TABLE 1. Infectious titers of the media from chimeric HCV RNA-transfected cells

Construct[a]	Core protein level (fmol/ml)	Infectivity (FFU/ml)
JFH-1 (wild type)	50.7 ± 4.1	$8.8 \times 10^3 \pm 5.7 \times 10^2$
JFH-1/GND	0	0
J6CF (wild type)	0	0
J6/N5BX-JFH1	0	0
J6/N3H+N5BX-JFH1	7.7 ± 1.7	$9.1 \times 10^1 \pm 4.1 \times 10^1$
JFH-1/N3H-J6	26.3 ± 3.6	$1.7 \times 10^1 \pm 1.2 \times 10^1$
JFH-1/N5B-J6	0.1 ± 0.0	$6.7 \times 10^0 \pm 4.1 \times 10^0$
JFH-1/3'UTR-J6	23.6 ± 2.9	$2.6 \times 10^3 \pm 7.1 \times 10^2$
JFH-1/N5BX-J6	0	0
JFH-1/N3H+N5B-J6	0	0
JFH-1/N3H+N5BX-J6	0	0

[a] Culture media were collected from the RNA-transfected cells 72 h after transfection.

(Fig. 2B), and currently, there is no clear explanation for this discrepancy. This will be further examined in a future study.

Importantly, we found that the J6/N3H+N5BX-JFH1 chimera produced infectious virus. These results strongly indicate that the NS3 helicase and NS5B-to-3'X regions of JFH-1 are important for autonomous replication of the replication-incompetent J6CF strain and for secretion of infectious chimeric virus, although the virus secretion efficiency and the infection efficiency of the secreted virus were low.

DISCUSSION

In the present study, we identified the regions that are important for efficient JFH-1 replication in Huh7 cells by using chimeric constructs with other genotype 2a clones. Via transient replication assays of JFH-1 and J6CF chimeras, both the NS3 helicase-coding (N3H) region and the NS5B-to-3'X (N5BX) region of JFH-1 were found to be important for replication (Fig. 2 and 3). This was also confirmed by full-length genomic RNA replication, but the replication level of J6/N3H+N5BX-JFH1 was lower than that of wild-type JFH-1 (Fig. 5B). The N5BX region of JFH-1 was the minimum essential region for subgenomic-replicon replication (Fig. 3B, N5BX-JFH-1), but in full-length RNA replication, the NS3 helicase-coding region of JFH-1 was also necessary (Fig. 5B). This contradiction might be explained by differences in RNA length, because shorter RNAs such as subgenomic replicons are likely to replicate even with a less powerful replication engine. Alternatively, there could be some negative element for replication in the J6CF structural-protein-coding region or some positive element in the *neo* encephalomyocarditis virus internal ribosome entry site region of the subgenomic replicon. Furthermore, J6 chimeric RNA with the minimum essential regions of JFH-1 (J6/N3H+N5BX-JFH1) caused Huh7 cells to secrete infectious chimeric virus particles. However, the infection efficiency of J6/N3H+N5BX-JFH1 was lower than that of wild-type JFH-1. First, this may be due to the low RNA replication level. With JFH-1 NS3 helicase and N5BX, J6CF was able to replicate, but the replication efficiency was lower than that of JFH-1 (Fig. 5B). Because J6CF replication could occur only with JFH-1 NS3 helicase and N5BX, more *cis*-acting replication elements (CREs) of JFH-1 may be needed for more efficient replication of J6CF. Second, the levels of virus assembly may be low. This chimera had only the NS3 helicase, NS5B, and 3'UTR regions of JFH-1, possibly omitting some regions important for efficient virus particle secretion. Given that the NS2 region of JFH-1 is reportedly important in virus assembly and release (39), the NS2 region may be a possible candidate. JFH-1/N3H-J6 RNA-transfected cells secreted a substantial amount of core protein; however, its infectivity was much lower (Table 1). The JFH-1 N3H region may be important for the infectivity of the secreted virus and/or for virus particle secretion itself. This will be determined in a future study.

Significance of JFH-1 N5BX for replication. We demonstrated the importance of both the NS5B-coding region and the 3'UTR in JFH-1 replication in the present study. There are several reports regarding CREs within the NS5B-coding region and 3'UTR of Con1 (9, 28, 52). The importance of the interaction between CREs in NS5B and the 3'UTR for replication has also been reported for the Con1 strain (9). The nucleotide sequences involved in the kissing-loop interaction were conserved between JFH-1, J6CF, and Con-1. However, mutations in other regions may affect this interaction by disrupting the RNA secondary structures. On the other hand, given that the NS5B-coding region encodes an RNA-dependent RNA polymerase, the enzymatic activities of the polymerase may differ among the tested strains. The sequence similarities of the JFH-1 and J6CF NS5B regions are 92.2% for the nucleotide sequence and 95.1% for the amino acid sequence. Out of 591 amino acids, only 29 amino acids differ, and the GDD motif that is highly conserved among RdRps is conserved. There are many reports regarding the interaction between NS5B and other viral or cellular proteins, and some of the interactions have been reported to play a role in replication (6, 10, 12, 15, 17, 27, 41–43, 45, 46). Furthermore, the importance of the membrane localization of NS5B with respect to replication has also been reported (29, 35). Mutations in J6CF NS5B may affect these roles. It is thus important to examine the RdRp activities of JFH-1 and J6CF NS5B proteins in vitro.

FIG. 5. Analysis of transient replication of genomic chimeric HCV RNA. (A) Structures of full-length chimeric HCV RNAs. Each chimeric full-length construct was prepared by the insertion of the restricted fragments as indicated. The restriction enzyme recognition sites used for the plasmid constructions are indicated. *C*, ClaI; *E*, EcoT22I; *B*, BsrGI; *S*, StuI; *X*, XbaI; wt, wild type. (B) Northern blot analysis of total RNA prepared from cells transfected with transcribed genomic HCV RNA. Numbers of synthetic JFH-1 RNA (control RNA), RNA isolated from naïve cells (Huh7), and hours after transfection (4, 10, 24, 48, and 72) are indicated. Arrowheads indicate full-length HCV RNA (HCV RNA) and 28S rRNA (28S). A representative autoradiogram (6-h exposure) of three independent experiments is presented. (C) HCV core protein secretion from the RNA-transfected cells. Transcribed wild-type or chimeric full-length HCV RNAs (10 μg) were transfected into Huh7 cells. Culture medium was harvested at 4, 10, 24, 48, and 72 h after transfection. The amounts of core proteins in the harvested culture medium were measured using an HCV core enzyme-linked immunosorbent assay. The assays were performed five times independently, and data are presented as means and standard deviations.

On the other hand, the effect of the 3'UTR is very surprising, especially since the nucleotide sequences of this region are very similar between JFH-1 and J6CF. In this study, the 3'UTR includes four parts: 22 nucleotides at the 3'-end NS5B region (as a result of the cloning strategy), 39 nucleotides of variable region, the poly(U/UC) region, and a 98-nucleotide 3'X region. There are a single synonymous nucleotide mutation in the 3'-end NS5B region and three nucleotide mutations in the variable region. The poly(U/UC) regions are 99 and 132 nucleotides in JFH-1 and J6CF, respectively. There are no mutations in the 3'X region in either strain. It is thus quite interesting to pursue the mechanisms of these mutations in the 3'UTR that affect the HCV RNA replication levels. Further studies are important for precise elucidation of the efficient replication mechanisms of JFH-1.

Significance of the JFH-1 NS3 helicase region for replication. In the present study, we demonstrated the importance of the JFH-1 NS3 helicase region, especially in full-length genomic RNA replication. It has been reported that an active NS3 helicase is required for replication of subgenomic replicons (25). The NS3 helicase domain possesses helicase activity and ATPase activity, and it has been reported that the characters of these enzymes differ among the genotypes and the strains (26). NS3 has also been reported to interact with positive- and negative-strand RNA 3'UTRs (1). One possible model of the role of NS3 in RNA replication is that NS3 helicase unwinds RNA secondary structures and/or a double-stranded RNA intermediate before RNA synthesis by NS5B (37). The sequence similarity of the NS3 helicase regions of JFH-1 and J6CF is rather high, 89.5% for the nucleotide sequence and 93.8% for the amino acid sequence, and out of 487 amino acids, only 30 amino acids differ. These mutations may affect the enzymatic activities of NS3 helicase.

