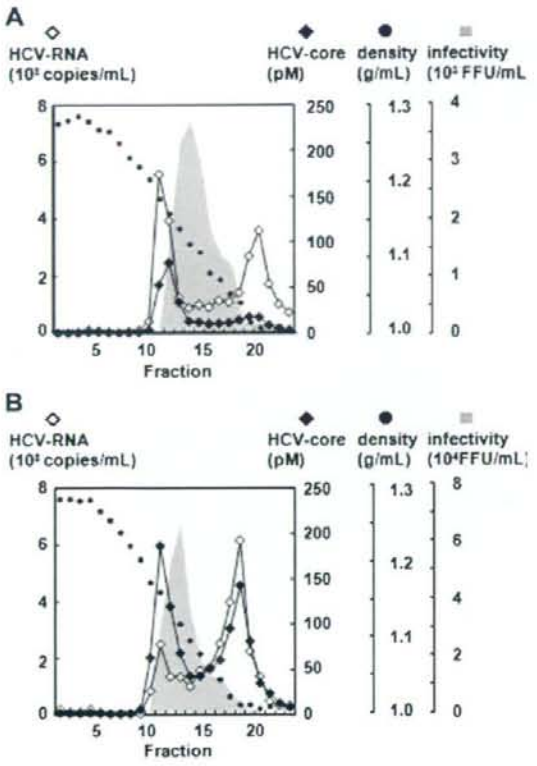

Fig. 2. Density gradient analysis of infectious HCV derived from Huh7 and IMY-N9 cells. Concentrated supernatants of Huh7 cells (A) and IMY-N9 cells (B) were layered on top of a preformed continuous 10–60% sucrose gradient in TNE buffer. The gradients were centrifuged in a SW41 rotor at 35,000 rpm for 16 h at 4 °C, and fractions (400 μL each) were collected from the bottom of the tube. The buoyant density (closed circles), HCV-core protein (closed diamonds), HCV-RNA (open diamonds) and infectivity for naïve Huh7.5.1 cells (shown in gray) was detected in each fraction as described in Materials and methods.

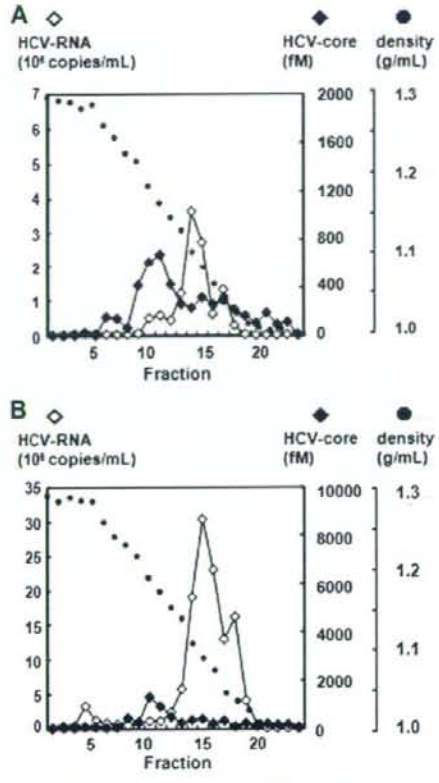

Fig. 3. Density gradient analysis of supernatants derived from Huh7 and IMY-N9 cells transfected with FGR-JFH1/deltaE12 RNA. Concentrated supernatants from Huh7 (A) and IMY-N9 (B) cells were analyzed by sucrose density gradient as described in the legend to Fig. 2. The buoyant density (closed circles), HCV-core protein (closed diamonds) and HCV-RNA (open diamonds) was analyzed in each fraction.

peak fractions containing the HCV-core protein and RNA from the supernatant of FGR-JFH1/deltaE12 cells were different from the peak fractions from that of FGR-JFH1 cells. Therefore it is possible that all of the peaks of HCV-core protein and RNA observed in the supernatant of FGR-JFH1 replicon cells may correlate to virus particles with different densities. However, the reason why they centrifuge at different densities is unclear. Interestingly, the supernatants from cells transfected with envelope-deleted replicon RNA exhibit non-identical HCV-core protein and RNA profiles on a sucrose density gradient. Envelope-deleted replicon RNA may have a decreased ability to form nucleocapsids although a detailed examination is necessary to establish this point.

We previously developed a method for infectious HCV production using the FGR-JFH1 [9], and have now succeeded in producing infectious HCV in the supernatant of cultured liver-derived cell lines harboring FGR-JFH1 RNA. Infectious HCV particles are useful for vaccine production and are considered good antigens for the generation of useful antibodies. Selection of an appropriate cell line is important for the production of HCV particles for vaccine development. The technique used in this study seemed to be appropriate for producing infectious HCV in various cell lines [8].

A second advantage of using HepG2 and IMY-N9 cells for the production of virus particles is that these parental cell lines, unlike the Huh7 cell line, do not express the CD81 molecule on the cell surface, however, the expression on cell clones used in this study was not confirmed. This means that the FGR-JFH1 replicon of these cell lines may have a single cycle of HCV production, encompassing replication, assembly, budding and secretion, and do not show HCV permissiveness. These cells should therefore be useful for the discovery of drugs targeted against HCV assembly and secretion.

Acknowledgments

This work was partially supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science and from the Ministry of Health, Labor, and Welfare of Japan by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation. Huh7 and Huh7.5.1 was a kind gift from Dr. Francis V. Chisari.

References

[1] T. Kato, T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, T. Wakita, Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon, Gastroenterology 125 (2003) 1808–1817.
[2] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.G. Krausslich, M. Mizokami, R. Bartenschlager, T.J. Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.

[3] J. Zhong, P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D.R. Burton, S.F. Wieland, S.L. Uprichard, T. Wakita, F.V. Chisari, Robust hepatitis C virus infection in vitro, Proc. Natl. Acad. Sci. USA 102 (2005) 9294–9299.
[4] B.D. Lindenbach, M.J. Evans, A.J. Syder, B. Wolk, T.L. Tellinghuisen, C.C. Liu, T. Maruyama, R.O. Hynes, D.R. Burton, J.A. McKeating, C.M. Rice, Complete replication of hepatitis C virus in cell culture, Science 309 (2005) 623–626.
[5] H. Nakabayashi, K. Taketa, K. Miyano, T. Yamane, J. Sato, Growth of human hepatoma cells lines with differentiated functions in chemically defined medium, Cancer Res. 42 (1982) 3858–3863.
[6] T. Date, T. Kato, M. Miyamoto, Z. Zhao, K. Yasui, M. Mizokami, T. Wakita, Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells, J. Biol. Chem. 279 (2004) 22371–22376.
[7] T. Kato, T. Date, M. Miyamoto, Z. Zhao, M. Mizokami, T. Wakita, Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon, J. Virol. 79 (2005) 592–596.
[8] S.L. Uprichard, J. Chung, F.V. Chisari, T. Wakita, Replication of a hepatitis C virus replicon clone in mouse cells, Virol. J. 3 (2006) 89.
[9] T. Date, M. Miyamoto, T. Kato, K. Morikawa, A. Murayama, D. Akazawa, J. Tanabe, S. Sone, M. Mizokami, T. Wakita, An infectious and selectable full-length replicon system with hepatitis C virus JFH-1 strain, Hepatol. Res. 37 (2007) 433–443.
[10] K. Aoyagi, C. Ohue, K. Iida, T. Kimura, E. Tanaka, K. Kiyosawa, S. Yagi, Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen, J. Clin. Microbiol. 37 (1999) 1802–1808.
[11] T. Takeuchi, A. Katsume, T. Tanaka, A. Abe, K. Inoue, K. Tsukiyama-Kohara, R. Kawaguchi, S. Tanaka, M. Kohara, Real-time detection system for quantification of hepatitis C virus genome, Gastroenterology 116 (1999) 636–642.
[12] T. Pietschmann, V. Lohmann, A. Kaul, N. Krieger, G. Rinck, G. Rutter, D. Strand, R. Bartenschlager, Persistent and transient replication of full-length hepatitis C virus genomes in cell culture, J. Virol. 76 (2002) 4008–4021.
[13] M. Monazahian, S. Kippenberger, A. Muller, H. Seitz, I. Bohme, S. Grethe, R. Thomssen, Binding of human lipoproteins (low, very low, high density lipoproteins) to recombinant envelope proteins of hepatitis C virus, Med. Microbiol. Immunol. 188 (2000) 177–184.
[14] R. Thomssen, S. Bonk, C. Propfe, K.H. Heermann, H.G. Kochel, A. Uy, Association of hepatitis C virus in human sera with beta-lipoprotein, Med. Microbiol. Immunol. 181 (1992) 293–300.
[15] S.U. Nielsen, M.F. Bassendine, A.D. Burt, C. Martin, W. Pumeechockchai, G.L. Toms, Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients, J. Virol. 80 (2006) 2418–2428.

Hepatitis C Virus JFH-1 Strain Infection in Chimpanzees Is Associated With Low Pathogenicity and Emergence of an Adaptive Mutation

Takanobu Kato,[1-3] Youkyung Choi,[4] Gamal Elmowalid,[1] Ronda K. Sapp,[1] Heidi Barth,[1] Akihiro Furusaka,[5] Shunji Mishiro,[2] Takaji Wakita,[3] Krzysztof Krawczynski,[4] and T. Jake Liang[1]

The identification of the hepatitis C virus (HCV) strain JFH-1 enabled the successful development of infectious cell culture systems. Although this strain replicates efficiently and produces infectious virus in cell culture, the replication capacity and pathogenesis *in vivo* are still undefined. To assess the *in vivo* phenotype of the JFH-1 virus, cell culture–generated JFH-1 virus (JFH-1cc) and patient serum from which JFH-1 was isolated were inoculated into chimpanzees. Both animals became HCV RNA-positive 3 days after inoculation but showed low-level viremia and no evidence of hepatitis. HCV viremia persisted 8 and 34 weeks in JFH-1cc and patient serum–infected chimpanzees, respectively. Immunological analysis revealed that HCV-specific immune responses were similarly induced in both animals. Sequencing of HCV at various times of infection indicated more substitutions in the patient serum–inoculated chimpanzee, and the higher level of sequence variations seemed to be associated with a prolonged infection in this animal. A common mutation G838R in the NS2 region emerged early in both chimpanzees. This mutation enhances viral assembly, leading to an increase in viral production in transfected or infected cells. *Conclusion:* Our study shows that the HCV JFH-1 strain causes attenuated infection and low pathogenicity in chimpanzees and is capable of adapting *in vivo* with a unique mutation conferring an enhanced replicative phenotype. (HEPATOLOGY 2008;48:732-740.)

Hepatitis C virus (HCV) infects approximately 170 million people worldwide and is a major causative agent of chronic liver diseases including cirrhosis and hepatocellular carcinoma.[1,2] However, the underlying biological mechanisms of pathogenesis and persistence are still not well understood. No vaccine protecting against HCV infection is currently available.[3] Therapy for HCV-related chronic hepatitis remains problematic, with limited efficacy, high cost, and substantial adverse effects.[1,4,5] Understanding the biology of this virus and the development of new therapies has been hampered by a lack of appropriate model systems for replication and infection of this virus.

Abbreviations: ALT, alanine aminotransferase; ELISpot, enzyme-linked immunosorbent spot; FFU, focus-forming unit; HCV, hepatitis C virus; HVR, hypervariable region; IFN-γ interferon gamma; JFH-1cc, cell culture generated JFH-1 virus; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription polymerase chain reaction; SFU, spot-forming unit; WT, wild-type.

From the [1]Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD; the [2]Department of Medical Sciences, Toshiba General Hospital, Shinagawa, Tokyo, Japan; the [3]Department of Virology II, National Institute of Infectious Diseases, Shinjuku, Tokyo, Japan; the [4]Division of Viral Hepatitis, Center for Disease Control and Prevention, Atlanta; and the [5]Department of Internal Medicine, Jikei University School of Medicine (Daisan), Komae, Tokyo, Japan.

Received February 4, 2008; accepted May 7, 2008.

TK and TW were partially supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science and from the Ministry of Health, Labor, and Welfare of Japan; and by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation. This work was supported by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases, NIH, and Division of Viral Hepatitis, Center for Disease Control and Prevention.

Dr. Furusaka is currently affiliated with Department of Gastroenterology and Hepatology, Tokyo Medical University, Tokyo, Japan.

Address reprint requests to: Dr. T. Jake Liang, Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892. E-mail: JakeL@bdg10.niddk.nih.gov; fax: 301-402-0491.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22422

Potential conflict of interest: Nothing to report.

Recent progress with a unique HCV genotype 2a strain, JFH-1, isolated from a case of fulminant hepatitis in Japan, has led to the development of a robust HCV infectious cell culture system.[6-9] This JFH-1 strain can replicate efficiently, produce the infectious viral particles, and show robust infection *in vitro*. However, in our previous report, the inoculation of cell culture–generated JFH-1 virus (JFH-1cc) induced only transient and attenuated infection in a chimpanzee.[8] The observed low virulence of this strain *in vivo* was unexpected but consistent, with an inverse relationship between *in vivo* and *in vitro* properties of cell culture adaptive mutations in the HCV replicon system.[10]

In this study, we performed an extensive analysis of the *in vivo* replication and pathogenicity of the JFH-1 strain by inoculating chimpanzees with JFH-1cc and patient serum from which the JFH-1 strain was isolated. Furthermore, we analyzed viral sequences during the infection to identify mutations that might represent *in vivo* adaptive mutations with unique phenotypes.

Materials and Methods

Cell Culture. Huh7 derivative cell lines Huh7.5 and Huh7.5.1 were provided by Charles Rice (Rockefeller University, New York, NY) and Francis Chisari (Scripps Research Institute, La Jolla, CA), respectively.[7,9] The Huh7 derivative clone Huh7-25 that lacks CD81 expression was reported previously.[11]

Inocula. The production of JFH-1cc has been reported previously.[12] Briefly, the full-length JFH-1 RNA was synthesized by *in vitro* transcription with linearized pJFH-1 plasmid and MEGAscript kit (Ambion, Austin, TX).[8] Ten micrograms full-length JFH-1 RNA was transfected into 3.0×10^6 Huh7 cells by electroporation, and the culture medium with JFH-1cc was harvested 5 days after transfection. The culture medium was passed through a 0.45-μm filter unit. The case of fulminant hepatitis C from which the JFH-1 strain was isolated has been reported previously.[6] An aliquot of acute-phase serum (point A as indicated by Kato et al.[6]) was used in this study. To determine the HCV RNA titers in these inocula, total RNA was extracted from 140 μL of these samples by QIAamp Viral RNA Kit (QIAGEN, Valencia, CA), and copy numbers of HCV RNA were determined by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR), as described previously.[13]

Infection Study in Chimpanzees. Housing, maintenance, and care of the chimpanzees used in this study conformed to the requirement for the humane use of animals in scientific research as defined by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention. Chimpanzee 10273 (CH10273, female, age 5, 20 kg) was inoculated intravenously with 100 μL serum (9.6×10^6 copies) from the fulminant hepatitis patient mixed with 400 μL Dulbecco's modified Eagle's medium culture medium. Chimpanzee 10274 (CH10274, female, age 5, 22 kg) was inoculated intravenously with 500 μL Dulbecco's modified Eagle's medium culture medium containing JFH-1cc (1.4×10^7 copies). Serum and liver biopsy samples of these animals were obtained at baseline and weekly after inoculation.