Furthermore, it has been reported that NS3 can stimulate NS5B RdRp activity (38). It has also been reported that the NS3 protease domain and NS5B stimulate NS3 helicase activity (53). Taken together, these findings show that not only the enzymatic activities themselves but also the combination or interaction of the NS3 and NS5B proteins could be important. However, it is still important to examine and compare the NS3 helicase enzymatic activities in vitro of JFH-1 and other HCV strains in a further study.

Replication in vitro and in vivo. We previously reported that JFH-1 RNA could replicate efficiently in Huh7 cells. Cell-cultured JFH-1 virus was also found to be infectious in chimpanzees; however, the virus was cleared immediately after transient viremia (48). In contrast, J6CF does not replicate in Huh7 cells, but it is infectious in chimpanzees (49). J6/JFH-1 chimeric RNA replicated efficiently in Huh7 cells (39) and Huh7-derived cell lines (30), and cell-cultured chimeric J6/JFH-1 virus was infectious in chimpanzees and in chimeric uPA-SCID mice (31). Replication efficiency in vitro may not necessarily correlate with that in vivo. The H77, Con-1, and HCV-N strains were infectious in chimpanzees (3, 5, 23, 50). However, the H77 and Con-1 strains need adaptive mutations for efficient replication in cultured cells (4, 24) and HCV-N replicates relatively efficiently in cultured cells (16). On the other hand, H77-S containing five adaptive mutations can produce infectious virus particles (51), but the Con-1 and HCV-N strains do not produce virus particles (16, 40). It is still unclear what viral or host factors are important for efficient replication and infectious-virus production in vitro and in vivo. However, understanding HCV replication mechanisms by using cell culture models is still important for elucidation of the HCV life cycle.

Significance of the regions responsible for JFH-1 replication. Using two HCV strains, JFH-1 and J6CF, which are very closely related but have different characteristics, we were able to determine which regions are important for replication in cultured cells. Replication of two other genotype 2a strains, JCH-1 and JCH-4, was also recovered by replacement of the N3H and N5BX regions of JFH-1 at the lower levels compared to replication of the J6 replicon (Fig. 3B and 4). This may be because J6CF is an infectious clone in chimpanzees, but the JCH-1 and JCH-4 strains are clinical isolates from chronic-hepatitis patients (21) and may include critical mutations in other important regions. Furthermore, replication of genotype 1 HCV replicons was not restored by the same procedure as that for genotype 2a replicons (Fig. 4). Functional complementation in the nonstructural region and 3'UTR may be difficult beyond the genotypes.

Obtaining virus particles is an important step in antiviral research. Although infection efficiency is improved in permissive cell lines, most HCV strains still cannot replicate or produce virus particles in cultured cells. Therefore, chimeric virus particles with the JFH-1 replication engine may be suitable substitutes. Furthermore, analyses using chimeric viruses that have structural proteins and other regions from various strains may give us new information regarding strain-specific effects on HCV life cycles. Consequently, applying the findings of the present study to replication-incompetent strains may be useful not only for analyses of virus strain specificity and precise analyses of the HCV life cycle but also for antiviral studies.

In conclusion, we analyzed the mechanism underlying efficient JFH-1 replication by using intragenotypic chimeras of JFH-1 and J6CF and clearly showed the importance of the JFH-1 NS3 helicase region and the NS5B-to-3'X region for efficient replication of HCV genotype 2a strains.

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REFERENCES

1. **Banerjee, R., and A. Dasgupta.** 2001. Specific interaction of hepatitis C virus protease/helicase NS3 with the 3'-terminal sequences of viral positive- and negative-strand RNA. J. Virol. **75**:1708–1721.
2. **Bartenschlager, R., and V. Lohmann.** 2000. Replication of hepatitis C virus. J. Gen. Virol. **81**:1631–1648.
3. **Beard, M. R., G. Abell, M. Honda, A. Carroll, M. Gartland, B. Clarke, K. Suzuki, R. Lanford, D. V. Sangar, and S. M. Lemon.** 1999. An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. Hepatology **30**:316–324.
4. **Blight, K. J., J. A. McKeating, J. Marcotrigiano, and C. M. Rice.** 2003.

Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J. Virol. 77:3181–3190.
5. **Bukh, J., T. Pietschmann, V. Lohmann, N. Krieger, K. Faulk, R. E. Engle, S. Govindarajan, M. Shapiro, M. St. Claire, and R. Bartenschlager.** 2002. Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. Proc. Natl. Acad. Sci. USA 99:14416–14421.
6. **Choi, S. H., K. J. Park, B. Y. Ahn, G. Jung, M. M. Lai, and S. B. Hwang.** 2006. Hepatitis C virus nonstructural 5B protein regulates tumor necrosis factor alpha signaling through effects on cellular IκB kinase. Mol. Cell. Biol. 26:3048–3059.
7. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362.
8. **Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina-Selby, P. J. Barr, et al.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA 88:2451–2455.
9. **Friebe, P., J. Boudet, J. P. Simorre, and R. Bartenschlager.** 2005. Kissing-loop interaction in the 3′ end of the hepatitis C virus genome essential for RNA replication. J. Virol. 79:380–392.
10. **Gao, L., H. Tu, S. T. Shi, K. J. Lee, M. Asanaka, S. B. Hwang, and M. M. Lai.** 2003. Interaction with a ubiquitin-like protein enhances the ubiquitination and degradation of hepatitis C virus RNA-dependent RNA polymerase. J. Virol. 77:4149–4159.
11. **Grakoui, A., C. Wychowski, C. Lin, S. M. Feinstone, and C. M. Rice.** 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. J. Virol. 67:1385–1395.
12. **Hamamoto, I., Y. Nishimura, T. Okamoto, H. Aizaki, M. Liu, Y. Mori, T. Abe, T. Suzuki, M. M. Lai, T. Miyamura, K. Moriishi, and Y. Matsuura.** 2005. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. J. Virol. 79:13473–13482.
13. **Hijikata, M., N. Kato, Y. Ootsuyama, M. Nakagawa, and K. Shimotohno.** 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc. Natl. Acad. Sci. USA 88:5547–5551.
14. **Hijikata, M., H. Mizushima, Y. Tanji, Y. Komoda, Y. Hirowatari, T. Akagi, N. Kato, K. Kimura, and K. Shimotohno.** 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc. Natl. Acad. Sci. USA 90:10773–10777.
15. **Hirano, M., S. Kaneko, T. Yamashita, H. Luo, W. Qin, Y. Shirota, T. Nomura, K. Kobayashi, and S. Murakami.** 2003. Direct interaction between nucleolin and hepatitis C virus NS5B. J. Biol. Chem. 278:5109–5115.
16. **Ikeda, M., M. Yi, K. Li, and S. M. Lemon.** 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J. Virol. 76:2997–3006.
17. **Ishido, S., T. Fujita, and H. Hotta.** 1998. Complex formation of NS5B with NS3 and NS4A proteins of hepatitis C virus. Biochem. Biophys. Res. Commun. 244:35–40.
18. **Kato, N., M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugimura, and K. Shimotohno.** 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. USA 87:9524–9528.
19. **Kato, T., T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, and T. Wakita.** 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 125:1808–1817.
20. **Kato, T., T. Date, M. Miyamoto, M. Sugiyama, Y. Tanaka, E. Orito, T. Ohno, K. Sugihara, I. Hasegawa, K. Fujiwara, K. Ito, A. Ozasa, M. Mizokami, and T. Wakita.** 2005. Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. J. Clin. Microbiol. 43:5679–5684.
21. **Kato, T., A. Furusaka, M. Miyamoto, T. Date, K. Yasui, J. Hiramoto, K. Nagayama, T. Tanaka, and T. Wakita.** 2001. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J. Med. Virol. 64:334–339.
22. **Kiyosawa, K., T. Sodeyama, E. Tanaka, Y. Gibo, K. Yoshizawa, Y. Nakano, S. Furuta, Y. Akahane, K. Nishioka, R. H. Purcell, et al.** 1990. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 12:671–675.
23. **Kolykhalov, A. A., E. V. Agapov, K. J. Blight, K. Mihalik, S. M. Feinstone, and C. M. Rice.** 1997. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science 277:570–574.
24. **Krieger, N., V. Lohmann, and R. Bartenschlager.** 2001. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J. Virol. 75:4614–4624.
25. **Lam, A. M., and D. N. Frick.** 2006. Hepatitis C virus subgenomic replicon requires an active NS3 RNA helicase. J. Virol. 80:404–411.
26. **Lam, A. M., D. Keeney, P. Q. Eckert, and D. N. Frick.** 2003. Hepatitis C virus NS3 ATPases/helicases from different genotypes exhibit variations in enzymatic properties. J. Virol. 77:3950–3961.
27. **Lan, S., H. Wang, H. Jiang, H. Mao, X. Liu, X. Zhang, Y. Hu, L. Xiang, and Z. Yuan.** 2003. Direct interaction between alpha-actinin and hepatitis C virus NS5B. FEBS Lett. 554:289–294.
28. **Lee, H., H. Shin, E. Wimmer, and A. V. Paul.** 2004. *cis*-Acting RNA signals in the NS5B C-terminal coding sequence of the hepatitis C virus genome. J. Virol. 78:10865–10877.
29. **Lee, K. J., J. Choi, J. H. Ou, and M. M. Lai.** 2004. The C-terminal transmembrane domain of hepatitis C virus (HCV) RNA polymerase is essential for HCV replication in vivo. J. Virol. 78:3797–3802.
30. **Lindenbach, B. D., 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, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science 309:623–626.
31. **Lindenbach, B. D., P. Meuleman, A. Ploss, T. Vanwolleghem, A. J. Syder, J. A. McKeating, R. E. Lanford, S. M. Feinstone, M. E. Major, G. Leroux-Roels, and C. M. Rice.** 2006. Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. Proc. Natl. Acad. Sci. USA 103:3805–3809.
32. **Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285:110–113.
33. **McLauchlan, J., M. K. Lemberg, G. Hope, and B. Martoglio.** 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J. 21:3980–3988.
34. **Miyamoto, M., T. Kato, T. Date, M. Mizokami, and T. Wakita.** 2006. Comparison between subgenomic replicons of hepatitis C virus genotypes 2a (JFH-1) and 1b (Con1 NK5.1). Intervirology 49:37–43.
35. **Moradpour, D., V. Brass, E. Bieck, P. Friebe, R. Gosert, H. E. Blum, R. Bartenschlager, F. Penin, and V. Lohmann.** 2004. Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication. J. Virol. 78:13278–13284.
36. **Nakabayashi, H., K. Taketa, K. Miyano, T. Yamane, and J. Sato.** 1982. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res. 42:3858–3863.
37. **Paolini, C., R. De Francesco, and P. Gallinari.** 2000. Enzymatic properties of hepatitis C virus NS3-associated helicase. J. Gen. Virol. 81:1335–1345.
38. **Piccininni, S., A. Varaklioti, M. Nardelli, B. Dave, K. D. Raney, and J. E. McCarthy.** 2002. Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. J. Biol. Chem. 277:45670–45679.
39. **Pietschmann, T., A. Kaul, G. Koutsoudakis, A. Shavinskaya, S. Kallis, E. Steinmann, K. Abid, F. Negro, M. Dreux, F. L. Cosset, and R. Bartenschlager.** 2006. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc. Natl. Acad. Sci. USA 103:7408–7413.
40. **Pietschmann, T., V. Lohmann, A. Kaul, N. Krieger, G. Rinck, G. Rutter, D. Strand, and R. Bartenschlager.** 2002. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J. Virol. 76:4008–4021.
41. **Shimakami, T., M. Hijikata, H. Luo, Y. Y. Ma, S. Kaneko, K. Shimotohno, and S. Murakami.** 2004. Effect of interaction between hepatitis C virus NS5A and NS5B on hepatitis C virus RNA replication with the hepatitis C virus replicon. J. Virol. 78:2738–2748.
42. **Shimakami, T., M. Honda, T. Kusakawa, T. Murata, K. Shimotohno, S. Kaneko, and S. Murakami.** 2006. Effect of hepatitis C virus (HCV) NS5B-nucleolin interaction on HCV replication with HCV subgenomic replicon. J. Virol. 80:3332–3340.
43. **Shirota, Y., H. Luo, W. Qin, S. Kaneko, T. Yamashita, K. Kobayashi, and S. Murakami.** 2002. Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. J. Biol. Chem. 277:11149–11155.
44. **Takamizawa, A., C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, and H. Okayama.** 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. J. Virol. 65:1105–1113.
45. **Tu, H., L. Gao, S. T. Shi, D. R. Taylor, T. Yang, A. K. Mircheff, Y. Wen, A. E. Gorbalenya, S. B. Hwang, and M. M. Lai.** 1999. Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. Virology 263:30–41.
46. **Uchida, M., N. Hino, T. Yamanaka, H. Fukushima, T. Imanishi, Y. Uchiyama, T. Kodama, and T. Doi.** 2002. Hepatitis C virus core protein binds to a C-terminal region of NS5B RNA polymerase. Hepatol. Res. 22:297–306.
47. **van den Hoff, M. J., A. F. Moorman, and W. H. Lamers.** 1992. Electroporation in 'intracellular' buffer increases cell survival. Nucleic Acids Res. 20:2902.
48. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11:791–796.
49. **Yanagi, M., R. H. Purcell, S. U. Emerson, and J. Bukh.** 1999. Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. Virology 262:250–263.
50. **Yanagi, M., R. H. Purcell, S. U. Emerson, and J. Bukh.** 1997. Transcripts

from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. Proc. Natl. Acad. Sci. USA 94:8738–8743.
51. **Yi, M., R. A. Villanueva, D. L. Thomas, T. Wakita, and S. M. Lemon.** 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. USA 103:2310–2315.
52. **You, S., D. D. Stump, A. D. Branch, and C. M. Rice.** 2004. A *cis*-acting replication element in the sequence encoding the NS5B RNA-dependent RNA polymerase is required for hepatitis C virus RNA replication. J. Virol. 78:1352–1366.
53. **Zhang, C., Z. Cai, Y. C. Kim, R. Kumar, F. Yuan, P. Y. Shi, C. Kao, and G. Luo.** 2005. Stimulation of hepatitis C virus (HCV) nonstructural protein 3 (NS3) helicase activity by the NS3 protease domain and by HCV RNA-dependent RNA polymerase. J. Virol. 79:8687–8697.
54. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA 102:9294–9299.

PROTOCOL

Cell culture and infection system for hepatitis C virus

Takanobu Kato[1], Tomoko Date[2], Asako Murayama[2], Kenichi Morikawa[2], Daisuke Akazawa[2,3] & Takaji Wakita[2]

[1]Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA. [2]Department of Virology II, National Institute of Infectious Diseases, Shinjuku, Tokyo 162-8640, Japan. [3]Pharmaceutical Research Lab, Toray Industries, Kanagawa 248-8555, Japan. Correspondence should be addressed to T.W. (wakita@nih.go.jp).

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Hepatitis C virus (HCV) infection causes chronic liver disease and is a worldwide health problem. Despite ever-increasing demand for knowledge on viral replication and pathogenesis, detailed analysis has been hampered by a lack of efficient viral culture systems. We isolated HCV genotype 2a strain JFH-1 from a patient with fulminant hepatitis. This strain replicates efficiently in Huh7 cells. Efficient replication and secretion of recombinant viral particles can be obtained in cell culture by transfection of *in vitro*–transcribed full-length JFH-1 RNA into Huh7 cells. JFH-1 virus generated in cell culture is infectious for both naive Huh7 cells and chimpanzees. The efficiency of viral production and infectivity of generated virus is substantially improved with permissive cell lines. This protocol describes how to use this system, which provides a powerful tool for studying viral life cycle and for the construction of antiviral strategies and the development of effective vaccines. Viral particles can be obtained in 12 days with this protocol.