Measurement of HCV RNA, anti-HCV, and Alanine Aminotransferase. HCV RNA in chimpanzees was quantitatively measured by nested RT-PCR with a sensitivity of detection of approximately 50 IU/mL (COBAS Amplicor; Roche Molecular Systems, Pleasanton, CA) and was quantified using Amplicor Monitor (Roche Molecular Systems). Serum samples were tested for anti-HCV (ORTHO version 3.0 enzyme-linked immunosorbent assay test system, Ortho-Clinical Diagnostics, Raritan, NJ). Serum alanine aminotransferase (ALT) values in chimpanzee's sera were established using a commercially available assay kit in accordance with the manufacturer's instructions (Drew Scientific, Dallas, TX). Cutoff values representing 95% confidence limit for the upper level of normal ALT activity were calculated individually for each chimpanzee using 10 pre-inoculation enzyme values obtained over a period of 4 to 6 weeks, and were 73 U/L in CH10274 and 76 U/L in CH10273.

HCV Sequencing. The total RNA was extracted from 280 μL chimpanzee sera collected at appropriate time points by the use of QIAamp viral RNA kit, and complementary DNA was synthesized by use of Superscript III (Invitrogen, Carlsbad, CA). The complementary DNAs were subsequently amplified with TaKaRa LA *Taq* DNA polymerase (Takara Mirus Bio, Madison, WI). Five separate fragments were amplified by nested PCR covering the entire open reading frame and a part of the 5'UTR of the JFH-1 strain as follows; nt 128-1829, nt 1763-4381, nt 4278-6316, nt 6172–7904, and nt 7670-9222. The sequence of each amplified fragment was determined directly. The fragment encompassing hypervariable region 1 (HVR-1) (nt 128-1829) was cloned into the pGEM-T easy vector (Promega, Madison, WI) and 10 clones from each time point were sequenced.

T-Cell Proliferation and Interferon-γ Enzyme-Linked Immunosorbent Spot Assays. The cryopreserved peripheral blood mononuclear cells (PBMCs) were used for immunological analysis. Standard T-cell proliferation assay was performed as described previously.[14] Cells were stimulated with recombinant HCV genotype 2a core or NS5a protein (Fitzgerald Industries Interna-

tional, Concord, MA) and pulsed with ^{3}H-thymidine (GE Healthcare BioSciences, Piscataway, NJ). T-cell stimulation was expressed as a stimulation index that was calculated as the ratio of average counts per minute (CPM) of antigen-stimulated proliferation over average CPM of the medium background. A sample was considered positive when the average stimulation index was greater than 5. The numbers of antigen-specific interferon gamma (IFN-γ)–producing cells were analyzed by enzyme-linked immunosorbent spot (ELISpot) assay. PBMCs were stimulated with recombinant protein antigens (HCV core and NS5a proteins) and HCV overlapping peptide pools (15mers overlapped by 10 amino acids) from core (38 peptides, amino acids 1-195) and NS3 (56 peptides, amino acids 1031-315)(Mimotopes, Raleigh, NC). The NS3 overlapping peptide pools were divided into two sets. The number of spots was counted by using a computer-assisted AID ELISpot Reader System and AID software version 3.5 (Autoimmune Diagnostika GmbH, Strassberg, Germany). Antigen-specific spot-forming unit (SFU) was calculated by subtracting the average of background values (four wells without antigen, typically fewer than 10 spots) from that of the antigen-stimulated sample. The sample was considered positive when the background-corrected SFU was greater than 10 and twice or more the mean SFU of the preinfection samples in the same animal.

To specifically evaluate the T-cell response against the NS2 region containing the G838R mutation, two peptides of 18 amino acids (NS2-G: ITLFTLTPGYKTLLGQCL and NS2-R: ITLFTLTPRYKTLLGQCL) were synthesized (Sigma-Genosys, The Woodlands, TX). PBMCs from both chimpanzees were stimulated with the wild-type (WT) and mutant peptides (2 μg/mL) and analyzed for IFN-γ production by IFN-γ ELISpot assays as described.

Production of JFH-1 G838R Mutant Virus. The full genome JFH-1 construct with G838R mutation in the NS2 region was generated by site-directed mutagenesis. The replication-deficient clone of JFH1 generated by introducing a point mutation into the GDD motif of the NS5B to abolish the RNA-dependent RNA polymerase activity was used as a negative control (JFH-1 GND).[8]

Quantification of HCV RNA and HCV Core Antigen. To determine the amount of HCV, total RNA was extracted with QIAamp Viral RNA Kit from 140 μL culture medium, or with RNeasy mini kit (QIAGEN, Valencia, CA) from cell pellet. Copy numbers of HCV RNA were determined by real-time quantitative RT-PCR as described. HCV core antigen (Ag) in culture supernatant was quantified by highly sensitive enzyme immunoassay (Ortho HCV core antigen ELISA Kit, Ortho Clinical Diagnostics, Tokyo, Japan).[15] To determine intracellular HCV core Ag, the cell pellet was resuspended with 100 μL radioimmune precipitation assay buffer containing 1% sodium dodecyl sulfate, 0.5% NP40, 10 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetra-acetic acid, 150 mM NaCl, and Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), then sonicated 10 minutes and subjected to the Ortho HCV core antigen enzyme-linked immunosorbent assay after centrifugation.

Titration of HCV Infectivity. To assess the intracellular infectivity, cells were harvested by treatment with trypsin-ethylenediaminetetra-acetic acid and pelleted by centrifugation. Cell pellets were resuspended with 500 μL Dulbecco's modified Eagle's medium with 10% fetal bovine serum and lysed by four freeze–thaw cycles. The supernatant was collected after centrifugation and passage through a 0.45-μm filter. These cell lysates and culture supernatants were serially diluted fivefold and inoculated into naïve Huh7.5.1 cells seeded at 1×10^4 cells/well in 96-well flat-bottom plates and assayed for focus-forming unit (FFU) by anti-core immunofluorescence as described previously.[16]

Statistical Analysis. Data from repeated experiments were averaged and expressed as mean ± standard deviation. Statistical analysis was performed using the Mann-Whitney test. P values of less than 0.05 were considered statistically significant.

Results

Clinical, Virological, and Immunological Profiles of JFH-1–Infected Chimpanzees. Chimpanzee 10273 (CH10273) was inoculated with patient serum containing 9.6×10^6 copies of HCV RNA. Chimpanzee 10274 (CH10274) was inoculated with 1.4×10^7 copies of JFH-1cc in culture medium. In both chimpanzees, HCV RNA became detectable in serum by RT-PCR 3 days after inoculation. Viremia was low, with titers of approximately 10^3 copies/mL. Serum ALT levels were within normal limits, and histological observation of liver biopsy showed no evidence of hepatitis (Fig. 1). In CH10273, HCV RNA in serum fluctuated but persisted for 34 weeks after inoculation, and anti-HCV was detected from 20 weeks after inoculation (Fig. 1A). In CH10274, serum HCV RNA disappeared at 9 weeks after inoculation, and no anti-HCV seroconversion was observed (Fig. 1B).

Immunological analysis for T-cell proliferation and IFN-γ production showed that HCV-specific immune responses were induced in both animals (Fig. 1). Their responses corresponded to the profiles of viremia and remained at low levels after disappearance of viremia. The

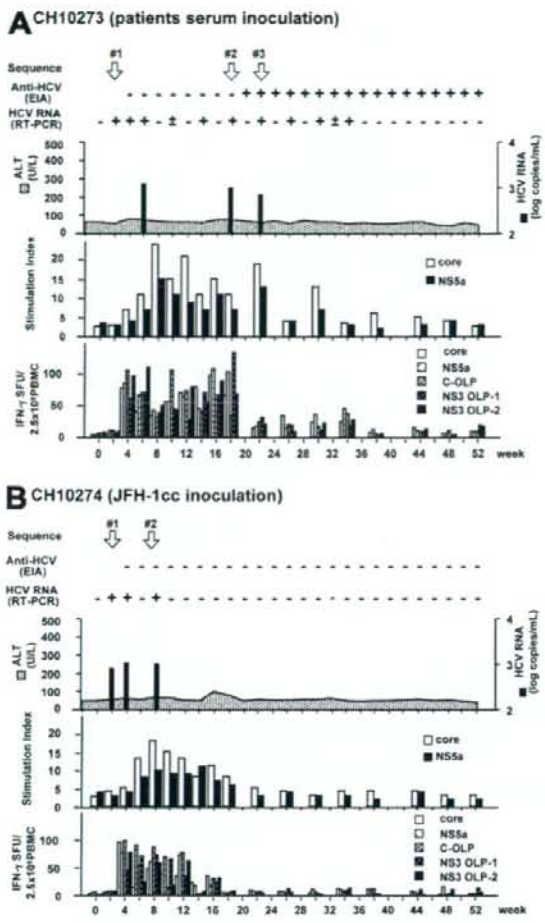

Fig. 1. Infection profiles and T cell immune responses in patient serum-inoculated and JFH-1cc-inoculated chimpanzees. (A) Chimpanzee CH10273 was inoculated with patient serum containing 9.6×10^6 copies of HCV. (B) Chimpanzee CH10274 was inoculated with JFH-1cc containing 1.4×10^7 copies of HCV. White arrows indicate the time points at which HCV sequences were determined. T cell proliferation assay results against HCV core and NS5a are shown as stimulation index (middle panel). IFN-γ responses against HCV core and NS5a proteins or overlapping peptide pools of core and NS3 are shown as SFU per 2.5×10^5 cells (bottom panel).