INTRODUCTION

Hepatitis C virus (HCV) is a chief causative agent of chronic liver disease and affects about 170 million people worldwide at present. This virus has the ability to cause persistent infection in susceptible hosts after parenteral transmission, and the underlying mechanisms are not well understood. No vaccine protecting against HCV infection is available. Therapy for HCV-related chronic hepatitis remains problematic, with limited efficacy, high cost and substantial adverse effects. Understanding the biology of this virus and developing new therapies have been hampered by a lack of appropriate model systems for replication and infection. Although many attempts have been made to establish an *in vivo* model that mimics HCV replication, sufficient replication has not been achieved. A unique HCV genotype 2a strain, JFH-1, was isolated from a Japanese patient with fulminant hepatitis[1]. This strain was found to replicate efficiently in cultured cells as a subgenomic replicon in the Huh7 human hepatoma cell line[2]. Using this strain, an efficient cell culture and infection system for HCV has been established[3,4].

Isolation of the JFH-1 strain

The HCV JFH-1 strain was isolated from a patient with fulminant hepatitis[1]. The patient was a 32-year-old man who was admitted with general fatigue, high-grade fever and liver dysfunction. He had high concentrations of serum aspartate aminotransferase and alanine aminotransferase, a low minimum prothrombin time and stage II encephalopathy. HCV RNA was detected by RT-PCR, and the patient was negative for antibodies to HCV. All other hepatitis virus markers were negative. The patient was diagnosed with HCV-associated fulminant hepatitis. HCV RNA was isolated from acute-phase serum and the entire genome was sequenced. The genome of this HCV strain, designated JFH-1, was analyzed phylogenetically and was classified as genotype 2a, but a slight deviation from other genotype 2a strains was identified[1].

Replication and viral secretion of JFH-1 strain in cell culture

For investigation of the replication capacity of this JFH-1 strain, a subgenomic JFH-1 replicon was constructed[2]. Colony formation efficiency of JFH-1 replicon was much higher than that of the prototype Con1 replicon or the adaptive mutants containing Con1 replicon in Huh7 cells. This JFH-1 replicon replicated not only in Huh7 cells but also in the HepG2 and IMY-N9 hepatocyte-derived cell lines and the HeLa and 293 non-hepatocyte-derived cell lines[5,6]. That difference may be due to the replication capacity of JFH-1. Notably, the JFH-1 replicon did not require an adaptive mutation to replicate in those cell lines[2,5,6]. Full-length HCV RNA containing multiple cell-culture adaptive mutations has been reported as not demonstrating active HCV infection[7]. High replication capacity without the need for adaptive mutations thus seems to be an important factor in development of an HCV infection system.

Taking advantage of that replication efficiency, full-length JFH-1 cDNA was constructed for use in assaying replication of viral RNA in transfected Huh7 cells[3]. JFH-1 consensus full-length cDNA has been cloned from RT-PCR fragments. For production of full-length JFH-1 RNA, the T7 promoter sequence was inserted immediately upstream of the full-length JFH-1 cDNA sequence and then used T7 RNA polymerase to transcribe the RNA. When synthesized full-length JFH-1 RNA was transfected into naive Huh7 cells, viral RNA replication and viral protein expression were found in transfected cells. Secretion of viral particles into culture medium was confirmed by sucrose density gradient assay. Viral RNA and all structural proteins (core, E1 and E2) were detected in fractions with a density of around 1.15–1.17 g ml^{-1}, suggesting the formation and secretion of complete viral particles. Immuno-electron microscopy was used to visualize viral particles; a spherical form of about 55 nm diameter was demonstrated by Bartenschlager's group (University of Heidelberg, Heidelberg, Germany)[3]. The *in vitro* infectivity of the cell culture generated JFH-1 virus was monitored by inoculation of naive Huh7 cells with the culture medium of JFH-1 RNA–transfected cells. Infectivity could be detected by indirect immunofluorescence microscopy, although the infectious titer was very low. The *in vivo* infectivity of this cell culture generated virus was also assessed by inoculation of a chimpanzee by Liang's group (National Institutes of Health, Bethesda, Maryland)[3]. The chimpanzee established transient viremia after inoculation of culture medium containing 8×10^3 copies of HCV RNA. These experiments confirmed both the *in vitro* and *in vivo* infectivity of cell culture–generated JFH-1 virus.

Permissive cell lines for HCV replication

To our knowledge, JFH-1 is the first HCV strain found to replicate efficiently and produce infectious virus particles in cultured cells. However, the efficiency of virus generation and infection was very low in the first study with standard Huh7 cells. That limitation was overcome with permissive cell lines[4,8]. Those cells were generated from replicon cell lines by eradication of the replicon with interferon-α and interferon-γ, and they are known to support HCV replication in replicon studies[4,9]. Efficient production of infectious JFH-1 virus in Huh7.5.1 cells or infectious chimeric virus in Huh7.5 cells have been independently reported by Chisari's group (Scripps Research Institute, La Jolla, California)[4] and Rice's group (Rockefeller University, New York, New York)[8]. All the groups used a similar procedure to produce virus particles. They also found that Huh7.5 and Huh7.5.1 cells were more susceptible to virus infection than were the standard Huh7 cells. These cell lines are capable of contributing to efficient viral production and infection in this system.

Biohazardous materials

Classifications for levels of infectious agents differ among countries. Furthermore, regulations regarding infectious agents differ among institutes and countries. Infectious HCV should be handled according to the applicable regulations. For example, HCV is infectious for humans and is designated a 'level 2' infectious agents in Japan and the United States and 'level 3' in France. HCV should thus be handled in a Biosafety Level 2 or Level 3 facility according to national regulations[10]. All liquid and solid wastes should be disposed of after autoclaving.

MATERIALS

REAGENTS

- Plasmid for full-length JFH-1 RNA transcription (pJFH1) ▲ **CRITICAL** The entire sequence of pJFH1 should be confirmed before use.
- Restriction enzyme *Xba*I (high concentration) and reaction buffer (NEBuffer 2; New England BioLabs, cat. no. R0145M)
- Nuclease-free water (Ambion, cat. no. 9938)
- 1 Kb Plus DNA Ladder (Invitrogen, cat. no. 10787-018)
- Ethidium bromide (10 mg ml^{-1}; Invitrogen, cat. no. 15585-011) ! **CAUTION** Ethidium bromide is mutagenic. Wear gloves when handling.
- Phenol–chloroform–isoamyl alcohol (25:24:1 (vol/vol/vol); Invitrogen, cat. no. 15593-031) ! **CAUTION** The phenol–chloroform–isoamyl alcohol mixture is harmful. Handle with appropriate safety equipment.
- Chloroform (Sigma, cat. no. C2432) ! **CAUTION** Chloroform is harmful. Handle with appropriate safety equipment.
- 3 M sodium acetate
- Ethanol (molecular biology grade), 99.5% and 70%
- Glycogen (molecular biology grade)
- Mung bean nuclease and buffer (New England BioLabs, cat. no. M0250S)
- Proteinase K (Invitrogen, cat. no. 25530-049)
- SDS (Sigma, cat. no. L4390) ! **CAUTION** SDS is harmful. Handle with appropriate safety equipment.
- λ DNA–*Hin*dIII fragment marker (Invitrogen, cat. no. 15612-013)
- MEGAscript T7 kit (Ambion, cat. no. 1334)
- TRIzol LS (Invitrogen, cat. no. 10296-010) ! **CAUTION** This reagent contains phenol and is harmful. Handle with appropriate safety equipment.
- RNA ladder (New England BioLabs, cat. no. N0362S)
- Huh7 cells
- Trypsin-EDTA (liquid; Invitrogen, cat. no. 25300-054)
- OptiMEM I reduced-serum medium (Invitrogen, cat. no. 31985-070)
- Cytomix buffer[11] (see REAGENT SETUP)
- Adenosine 5′-triphosphate (ATP; Sigma, cat. no. A-7699)
- L-glutathione (Sigma, cat. no. G-6529)
- Complete medium (see REAGENT SETUP)
- DMEM (low-glucose; Invitrogen, cat. no. 12567-014)
- FBS (appropriate lot for Huh7 cell and their derivatives)
- Penicillin-streptomycin (100× liquid; Invitrogen, cat. no. 15140-122)
- Nonessential amino acids solution (100×; Invitrogen, cat. no. 11140-050)
- L-glutamine (100×; Invitrogen, cat. no. 25030-081)
- HEPES (1-M solution; Invitrogen, cat. no. 11344-041)
- PBS (–) (see REAGENT SETUP)
- KCl (1-M solution; Sigma, cat. no. 60142)
- KH_2PO_4 (Sigma, cat. no. P9791)
- Methanol (molecular biology grade), 100%
- **Immunofluorescence (IF) buffer** (see REAGENT SETUP)
- Bovine serum albumin (Sigma, cat. no. A2153)
- EDTA (Sigma, cat. no. E5134)
- Antibody to HCV (anti-HCV; e.g., anti–Core C7-50; Affinity BioReagents, cat. no. MA1-080)
- Fluorescent dye–conjugated anti–mouse IgG (Alexa Fluor 488; Invitrogen, cat. no. A11029)

EQUIPMENT

- Agarose gel and apparatus for electrophoresis
- Spectrophotometer (Beckman)
- Electroporation cuvette (0.4-cm gap width; Thermo Hybrid, cat. no. EPECU104)
- Gene Pulser II (Bio-Rad)
- 10-cm culture dish (Corning, cat. no. 430167)
- Bottletop filter unit (0.22-μm PES; Corning, cat. no. 431096)
- Syringe-top disk filter (0.22-μm and 0.45-μm; Millipore, cat. nos. SLGS 033 SS and SLHV 033 RS)
- Amicon Ultra-15 (100,000 NMWL membrane; Millipore, cat. no. UFC9 100 08)
- Poly-D-lysine–coated 96-well plate (Corning, cat. no. 3665)
- Fluorescent microscope

REAGENT SETUP

TE buffer This buffer is 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0.

ATP solution To prepare, dissolve 0.28 g ATP in 5 ml nuclease-free water. Sterilize with a 0.22-μm syringe-top filter unit.

L-glutathione solution To prepare, dissolve 0.38 g L-glutathione in 5 ml nuclease-free water. Sterilize with a 0.22-μm syringe-top filter unit.

▲ **CRITICAL** ATP and L-glutathione solutions should be added just before use.

Cytomix buffer Just before use, mix 1 ml cytomix solution, 20 μl ATP solution and 20 μl L-glutathione solution[11]. ▲ **CRITICAL** Cytomix solution should be prepared so it is RNase free.

Complete medium for cell culture This is low-glucose DMEM containing 10% FBS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM L-glutamine, 100 nM nonessential amino acids and 10 mM HEPES. Sterilize with a 0.22-μm bottletop filter unit.

PBS (–) This buffer is 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl and 1.47 mM KH_2PO_4.

IF buffer This buffer is PBS (–) containing 1% bovine serum albumin and 2.5 mM EDTA.

PROCEDURE

Preparation of template for reverse transcription

1| Obtain the pJFH1 plasmid in appropriate quality and quantity (2 μg μl^{-1}). If necessary, include negative control constructs such as a replication-incompetent construct (pJFH1/GND)[3], an envelop region–deletion construct (pJFH1/ΔE1-E2)[3] or a subgenomic replicon (pSGR-JFH1)[2].

2| Digest pJFH1 DNA with restriction enzymes by combining reagents as follows:

Reagent	Amount
pJFH1 DNA (2 μg μl^{-1})	8 μl (16 μg)
NEBuffer 2 (10x)	5 μl (1× final concentration)
*Xba*I (100 U μl^{-1})	1 μl (100 U)
Nuclease-free water	36 μl
Total volume	50 μl

3| Incubate digestion reactions for 1–2 h in a heat block at 37 °C.

4| Check complete digestion of pJFH1 DNA by separating 0.5 μl of a digested sample, along with the 1 Kb Plus DNA Ladder, by electrophoresis through a 1% agarose gel containing 0.1 μg ml^{-1} ethidium bromide.
? TROUBLESHOOTING

5| Mix the digestion product with 50 μl TE buffer. Pipet into the tube 100 μl phenol–chloroform–isoamyl alcohol (25:24:1 (vol/vol/vol); phenol–chloroform–isoamyl alcohol extraction).

6| Shake vigorously and centrifuge for 15 min at 12,000*g* at 20–25 °C (room temperature).

7| Transfer the aqueous phase to a new tube.

8| Pipet 100 μl chloroform into the tube. Shake vigorously and centrifuge for 5 min at 12,000*g* at room temperature (chloroform extraction).

9| Transfer the aqueous phase to a new tube and pipet 1/10 volume 3 M sodium acetate, 2.5 volume 99.5% ethanol and 1/100 volume glycogen into the tube (ethanol precipitation).

10| Store for 20 min at −80 °C.

11| Centrifuge for 20 min at 12,000*g* and 4 °C.

12| Discard the supernatant and wash the pellet with 500 μl 70% ethanol.

13| Centrifuge for 15 min at 12,000*g* and 4 °C.

14| Discard the supernatant and dry the pellet at room temperature.

15| Resuspend the pellet in 43 μl nuclease-free water.
■ **PAUSE POINT** DNA solution can be stored at −80 °C until further use.

16| Treat with Mung bean nuclease by combining reagents as follows:

Reagent	Amount
Digested DNA solution	43 μl
Mung bean nuclease buffer (10×)	5 μl (1× final concentration)
Mung bean nuclease (10 U μl^{-1})	2 μl (20 U)
Total volume	50 μl

17| Incubate for 30 min in a heat block at 30 °C.

18| Treat with proteinase K by combining reagents as follows:

Reagent	Amount
Mung bean nuclease–treated solution	50 μl
10% (wt/vol) SDS	10 μl
Proteinase K solution (20 μg μl^{-1})	2 μl (40 μg)
Nuclease-free water	138 μl
Total volume	200 μl

19| Incubate for 1 h in a heat block at 50 °C.

20| Extract with 200 μl phenol–chloroform–isoamyl alcohol (25:24:1) as described in Steps 5–7.

21| Extract with 200 μl chloroform as described in Step 8.

22| Precipitate DNA by ethanol precipitation as described in Steps 9–14.

23| Resuspend the pellet in 11 μl nuclease-free water.

24| Pipet 1 μl of the resuspended solution into 49 μl TE buffer (50-fold dilution). Estimate DNA concentration by separating 10 μl of diluted sample, along with a λDNA–*Hin*dIII fragment marker, by electrophoresis through a 1% agarose gel containing 0.1 μg ml^{-1} ethidium bromide.
▲ **CRITICAL STEP** It is important to confirm the size and yield of the digested DNA fragment.
■ **PAUSE POINT** DNA solution can be store at −80 °C until further use.
? TROUBLESHOOTING

RNA transcription and purification
25| Transcribe RNA with a MEGAscript kit. First, combine reagents as follows:

Reagent	Amount
Linear template DNA	2–4 μl (500 ng to 1 μg)
ATP solution	2 μl
CTP solution	2 μl
GTP solution	2 μl
UTP solution	2 μl
Reaction buffer (10×)	2 μl
Enzyme mix	2 μl
Nuclease-free water	4–6 μl
Total volume	20 μl

26| Incubate for 3 h in an incubator at 37 °C.

27| Add 1 μl DNase (included in the kit). Incubate for 15 min in an incubator at 37 °C.

28| Add 115 μl nuclease-free water and 15 μl stop solution (included in the kit).

29| Add 100 μl nuclease-free water and purify RNA with 750 μl TRIzol LS according to the instructions of the manufacturer.

30| Resuspend the RNA pellet in 11 μl nuclease-free water.