T-cell proliferative responses against the HCV core and NS5a proteins became positive 4 weeks after inoculation and continued up to 30 and 18 weeks in CH10273 and CH10274, respectively. Likewise, the IFN-γ responses against HCV structural and nonstructural antigens were detected 4 weeks after inoculation and maintained 34 weeks and 16 weeks in CH10273 and CH10274, respectively (Fig. 1).

HCV Sequence Analysis. To investigate the difference and evolution of infected viruses, HCV sequences in both chimpanzees were determined directly at multiple time points as indicated in Fig. 1. In CH10273, HCV sequences were determined with sera collected at weeks 2, 19, and 23. Nineteen synonymous and six nonsynonymous mutations were already observed at week 2, and the number of mutations increased gradually with time (Table 1). Conversely, CH10274 showed no mutation at the earliest time point of infection (week 2) but subsequently developed four synonymous and seven nonsynonymous mutations at week 7 (Table 1). The mutated amino acids in the JFH-1 genome were distributed in E2, NS2, NS5a, and NS5b regions (Fig. 2A). Among these mutations, only one mutation, G838R in NS2, was identified as a common mutation between the two chimpanzees. To assess the complexity of the quasispecies, the amplified fragment encompassing HVR-1 was cloned and 10 clones in each time point were sequenced. In both animals, HVR populations of isolated HCV indicated similarly low complexity of heterogeneity (Fig. 2B). HCV clones isolated from CH10273 contained one HVR-1 mutation N397S at the earliest time point of infection, and this mutation could not be found in clones of the inoculum (Fig. 2B). To exclude the possibility of PCR artifact, sequences were confirmed by independent analyses. To ensure that the common NS2 mutant was not present as a minor species at the earliest time point of CH10274 (week 2), cloning (15 clones) and sequencing was performed and showed the WT sequence.

Effect of the NS2 Mutation on HCV Life Cycle. To assess whether this NS2 mutation could be a result of cytotoxic T-lymphocyte escape, which has been described in acutely HCV-infected chimpanzees,[17] we tested the T cell response of PBMCs from various time points during the infection against 18-mer peptides encompassing this region (both the WT and mutant sequences were tested). No T cell response could be detected against either the WT or mutant peptides throughout the infection, therefore making cytotoxic T-lymphocyte escape mutation highly unlikely. To assess the phenotype of the observed common mutation, G838R in the NS2 region, JFH-1 construct with this mutation was generated (JFH-1

Table 1. Sequence Evolution of JFH-1 in Chimpanzees

	Synonymous Mutations*	Non-synonymous Mutations*	Total
CH10273			
#1 (week 2)	19	6	25
#2 (week 19)	33	15	48
#3 (week 23)	35	17	52
CH10274			
#1 (week 2)	0	0	0
#2 (week 7)	4	7	11

*Compared with the consensus JFH-1 sequence.

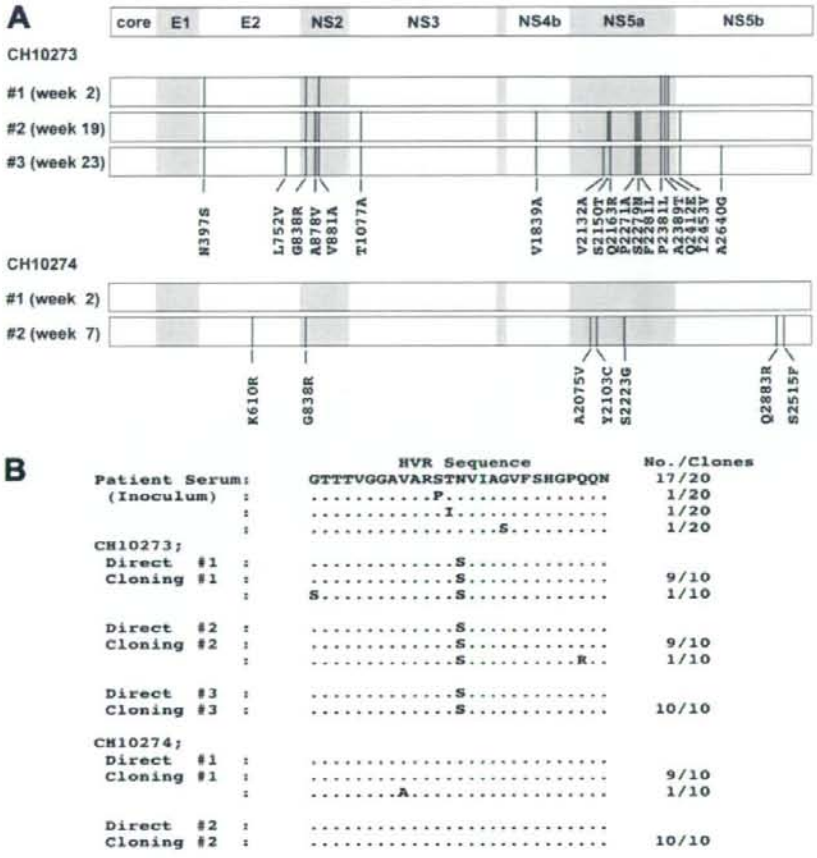

Fig. 2. HCV sequence analyses. (A) Distribution of amino acid substitutions in patient serum–inoculated (CH10273) and JFH-1cc-inoculated (CH10274) chimpanzees. Positions of amino acid substitutions are indicated as vertical bars, and the mutated amino acids are shown at the bottom of each panel. The amino acid numbers correspond to the JFH-1 sequence. (B) HVR-1 populations in patient serum (inoculum) and chimpanzees. HVR-1 sequence in patient serum has been reported previously.[6] HVR-1 sequences determined by direct sequencing (Direct) or cloning and sequencing (Cloning) (10 clones at each time) in each animal are shown. Investigated time points (#1, 2, and 3) are indicated in Fig. 1. Identical amino acids are indicated as dots.

G838R). Viral replication and production of the JFH-1 G838R mutant was compared with that of the WT JFH-1 (JFH-1 WT) by transfecting the *in vitro* transcribed full-length genome RNA into Huh7.5.1 cells. HCV RNA levels in culture media of JFH-1 WT and JFH-1 G838R transfected cells were $2.96 \times 10^6 \pm 1.63 \times 10^5$ and $1.69 \times 10^7 \pm 3.61 \times 10^5$ copies/mL on day 3, and $2.67 \times 10^6 \pm 3.69 \times 10^5$ and $1.14 \times 10^7 \pm 2.23 \times 10^5$ copies/mL on day 5, respectively ($P < 0.05$)(Fig. 3A). In JFH-1 WT and JFH-1 G838R transfected cells, intracellular HCV RNA levels were $1.14 \times 10^8 \pm 1.36 \times 10^7$ and $3.66 \times 10^8 \pm 1.20 \times 10^7$ copies/well on day 3, and $1.67 \times 10^8 \pm 3.94 \times 10^7$ and $2.23 \times 10^8 \pm 1.90 \times 10^7$ copies/well on day 5, respectively ($P < 0.05$) (Fig. 3A). Thus, JFH-1 G838R could produce HCV RNA approximately fivefold higher than the JFH-1 WT in culture media and transfected cells (days 3 and 5, $P < 0.05$).

To confirm this observation, an infection study was also conducted with cell culture–generated viruses. After transfection of JFH1 WT and JFH-1 G838R genome RNA, viruses in culture media were harvested, and FFU of these viruses were titrated. The same titer of JFH1 WT or JFH-1 G838R viruses was inoculated into naïve Huh7.5.1 cells (9×10^2 FFU, multiplicity of infection = 0.003). After infection, HCV RNA titer in culture medium and infected cells was determined. Consistent with the transfection study, HCV RNA levels in culture media of JFH-1 G838R virus–infected cells were threefold to sixfold higher than those of JFH-1 WT virus (days 3 and 5, $P < 0.05$; Fig. 3B). Intracellular HCV RNA level on

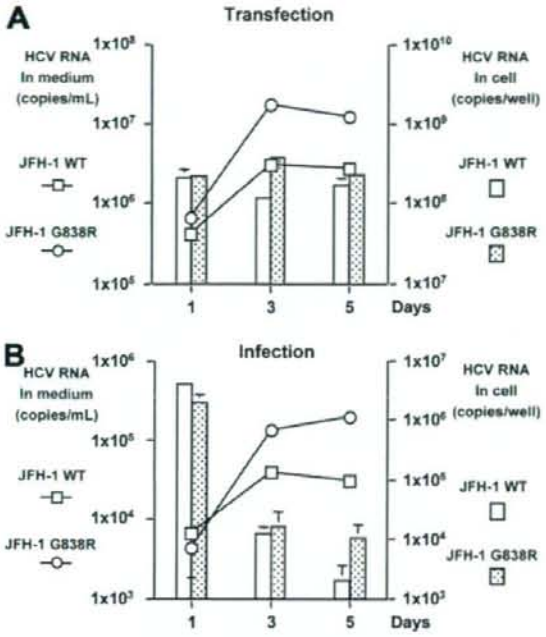

Fig. 3. Comparison of viral replication between JFH-1 WT and JFH-1 G838R in Huh 7.5.1 cells. At various times, HCV RNA was measured in culture media and cells by transfecting the same amount of *in vitro* transcribed full genome RNA (A) and by infecting the same FFU of JFH-1cc at a multiplicity of infection of 0.003 (B). Means of triplicate samples ± standard deviations are shown.

day 5 also appeared to be higher (fivefold) in JFH-1 G838R–infected cells ($P < 0.05$). Based on these data, JFH-1 G838R replicates more efficiently than the WT.

To further investigate the mechanism of this enhanced replication, we reasoned that this mutation could affect any of the viral RNA synthesis, assembly, or secretion steps. To distinguish among these possibilities, we used Huh7-25 cells, a Huh7 cells–derived cell line lacking CD81 expression.[11] This cell line cannot be reinfected by HCV but can support and produce infectious HCV on transfection with the HCV genome, therefore allowing us to address this question without the confounding effect of reinfection. HCV RNA levels of JFH-1 G838R–transfected cells in culture media were eightfold higher on day 1 and threefold higher on day 3 compared with those of JFH-1 WT transfected cells (Fig. 4A, $P < 0.05$). On day 5, the HCV RNA level was still higher in JFH-1 G838R–transfected cells, but the difference was less. The HCV RNA levels of the replication-deficient clone, JFH-1 GND, transfected cells were substantially lower than both NS2 mutant-transfected and WT-transfected cells (Fig. 4A). Similarly, HCV core Ag in culture media showed a significant difference between JFH-1 WT–transfected and JFH-1 G838R–transfected cells (days 1, 3, and 5, $P < 0.05$)(Fig. 4B). HCV core Ag of JFH-1 GND–transfected cells was under the detection limit. In contrast to culture media data, intracellular HCV RNA and core Ag levels in JFH-1 G838R–transfected cells were similar to or slightly lower than those of JFH-1 WT–transfected cells. Therefore, the G838R mutation does not appear to affect RNA replication and probably enhances either the assembly or secretion step.

To distinguish between these two possible effects, we determined the infectivity titer of intracellular viral particles in transfected cells as reported previously.[18] On day 3 after transfection, the intracellular infectivity titer in JFH-1 G838R–transfected cells was approximately four-

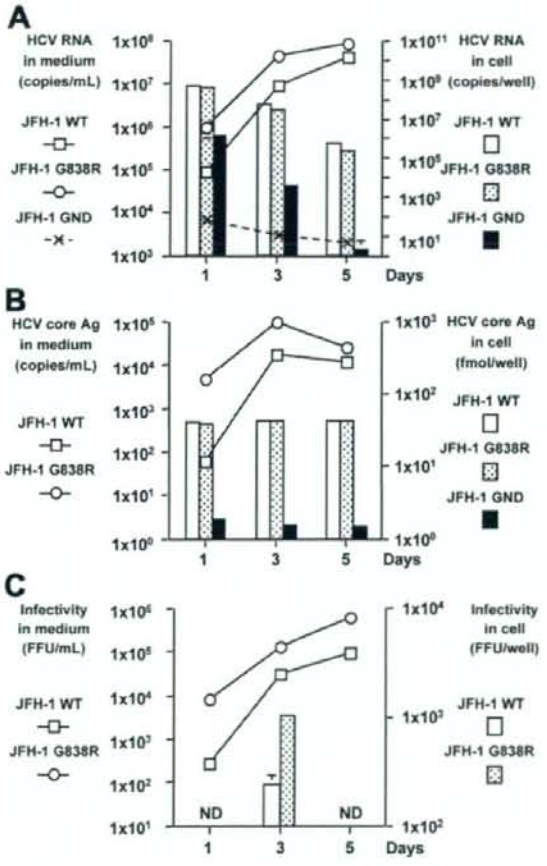

Fig. 4. Comparison of viral replication among JFH-1 WT, JFH-1 G838R, and JFH-1 GND in Huh 7-25 cells. At various times, HCV production was assessed in culture media and cells by transfecting the same amount of *in vitro* transcribed full genome RNA. HCV RNA titer (A), HCV core Ag level (B), and infectivity titers (C) are shown. The data are expressed as means of triplicate samples ± standard deviations. ND, not done.

Table 2. Specific Intectivity and Virus Secretion in Huh7-25 Cells

Clone	Intracellular HCV RNA (copies/well)	Intracellular Intectivity* (FFU/well)	Specific Intracellular Infectivity* (FFU/copies)	Extracellular HCV RNA* (copies/well)	Extracellular Infectivity* (FFU/well)	Specific Exracellular Infectivity (FFU/copies)	Infectious Virus Secretion (extra/intra)
JFH-1 WT	$4.40 \times 10^7 \pm 1.58 \times 10^7$	$2.27 \times 10^2 \pm 5.17 \times 10^1$	$1.09 \times 10^{-5} \pm 2.58 \times 10^{-6}$	$1.83 \times 10^7 \pm 1.95 \times 10^6$	$6.17 \times 10^3 \pm 9.61 \times 10^2$	$3.37 \times 10^{-4} \pm 1.38 \times 10^{-3}$	7.20 ± 2.83
JFH-1 G838R	$2.19 \times 10^7 \pm 1.11 \times 10^6$	$9.89 \times 10^2 \pm 5.02 \times 10^1$	$9.05 \times 10^{-5} \pm 2.76 \times 10^{-6}$	$5.14 \times 10^7 \pm 3.48 \times 10^6$	$2.69 \times 10^4 \pm 6.96 \times 10^3$	$5.33 \times 10^{-4} \pm 1.83 \times 10^{-4}$	6.87 ± 2.07

The data are from day 3 after HCV RNA transfection of the Huh7-25 cells.
*$P < 0.05$ comparing JFH-1 WT and G838R.

fold higher than that in JFH-1 WT–transfected cells ($P < 0.05$, Fig. 4C and Table 2). Moreover, specific intracellular infectivity of JFH-1 G838R–transfected cells was approximately eightfold higher than that in JFH-1 WT–transfected cells ($P < 0.05$, Table 2). Specific infectivity in culture medium was determined as the ratio of infectious virus (FFU) over HCV RNA copies. Specific infectivity of the JFH-1 G838R viruses was not significantly different from that of JFH-1 WT (Table 2). Finally, the rate of secretion was determined by the ratio of extracellular FFU over the intracellular FFU (Table 2), and no difference was observed between JFH-1 WT and G838R-transfected cells. Based on these data, the G838R mutation in JFH-1 enhances the assembly step of HCV.