31| Dilute 1 μl RNA solution with 19 μl nuclease-free water. After denaturing for 5 min at 65 °C, confirm the size of the synthesized RNA by separating 1 μl of diluted sample, along with an RNA ladder, by electrophoresis through a 1% agarose gel containing 0.1 μg ml^{-1} ethidium bromide. Determine the RNA concentration with a spectrophotometer after a further 50-fold dilution (final dilution, 1,000-fold).
▲ **CRITICAL STEP** It is important to confirm that the size and yield of the purified RNA are appropriate.
■ **PAUSE POINT** RNA solution can be store at −80 °C for a few months. Avoid repeated cycles of freezing and thawing.
? TROUBLESHOOTING

Transfection (electroporation)
32| Prepare Huh7 cells or the permissive cell lines Huh7.5 or Huh7.5.1. Trypsinize cells and wash with OptiMEM I reduced-serum medium.

33| Resuspend 3.0×10^6 cells (for Huh7) or 7.5×10^6 cells (for Huh7.5 or Huh7.5.1) with 400 μl Cytomix buffer[11].

34| Mix 10 μg RNA with the 400 μl cell suspension and transfer to an electroporation cuvette.

35| Electroporate the cells with a Gene Pulser II apparatus in conditions of 260 V and 950 μF.

36| Transfer the transfected cells into two 10-cm culture dishes, each containing 8 ml complete medium.

37| Incubate the dishes for 24 h at 37 °C and 5% CO_2.

38| Remove culture medium and wash the transfected cells three times with PBS (−), then add fresh complete medium.

Figure 1 | Infected foci of cell culture–generated HCV-infected cells. Huh 7.5.1 cells were seeded at a density of 1×10^4 cells per well in 96-well plates. Each well was inoculated with serially diluted virus solution. At 3 d after infection, inoculated cells were fixed with 100% methanol and were then stained with antibody to core protein (2H9; ref. 3). This image includes six foci. Original magnification, ×200.

39| Transfected cells should be passaged every 2–3 d before the cells become confluent.

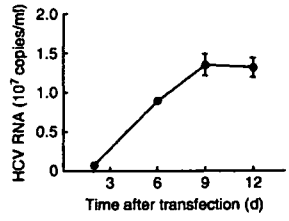

Figure 2 | Transient HCV RNA secretion by transfected Huh7 cells.

Collect the generated HCV

40| Collect culture medium 72 h after transfection and add fresh medium to the cells, then repeat culture medium collection every 2–3 d after cell passage until virus production is decreased.

41| Remove cell debris by low-speed centrifugation (20 min at 1,000*g*).

42| Pass the culture medium through a 0.45-μm syringe-top filter unit.

43| If necessary, concentrate medium using an Amicon Ultra-15 device. First, pipet filtered culture medium into the Amicon Ultra-15 device (maximum volume is 15 ml). Centrifuge for 30 min at 3,000*g* and 4 °C. Culture medium with virus can be concentrated until the medium reaches 50× concentration.

Titration of generated HCV

44| Check the titer of HCV RNA by quantitative RT-PCR after extracting RNA from medium, as described before[12].
? TROUBLESHOOTING

45| To determine the infectious titer, prepare Huh7, Huh7.5 or Huh7.5.1 cells at a density of 1×10^4 cells per well in poly-D-lysine–coated 96-well plates 24 h before inoculation.

46| Prepare inoculum with serial tenfold dilutions of culture medium containing virus. If a positive control sample is needed, include virus stock solution that has been titrated.

47| Aspirate medium from cells in 96-well plates.

48| Inoculate cells with 100 μl diluted culture medium containing virus. Inoculation of each diluted culture medium should be done in more than triplicate.

49| Incubate the inoculated culture plates for 4 h at 37 °C and 5% CO_2.

50| Remove the inoculum and add 100 μl fresh medium to each well.

51| Incubate the culture plates for 72 h at 37 °C and 5% CO_2.

52| Fix the cells for 20 min at −20 °C with 100% methanol.

53| Incubate the cells for 1 h at room temperature with IF buffer.

54| Incubate the cells for 1 h with anti-HCV at the appropriate concentration (e.g., Anti-Core C7-50, 1:300 dilution).

55| Aspirate the antibody solution and add 100 μl PBS (−) to each well.

56| Aspirate the PBS (−). Repeat Steps 55 and 56 three times (washing step).

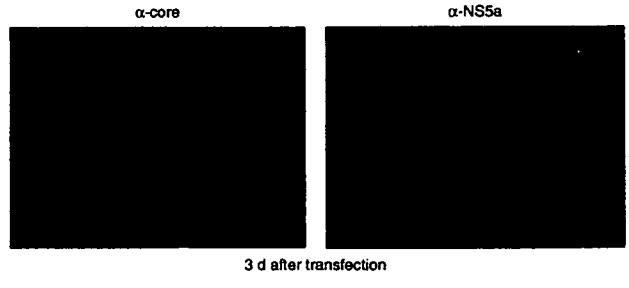

Figure 3 | Immunofluorescence microscopy of HCV proteins in Huh7 cells transfected with JFH-1 RNA. Transfected cells were seeded on coverslips 2 d after transfection, and HCV proteins were detected with antibody to core protein (α-core; 2H9; ref. 3) and to nonstructural protein 5A (α-NS5a; mouse polyclonal serum; ref. 5). Original magnification, ×300.

57| Incubate the cells for 1 h with fluorescent dye–conjugated anti-mouse IgG at the appropriate concentration (e.g., Alexa 488–anti-mouse IgG, 1:1,000 dilution).

58| Wash cell three times with PBS (−) as in Steps 55–56.

59| Using a fluorescence microscope, select the appropriate well for counting infected foci. In general, the well that was inoculated at the highest dilution among wells showing infectivity should be selected. Count the infected cell foci in the selected well (**Fig. 1**) and multiply the number of infected foci by the dilution factor. The infectious titer is calculated from the average of triplicate procedures.
? TROUBLESHOOTING

Template preparation (Steps 1–24): 6–7 h
RNA transcription and purification (Steps 25–31): 5 h
Transfection (Steps 32–39): 2 h
Collection (Steps 40–43): 3 d
Titration (Steps 44–59): 6 d

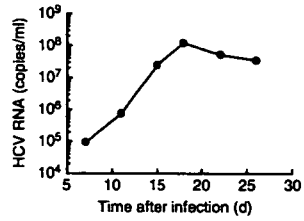

Figure 4 | HCV RNA titers in culture supernatants of inoculated Huh7.5.1 cells.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Cause	Solution
Step 4	Incomplete digestion	Inappropriate quality and quantity of plasmid DNA	Check quality and quantity of plasmid DNA
		Ruined enzyme	Use new enzyme
		Incubation time too short	Prolong incubation time
Step 24	Insufficient template DNA	Sample lost during procedures	Repeat digestion and purification steps more carefully
		Incorrect plasmid DNA concentration	Check plasmid DNA concentration
Step 31	Low yield of synthesized RNA	Contamination of RNase, SDS or EDTA	Repeat proteinase K treatment and purification step
		Insufficient template	Increase amount of template
		Incubation time too short	Prolong incubation time
	Synthesized RNA shows smear band	Contamination of RNase	Repeat proteinase K treatment and purification step
Steps 44 and 59	Low yield of generated HCV	Low transfection efficiency	Check transfection efficiency by indirect immunofluorescence

ANTICIPATED RESULTS

Huh7 cells transfected with synthesized full-length JFH-1 RNA were passaged at 2, 6, 9 and 12 d after transfection. At each time point, culture media from three independent transfected cells were collected and HCV RNA in culture media was measured in culture media by quantitative RT-PCR. HCV RNA titers in culture medium continuously increased up to 9 d after transfection (**Fig. 2**). At 72 h after transfection, HCV had spread to 60–80% of cells (**Fig. 3**). With this protocol, approximately 1×10^5 copies per ml of HCV can be obtained 72 h after transfection of 10 μg JFH-1 RNA into Huh7 cells, and 6 d after transfection, up to 1×10^7 copies per ml can be obtained. However, virus production is not always consistent and should be confirmed by HCV RNA titration or by counting infected foci in each experiment.