Discussion

Although HCV-associated fulminant hepatitis is rare, several cases have been reported.[6,19-25] The HCV JFH-1 strain was isolated from one of these cases, and its unique characteristic of robust replication in cell culture might be related to the cause of fulminant hepatitis. Previously, HCV from a patient with fulminant liver failure has been shown to cause severe acute hepatitis with high viremia in a chimpanzee, although its molecular clone could not replicate in culture cells and did not induce severe hepatitis in the chimpanzee.[26,27] In our previous study, JFH-1cc induced a transient and attenuated infection in a chimpanzee.[8] The infection profile was different from the typical course of HCV infection either with patient sera or infectious RNA molecules in chimpanzees.[28-32] Because this observation was unexpected, we reasoned that the lower virulence of this strain *in vivo* might be related to the age of the chimpanzee. The chimpanzee used in the previous study was older (>25 years of age), and older chimpanzees typically do not develop significant disease on HCV infection. Another possible cause was the characteristics of the viral inoculum. JFH-1cc inoculated in the chimpanzee was monotypic because it was generated in culture cells. The original JFH-1 virus replicating in the fulminant hepatitis patient existed as a mixture of various viral species and might induce a different outcome *in vivo*. Thus, to elucidate the pathogenesis and replication capacity of the original JFH-1 strain *in vivo*, the patient serum and the JFH-1cc were inoculated into juvenile chimpanzees (5 years old). However, both chimpanzees showed attenuated infection with low-titer viremia, no ALT elevation, and absence of histological hepatitis during the acute phase of infection. Therefore, the manifestation of fulminant hepatitis of the original patient was likely a result of host factors, with the caveat that humans and chimpanzees might respond differently to HCV infection.

Similar to our previous study, the chimpanzee inoculated with monotypic JFH-1cc showed a short duration of infection and absence of seroconversion. Conversely, the chimpanzee inoculated with the patient serum showed a longer course of infection and developed anti-HCV antibodies. Immunological analysis with T-cell proliferation and IFN-γ ELISpot assays showed that HCV-specific immune responses were similarly induced in both animals and abated with the disappearance of viremia. Consistent with the longer viremia, the chimpanzee inoculated with the patient serum had a longer duration of detectable HCV immune response (Fig. 1). These differences could be explained by the sequence variations of the infecting HCV. In the chimpanzee inoculated with the patient serum, the infecting HCV showed a low sequence complexity but exhibited some sequence diversity already at week 2. The infecting HCV had a sequence alteration in the HVR-1 (N397S), but this sequence alteration could not be found in any of the 20 clones of the inoculum (Fig. 2B).[6] In addition, the NS2 G838R mutation was also not detected by cloning (six clones) and sequencing of the inoculum. Thus, this infecting HCV was probably selected from a minor species in the patient serum. It has been reported that minor clones in human serum were

selected during HCV infection in chimpanzees.[33] The selected clones were in the lighter fraction of the sucrose density gradient of the inoculum, which is devoid of immunoglobulins. Similar selection might have occurred in our study. The dominant clones in the inoculum might not be infectious because of binding to neutralizing antibodies. As a result, the infection-competent minor clone, selected during the infection, became the dominant species. Furthermore, this infecting minor clone could persist longer, although the characteristics of this clone and mechanisms for persistence are still unknown. HCV clones in CH10273 showed several other mutations at 2 weeks postinfection and accumulated additional mutations in E2, NS2, NS3, NS4b, NS5a, and NS5b regions over time (Fig. 2). Some of these regions contain known T-cell epitopes, although the major histocompatibility complex haplotype of this animal is unknown. In this chimpanzee, heterogeneity of the inoculating viruses might have contributed to the emergence of escape mutants from the host immune system, resulting in a prolonged infection. Similar observations have been reported in acute HCV infection in chimpanzees and humans.[34-36]

In HCV strains isolated from these two chimpanzees, one common mutation G838R in the NS2 region was identified. This mutation has not been reported among the adaptive mutations emerged in the JFH-1 virus passaged in cell culture.[37-39] This mutation likely arose *de novo* because one of the chimpanzees was inoculated with a molecular clone, and the week 2 sample did not harbor this mutation. NS2 is a membrane-associated cysteine protease, composed of three transmembrane domains and a protease domain.[40] Although the NS2 region is dispensable for RNA replication, it is essential for production of infectious virus in cultured cells.[41-43] Furthermore, the significance of this region has been shown in the establishment of replication-competent and infection-competent intergenotypical chimeric viruses.[44,45] The identified common mutation G838R was at the end of the first transmembrane domain,[46] and mutations in the transmembrane domains have been shown to improve the yield of infectious virus production in several studies.[45,47] Thus, some advantage of this mutation in HCV replication and production could be expected. This mutation was shown to enhance HCV production in Huh7.5.1 cells. Detailed analysis with CD81-negative Huh7-25 cell demonstrated that viral assembly was affected by this mutation. Production of infectious virus in JFH-1 G838R–transfected cells was eightfold higher than that in the JFH-1 WT–transfected cells. Thus, this mutation enhances the assembly of infectious virus particle in cultured cells, and as a result, increases infectious virus production in the culture medium. This mutation represents the first identified *in vivo* adapted mutation that is not immunologically mediated and probably confers a replication advantage to the virus *in vivo*. This adaptive mutation, unlike the other adaptive mutations reported *in vitro* with poor infectivity *in vivo*, likely results from a highly biologically relevant event in the dynamic interaction between HCV and host. Finally, it is possible that compensatory mutations in other regions of the virus may contribute to the overall biological adaptive response of the virus *in vivo*.

This study demonstrates that the HCV JFH-1 strain either generated in cell culture as a monotypic virus or obtained from patient serum is associated with attenuated infection in chimpanzees; however, the virus can rapidly evolve with adaptive mutations to facilitate propagation of the virus in a susceptible host.

Acknowledgment: The authors thank Charles Rice and Francis Chisari for providing the cell lines and the veterinary staff for technical assistance in the chimpanzee experiment.

References

1. Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann Intern Med 2000; 132:296-305.
2. Feld JJ, Liang TJ. Hepatitis C: identifying patients with progressive liver injury. HEPATOLOGY 2006;43(Suppl):S194-S206.
3. Shiina M, Rehermann B. Hepatitis C vaccines: Inducing and challenging memory T cells. HEPATOLOGY 2006;43:1395-1398.
4. De Francesco R, Migliaccio G. Challenges and successes in developing new therapies for hepatitis C. Nature 2005;436:953-960.
5. Di Bisceglie AM, Hoofnagle JH. Optimal therapy of hepatitis C. HEPATOLOGY 2002;36(Suppl):S121-S127.
6. Kato T, Furusaka A, Miyamoto M, Date T, Yasui K, Hiramoto J, et al. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J Med Virol 2001;64:334-339.
7. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A 2005;102:9294-9299.
8. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
9. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.
10. Bukh J, Pietschmann T, Lohmann V, Krieger N, Faulk K, Engle RE, et al. Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. Proc Natl Acad Sci U S A 2002;99:14416-14421.
11. Akazawa D, Date T, Morikawa K, Murayama A, Miyamoto M, Kaga M, et al. CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. J Virol 2007;81:5036-5045.
12. Kato T, Date T, Murayama A, Morikawa K, Akazawa D, Wakita T. Cell culture and infection system for hepatitis C virus. Nat Protoc 2006;1: 2334-2339.
13. Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-Kohara K, et al. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636-642.

14. Elmowalid GA, Qiao M, Jeong SH, Borg BB, Baumert TF, Sapp RK, et al. Immunization with hepatitis C virus-like particles results in control of hepatitis C virus infection in chimpanzees. Proc Natl Acad Sci U S A 2007;104:8427-8432.
15. Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, et al. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J Clin Microbiol 1999;37:1802-1808.
16. Kato T, Matsumura T, Heller T, Saito S, Sapp RK, Murthy K, et al. Production of infectious hepatitis C virus of various genotypes in cell cultures. J Virol 2007;81:4405-4411.
17. Erickson AL, Kimura Y, Igarashi S, Eichelberger J, Houghton M, Sidney J, et al. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. Immunity 2001;15:883-895.
18. Gastaminza P, Kapadia SB, Chisari FV. Differential biophysical properties of infectious intracellular and secreted hepatitis C virus particles. J Virol 2006;80:11074-11081.
19. Wright TL, Hsu H, Donegan E, Feinstone S, Greenberg H, Read A, et al. Hepatitis C virus not found in fulminant non-A, non-B hepatitis. Ann Intern Med 1991;115:111-112.
20. Liang TJ, Jeffers L, Reddy RK, Silva MO, Cheinquer H, Findor A, et al. Fulminant or subfulminant non-A, non-B viral hepatitis: the role of hepatitis C and E viruses. Gastroenterology 1993;104:556-562.
21. Yoshiba M, Dehara K, Inoue K, Okamoto H, Mayumi M. Contribution of hepatitis C virus to non-A, non-B fulminant hepatitis in Japan. HEPATOLOGY 1994;19:829-835.
22. Villamil FG, Hu KQ, Yu CH, Lee CH, Rojter SE, Podesta LG, et al. Detection of hepatitis C virus with RNA polymerase chain reaction in fulminant hepatic failure. HEPATOLOGY 1995;22:1379-1386.
23. Vento S, Cainelli F, Mirandola F, Cosco L, Di Perri G, Solbiati M, et al. Fulminant hepatitis on withdrawal of chemotherapy in carriers of hepatitis C virus. Lancet 1996;347:92-93.
24. Farci P, Alter HJ, Shimoda A, Govindarajan S, Cheung LC, Melpolder JC, et al. Hepatitis C virus-associated fulminant hepatic failure. N Engl J Med 1996;335:631-634.
25. Fukai K, Yokosuka O, Fujiwara K, Tagawa M, Imazeki F, Saisho H, et al. Etiologic considerations of fulminant non-A, non-B viral hepatitis in Japan: analyses by nucleic acid amplification method. J Infect Dis 1998;178:325-333.
26. Farci P, Munoz SJ, Shimoda A, Govindarajan S, Wong DC, Coiana A, et al. Experimental transmission of hepatitis C virus-associated fulminant hepatitis to a chimpanzee. J Infect Dis 1999;179:1007-1011.
27. Sakai A, Takikawa S, Thimme R, Meunier JC, Spangenberg HC, Govindarajan S, et al. In vivo study of the HC-TN strain of hepatitis C virus recovered from a patient with fulminant hepatitis: RNA transcripts of a molecular clone (pHC-TN) are infectious in chimpanzees but not in Huh7.5 cells. J Virol 2007;81:7208-7219.
28. Kolykhalov AA, Agapov EV, Blight KJ, Mihalik K, Feinstone SM, Rice CM. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science 1997;277:570-574.
29. Hong Z, Beaudet-Miller M, Lanford RE, Guerra B, Wright-Minogue J, Skelton A, et al. Generation of transmissible hepatitis C virions from a molecular clone in chimpanzees. Virology 1999;256:36-44.
30. Beard MR, Abell G, Honda M, Carroll A, Gartland M, Clarke B, et al. An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. HEPATOLOGY 1999;30:316-324.
31. Thomson M, Nascimbeni M, Gonzales S, Murthy KK, Rehermann B, Liang TJ. Emergence of a distinct pattern of viral mutations in chimpanzees infected with a homogeneous inoculum of hepatitis C virus. Gastroenterology 2001;121:1226-1233.
32. Major ME, Mihalik K, Puig M, Rehermann B, Nascimbeni M, Rice CM, et al. Previously infected and recovered chimpanzees exhibit rapid responses that control hepatitis C virus replication upon rechallenge. J Virol 2002;76:6586-6595.
33. Kojima M, Osuga T, Tsuda F, Tanaka T, Okamoto H. Influence of antibodies to the hypervariable region of E2/NS1 glycoprotein on the selective replication of hepatitis C virus in chimpanzees. Virology 1994;204:665-672.
34. Farci P, Shimoda A, Coiana A, Diaz G, Peddis G, Melpolder JC, et al. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science 2000;288:339-344.
35. Erickson AL, Kimura Y, Igarashi S, Eichelberger J, Houghton M, Sidney J, et al. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. Immunity 2001;15:883-895.
36. Timm J, Lauer GM, Kavanagh DG, Sheridan I, Kim AY, Lucas M, et al. CD8 epitope escape and reversion in acute HCV infection. J Exp Med 2004;200:1593-1604.
37. Delgrange D, Pillez A, Castelain S, Cocquerel L, Rouille Y, Dubuisson J, et al. Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins. J Gen Virol 2007;88:2495-2503.
38. Kaul A, Woerz I, Meuleman P, Leroux-Roels G, Bartenschlager R. Cell culture adaptation of hepatitis C virus and in vivo viability of an adapted variant. J Virol 2007;81:13168-13179.
39. Russell RS, Meunier JC, Takikawa S, Faulk K, Engle RE, Bukh J, et al. Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus. Proc Natl Acad Sci U S A 2008;105:4370-4375.
40. Lorenz IC, Marcotrigiano J, Dentzer TG, Rice CM. Structure of the catalytic domain of the hepatitis C virus NS2–3 protease. Nature 2006;442:831-835.
41. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110-113.
42. Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, et al. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 2003;125:1808-1817.
43. Jones CT, Murray CL, Eastman DK, Tassello J, Rice CM. Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. J Virol 2007;81:8374-8383.
44. Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, et al. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc Natl Acad Sci U S A 2006;103:7408-7413.
45. Yi M, Ma Y, Yates J, Lemon SM. Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. J Virol 2007;81:629-638.
46. Yamaga AK, Ou JH. Membrane topology of the hepatitis C virus NS2 protein. J Biol Chem 2002;277:33228-33234.
47. Murray CL, Jones CT, Tassello J, Rice CM. Alanine scanning of the hepatitis C virus core protein reveals numerous residues essential for production of infectious virus. J Virol 2007;81:10220-10231.

Journal of General Virology (2008), **89**, 1587–1592 DOI 10.1099/vir.0.83618-0

Short Communication

Virological characterization of the hepatitis C virus JFH-1 strain in lymphocytic cell lines

Kyoko Murakami,[1] Toshiro Kimura,[1] Motonao Osaki,[1] Koji Ishii,[1] Tatsuo Miyamura,[1] Tetsuro Suzuki,[1] Takaji Wakita[1] and Ikuo Shoji[1,2]

Correspondence
Ikuo Shoji
ishoji@med.kobe-u.ac.jp

[1]Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

[2]Division of Microbiology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan

While hepatocytes are the major site of hepatitis C virus (HCV) infection, a number of studies have suggested that HCV can replicate in lymphocytes. However, *in vitro* culture systems to investigate replication of HCV in lymphocytic cells are severely limited. Robust HCV culture systems have been established using the HCV JFH-1 strain and Huh-7 cells. To gain more insights into the tissue tropism of HCV, we investigated the infection, replication, internal ribosome entry site (IRES)-dependent translation and polyprotein processing of the HCV JFH-1 strain in nine lymphocytic cell lines. HCV JFH-1 failed to infect lymphocytes and replicate, but exhibited efficient polyprotein processing and IRES-dependent translation in lymphocytes as well as in Huh-7 cells. Our results suggest that lymphocytic cells can support HCV JFH-1 translation and polyprotein processing, but may lack some host factors essential for HCV JFH-1 infection and replication.