Huh7.5.1 cells (8×10^4 cells per well) were infected with concentrated culture medium from RNA-transfected cells containing 1×10^8 copies of HCV RNA. Inoculated cells were serially passaged and culture supernatants were collected at 7, 11, 15, 18, 22 and 26 d after infection. Amounts of HCV RNA in culture medium were determined by quantitative RT-PCR. By inoculation of cell culture–generated HCV into naive Huh 7.5.1 cells, infection and production of HCV could be monitored and maintained for 26 d or more after inoculation (**Fig. 4**).

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1. Kato, T. *et al.* Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. *J. Med. Virol.* **64**, 334–339 (2001).
2. Kato, T. *et al.* Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* **125**, 1808–1817 (2003).
3. Wakita, T. *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* **11**, 791–796 (2005).
4. Zhong, J. *et al.* Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. USA* **102**, 9294–9299 (2005).
5. Date, T. *et al.* Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells. *J. Biol. Chem.* **279**, 22371–22376 (2004).
6. Kato, T. *et al.* Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon. *J. Virol.* **79**, 592–596 (2005).
7. Bukh, J. *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. USA* **99**, 14416–14421 (2002).
8. Lindenbach, B.D. *et al.* Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623–626 (2005).
9. Blight, K.J., McKeating, J.A. & Rice, C.M. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J. Virol.* **76**, 13001–13014 (2002).
10. World Health Organization. Laboratory biosafety manual. 3rd edn. (World Health Organization, Geneva, Switzerland, 2004).
11. van den Hoff, M.J.B., Moorman, A.F.M. & Lamers, W.H. Electroporation in 'intracellular' buffer increases cell survival. *Nucleic Acids Res.* **20**, 2902 (1992).
12. Takeuchi, T. *et al.* Real-time detection system for quantification of hepatitis C virus genome. *Gastroenterology* **116**, 636–642 (1999).

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CD81 Expression Is Important for the Permissiveness of Huh7 Cell Clones for Heterogeneous Hepatitis C Virus Infection▽

Daisuke Akazawa,[1,2] Tomoko Date,[2] Kenichi Morikawa,[2] Asako Murayama,[2] Michiko Miyamoto,[3] Minako Kaga,[2] Heidi Barth,[4] Thomas F. Baumert,[4]† Jean Dubuisson,[5] and Takaji Wakita[2]*

Pharmaceutical Research Laboratories, Toray Industries, Inc., Kanagawa, Japan[1]; Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan[2]; Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan[3]; Department of Medicine II, University of Freiburg, Freiburg, Germany[4]; and CNRS-UPR2511, Institut de Biologie de Lille, Institut Pasteur de Lille, Lille, France[5]

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Huh7 cells constitute a permissive cell line for cell culture of hepatitis C virus (HCV) particles. However, our Huh7 line shows limited permissiveness for HCV. Thus, in this study we set out to determine which host factors are important for conferring permissiveness. To analyze the limited permissiveness of our Huh7 cells, 70 clones were obtained after single-cell cloning of parental Huh7 cells. The cloned Huh7 cells exhibited various levels of HCV pseudoparticles and JFH-1 virus infection efficiency, and some clones were not permissive. A subgenomic replicon was then transfected into the cloned Huh7 cells. While the replication efficiencies differed among the cloned Huh7 cells, these efficiencies did not correlate with infectious permissibility. Flow cytometry showed that CD81, scavenger receptor class B type I, and low-density-lipoprotein receptor expression on the cell surfaces of the Huh7 clones differed among the clones. Interestingly, we found that all of the permissive cell clones expressed CD81 while the nonpermissive cell clones did not. To confirm the importance of CD81 expression for HCV permissiveness, CD81 was then transiently and stably expressed on a nonpermissive Huh7 cell clone, which was consequently restored to HCV infection permissiveness. Furthermore, permissiveness was down-regulated upon transfection of CD81 silencing RNA into a CD81-positive cell clone. In conclusion, CD81 expression is an important determinant of HCV permissiveness of Huh7 cell clones harboring different characteristics.

Hepatitis C virus (HCV) is a worldwide human pathogen, and most infected patients progress to chronic liver disease. The primary therapy for HCV is treatment with pegylated interferon and ribavirin; however, these agents do not cause a marked decline in the virus titers of all treated patients. Thus, the elucidation of native virus-host interactions is necessary to develop new, more effective therapies. However, the lack of a robust cell culture system to produce infectious virions has hampered research. That said, in a great boon to HCV research, a cell culture system that allows the whole life cycle of HCV to be investigated was recently developed (22, 34, 41).

HCV is an enveloped virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family (23). Cell attachment of flaviviruses generally leads to endocytosis of bound virions. Several molecules have been proposed as cell entry receptors of HCV; most of these have been identified based on binding with soluble recombinant E2 protein or HCV-like particles (2, 3, 12, 25, 30, 31). Putative HCV receptors include CD81 (30), low-density-lipoprotein (LDL) receptor (1), scavenger receptor class B type I (SR-BI) (31), and several molecules that induce concentration of viral particles at the cell surface. Infectious HCV pseudoparticles (HCVpp) harboring E1E2 glycoproteins (5, 11) have substantiated the functional roles of the candidate receptors CD81 and SR-BI in HCV entry (5, 6, 15). The importance of CD81 for HCV entry was recently confirmed using cell-cultured HCV particles (22, 34). Furthermore, CD81 is important for postattachment of HCV particles on Huh7 cells (19, 28).

Huh7 and its interferon-cured cells are considered permissive cell lines for HCV particles (22, 34, 41), but our Huh7 cell line shows limited permissiveness. In the present study, we performed single-cell cloning of Huh7 cells and then analyzed heterogeneity. To investigate the host factors important for HCV infection, the Huh7 cell clones were then transiently infected with JFH-1 virus and comparisons of efficiency of replication and expression of candidate receptors were performed.

MATERIALS AND METHODS

Cell culture and single-cell cloning. Parental Huh7 cells, Huh7.5.1 cells (41) (a generous gift from Francis V. Chisari), and Huh7 cell clones were cultured at 37°C in 5% CO_2. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, as described previously (18). Parental Huh7 cells were diluted with medium and seeded into 96-well plates at 0.3 cells per well. Seventy single-cell-derived clones were then selected, and after 3 weeks their cells were passaged. The resultant Huh7 cell clones were stored at −80°C until use.

Plasmids. pJFH-1 (34), pSGR-JFH1/Luc, pSGR-JFH1/Luc-GND (17), and pFGR-JFH1 (10) were generated as previously reported. pFGR-J6/N2X-JFH1 was generated by replacement of the JFH-1 structural region (a core coding region to the BclI site) with pJ6CF (35) (a generous gift from Jens Bukh). pFGR-JFH1/EGFP and pFGR-J6/N2X-JFH1/EGFP were generated by replacement of the neomycin-resistant gene of pFGR-JFH1 and that of pFGR-J6/N2X-JFH1 with the enhanced green fluorescent protein (EGFP) gene from pEGFP-N3 (Clontech, Mountain View, CA). pcDNA3.1-CD81 and the vesicular stomatitis virus (VSV) G protein-expressing construct pCAG-VSVG (27) were

* 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: wakita@nih.go.jp.

† Present address: Inserm Unité 748, Université Louis Pasteur, 3 rue Koeberlé, 67000 Strasbourg, France.

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kind gifts from Yoshiharu Matsuura (Osaka University, Suita, Japan). The JFH-1 E1E2 expression construct pcDNAdeltaC-E1-E2(JFH1) was a kind gift from Thomas Pietschmann (University of Heidelberg, Heidelberg, Germany), while the murine leukemia virus packaging construct and the luciferase-based transfer vector construct have been described previously (5).