Received 25 November 2007
Accepted 18 March 2008

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Choo *et al.*, 1989; Saito *et al.*, 1990). Infection with HCV is frequently associated with B-cell-related diseases, such as mixed cryoglobulinaemia and non-Hodgkin's lymphoma (Hausfater *et al.*, 2000). A number of studies have suggested that HCV can replicate not only in hepatocytes, but also in lymphocytes (Ducoulombier *et al.*, 2004; Karavattathayyil *et al.*, 2000, Lerat *et al.*, 1998), whereas the determinants of HCV tropism are still unknown. The development of HCV strain JFH-1, which generates infectious HCV in culture, has made an important contribution to the study of the HCV life cycle (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). The HCV life cycle is divided into several steps. After entry into the cell and uncoating, the HCV life cycle leads to translation, polyprotein processing, RNA replication, virion assembly, transport and release. The JFH-1 subgenomic replicon can replicate in non-hepatic cell lines, such as HeLa cells and 293 cells, suggesting that the host factors required for HCV replication are not hepatocyte-specific (Kato *et al.*, 2005b). The SB strain of HCV (genotype 2b strain) was isolated from an HCV-infected non-Hodgkin's B-cell lymphoma and has been reported to infect B and T cells (Kondo *et al.*, 2007; Sung *et al.*, 2003). The virus titres of the SB strain in lymphocytes were, however, lower than those of JFH-1 in Huh-7 cells and the expression of HCV proteins was not confirmed (Kondo *et al.*, 2007). It is unknown whether HCV JFH-1 can infect and replicate in lymphocytes. To gain more insight into the tissue tropism of HCV infection, we investigated the infection, replication, IRES-dependent translation and polyprotein processing of the JFH-1 strain in nine lymphocytic cell lines.

We first sought to determine whether HCV JFH-1 can infect lymphocytic cell lines. We chose nine lymphocytic cell lines derived from Burkitt's lymphoma, the EBV-immortalized human B cell line, lymphoblasts and acute T-cell leukaemia. C1R, IB4, Namalwa, P3HR1 and Raji cells were Epstein–Barr virus (EBV)-positive (Table 1). Infectious HCV was generated from HCV JFH-1 RNA in Huh-7 cells (Shirakura *et al.*, 2007; Wakita *et al.*, 2005) and the calculation of the 50% tissue culture infectious dose ($TCID_{50}$) was based on methods described previously (Lindenbach *et al.*, 2005). These cell lines (1×10^5 cells per well of a six-well plate) were incubated with 2 ml inoculum (5×10^3 or 5×10^4 $TCID_{50}$ ml^{-1}) for 3 h, washed three times with PBS, and cultured in fresh medium. The culture medium was changed every 2 days. Cells were harvested at 0 (3 h post-infection [p.i.]), 4 and 8 day p.i. HCV core antigen within cells was quantified by immunoassay (Ortho HCV-core ELISA kit; Ortho-Clinical Diagnostics). As shown in Fig. 1(a), increasing the HCV titre of the inoculum resulted in a 7.2-fold increase in the levels of HCV core protein in Huh-7 cells at 3 h p.i. Increasing the HCV titre of the inoculum resulted in a 1.5- to 3.2-fold increase in the levels of the core protein in C1R, BL41,

Table 1. Summary of the virological characterization of HCV JFH-1 in lymphocytes

Name	Source	EBV	Transfection			Concentration of G418 for selection (μg ml^{-1})	HCVcc infection	HCV-RNA replication	Translation*		Polyprotein processing†
			Buffer	Program	Efficiency				HCV-IRES	EMCV-IRES	
Bjab	Burkitt's lymphoma	−	T	T−16	>90 %	600–800	−	−	+	++	+
BL41	Burkitt's lymphoma	−	V	I−10	60–70 %	1000	−	−	+	++	ND
C1R	B lymphoblast	+	V	T−20	70–80 %	100	−	−	++++	++++	+
IB4	Lymphoblastoid	+	V	T−20	80–90 %	1000	−	−	+++	++++	+
Jurkat	Acute T cell leukaemia	−	V	I−10	60–70 %	600	−	−	++	+	ND
Namalwa	Burkitt's lymphoma	+	V	M−13	60–70 %	600–800	−	−	+++	++++	+
P3HR1	Burkitt's lymphoma	+	V	A−23	60–70 %	800	−	−	+++	++++	ND
Raji	Burkitt's lymphoma	+	V	T−27	70–80 %	800	−	−	++	++++	+
Ramos	Burkitt's lymphoma	−	V	M−13	40–60 %	400	−	−	+	+	ND
Huh7	Hepatoma	−	T	T−14	70–80 %	500	+	+	+++	+++	+

*+, <0.25 fold IRES activity of Huh-7; ++, 0.25–0.75 fold; +++, 0.75–1.5-fold; ++++, >1.5-fold.
†ND, Not determined.

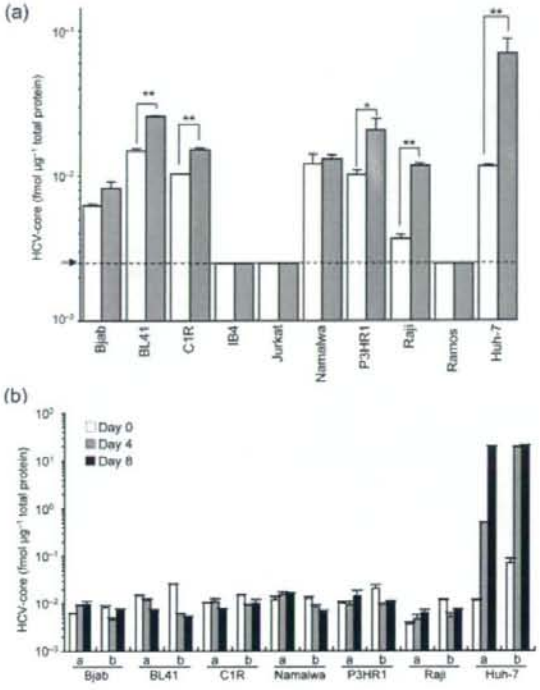

Fig. 1. HCV infection assay. (a) HCV core protein levels 3 h after infection. A total of 1×10^5 cells were infected with 2 ml of the inoculum (5×10^3 [white bars] or 5×10^4 [grey bars] $TCID_{50}$ ml^{-1}) for 3 h at 37 °C and harvested at 3 h p.i. HCV core protein in cell lysate was quantified by ELISA. The average values with standard deviations from triplicate samples are shown. The cut-off value of the immunoassay is indicated by an arrow and a dotted line. The difference between low m.o.i. (white bars) and high m.o.i. (grey bars) was significant (*, $P<0.05$; **, $P<0.01$, Student's *t*-test). (b) Time-course of HCV core protein levels after infection. In total, 1×10^5 cells were infected with 2 ml of the inoculum (5×10^3 [a] or 5×10^4 [b] $TCID_{50}$ ml^{-1}) for 3 h and harvested at 0, 4 and 8 days p.i. HCV core protein in cell lysate was quantified by ELISA. Average values ± SD from triplicate samples are shown.

P3HR1 and Raji cells, suggesting that HCV can bind to these cell lines (Fig. 1a). In contrast, the levels of HCV core protein in IB4, Jurkat and Ramos cells at 3 h p.i. were below the detection limits and there were no significant differences in the levels of the core protein in Bjab cells and Namalwa cells, suggesting that HCV binding to these cells was very inefficient (Fig. 1a). Moreover, the levels of HCV core protein increased in Huh-7 cells but, in the case of all lymphocytic cell lines, including Raji cells, the core titre did not increase at day 4 and 8 p.i., suggesting that HCV JFH-1 does not infect and/or replicate efficiently in these lymphocytic cell lines (Fig. 1b).

To assess the replication of JFH-1 in our lymphocytic cell lines, we utilized the HCV replicon system. To visualize the