RNA synthesis. RNA synthesis was performed as described previously (17, 34). Briefly, the plasmids pJFH-1, pSGR-JFH1/Luc, pSGR-JFH1/Luc-GND, pFGR-JFH1/EGFP, and pFGR-J6/N2X-JFH1/EGFP were digested with XbaI and treated with mung bean nuclease (New England Biolabs, Beverly, MA). Digested plasmid DNA fragments were then purified and used as templates for RNA synthesis. HCV RNA was synthesized in vitro by use of a MEGAscript T7 kit (Ambion, Austin, TX). Synthesized RNA was treated with DNase I, followed by acid phenol extraction to remove any remaining template DNA.

Replication assay of JFH-1 subgenomic replicon. Replication of a JFH-1 subgenomic replicon (SGR-JFH1) in Huh7 cells was detected as described previously (17). Briefly, 2 μg of reporter replicon RNA transcribed from pSGR-JFH1/Luc and pSGR-JFH1/Luc-GND was transfected into 2×10^6 Huh7 cells by electroporation. Transfected cells were immediately transferred to culture medium and seeded into six-well culture plates.

Cells were harvested serially at 4 h (day 0), 24 h (day 1), and 48 h (day 2) after transfection and lysed with 200 μl of cell culture lysis reagent (Promega, Madison, WI). Debris was then removed by centrifugation. Luciferase activities were quantified by use of LUMAT LB9507 (EG & G Berthold, Bad Wildbad, Germany) and a luciferase assay system (Promega). Assays were performed in duplicate, and the results at 24 and 48 h after transfection were normalized and expressed as the relative luciferase activities (RLA) compared to the luciferase activity at 4 h after transfection. The replication efficiency of each cell is indicated in Fig. 3 and Table 1 as follows: −, RLA below 1; ±, RLA between 1 and 10; +, RLA between 10 and 50; ++, RLA between 50 and 100; +++, RLA over 100.

Production of infectious HCV particles. HCV particles derived from JFH-1 were prepared as described previously (34). Briefly, in vitro-synthesized RNA was transfected into Huh7 cells by electroporation. Cell culture supernatants were collected 72 h after transfection and passed through a 0.45-μm filter. Filtrated culture medium was then pooled and concentrated using Amicon Ultra-15 (100,000 molecular weight cutoff; Millipore, Bedford, MA). The infectious titer was 1.6×10^4 focus-forming units (ffu) per ml and was determined by immunofluorescence detection of infected foci following infection of naïve parental Huh7 cells. RNA quantification was performed by real-time detection reverse transcription-PCR analysis, as described previously (32), using an ABI Prism 7700 sequence detector system (Applied Biosystems Japan, Tokyo, Japan). The titer was determined to be 4.3×10^8 RNA copies/ml. Concentrated culture medium samples were stored at −80°C until use.

HCV particle infection and immunofluorescence. Parental Huh7 cells and Huh7 cell clones were seeded at 1×10^4 cells/well in poly-D-lysine-coated 96-well plates (Corning, Inc., Corning, NY), cultured overnight, and then inoculated with serially diluted culture medium containing infectious HCV particles. At 48 h after inoculation, the cells were fixed in methanol for 15 min at −20°C, and the infected foci were visualized by immunofluorescence as described below.

Cells were permeabilized and blocked for 1 h with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) containing 0.3% (vol/vol) Triton X-100. The cells were then washed five times with phosphate-buffered saline (PBS), and anticore monoclonal antibody 2H9 (34) was added at 50 μg/ml in BlockAce. After incubation for 1 h at room temperature, the cells were washed and incubated with a 1:400 dilution of AlexaFluor 488-conjugated or AlexaFluor 546-conjugated anti-mouse immunoglobulin G (IgG) (Molecular Probes, Eugene, OR) with BlockAce. The cells were then washed and examined by fluorescence microscopy (Olympus, Tokyo, Japan). Quantification of infectivity was performed by counting the infected foci, and the assay was performed in triplicate. The infectivity of each clone is indicated in Fig. 3 and Table 1 as follows: −, no infected foci; ±, between 1 and 5 foci; +, between 5 and 10 foci; ++, between 10 and 50 foci; +++, over 50 foci.

Stable and transient expression of CD81. A trypsinized CD81-negative clone (clone 25) was washed with Opti-MEM I (Invitrogen, Carlsbad, CA) and resuspended at 5×10^6 cells/ml in Cytomix buffer (18). pcDNA3.1-CD81 plasmid DNA (75 μg) was mixed with 400 μl of cell suspension and the mix then transferred to an electroporation cuvette (Precision universal cuvette; Thermo Hybrid, Middlesex, United Kingdom). The cells were then pulsed at 220 V and 950 μF with a Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred to a 75-cm^2 flask (Corning) and incubated at 37°C/5% CO_2. After 3 days, the cells were passaged and seeded into 10-cm dishes, and G418 (0.8 mg/ml) (Nacalai Tesque, Kyoto, Japan) was then added to the culture medium. Culture medium supplemented with G418 was replaced twice a week. Three weeks later, the colonies were observed and then the cells were trypsinized. CD81-positive cells were obtained and confirmed by the same method as for single-cell cloning of parental Huh7 cells. We obtained a clone, Huh7-25-CD81, in which CD81 was stably expressed.

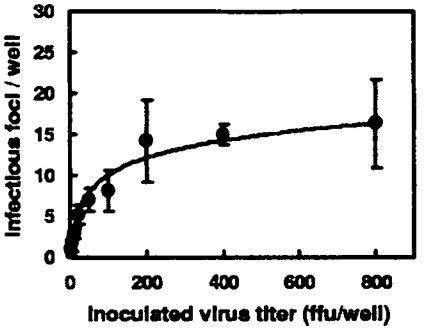

FIG. 1. Infectivity of JFH-1 virus in parental Huh7 cell. Parental Huh7 cells were seeded at 1×10^4 cells/well and infected with JFH-1 virus at 3 to 800 ffu/well for 48 h in 96-well plates. Infected cells were visualized with immunofluorescence using anticore antibody (2H9), and the foci were counted. All experiments were performed in triplicate, and the data are shown as means ± standard deviations.

For transient CD81 expression, 6 μg of pcDNA3.1-CD81 plasmid was transfected into Huh7 clone 25 (Huh7-25) cells (2.5×10^6) by using FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis, IN). After 24 h, cells were passaged and an aliquot used for flow cytometric analysis and virus infection. As controls, Huh7-25 and Huh7-25-CD81 cells were similarly treated by only FuGENE6 and used for flow cytometric analysis and virus infection.

Pseudotype production and infection. Murine leukemia virus pseudotypes were generated according to methods described previously (5). Briefly, the Gag-Pol packaging construct (3.1 μg), the transfer vector construct (3.1 μg), and the JFH-1 glycoprotein or the VSV-G protein-expressing construct (1 μg) DNAs were transfected into 2.5×10^6 293T cells seeded the day before in 10-cm dishes by use of FuGENE6 transfection reagent (Roche Diagnostics). For the negative control, the constructs (except for the glycoprotein-expressing construct) were similarly transfected. The medium (8 ml/dish) was replaced 6 h after transfection. Supernatants containing the pseudotypes were collected 48 h later and passed through a 0.45-μm filter. The supernatants were stored at −80°C until use.

Target cells were seeded into 48-well plates at a density of 2×10^4 cells/well and incubated overnight at 37°C. A 100-μl aliquot of each diluted supernatant containing pseudotypes was added to each well and incubated for 3 h. The supernatants were removed, and the cells were incubated in regular medium for 72 h at 37°C. Cells were washed once with PBS and lysed with 40 μl/well of cell culture lysis reagent. Luciferase activities were quantified using a luciferase assay system (Promega) as described above. Assays were performed in triplicate. All Huh7 cell clones showed infectivity by the VSV-G pseudoparticle, and infectivity by HCVpp was indicated by the luciferase activity (relative luciferase units [RLU]), determined by subtraction from the activity of the nonenveloped control. The infectivity of each clone is indicated in Table 1 as follows: −, luciferase activity below 1 RLU; ±, activity between 1 and 5,000 RLU; +, activity between 5,000 and 30,000 RLU; ++, activity between 30,000 and 100,000 RLU; +++, activity over 100,000 RLU.

RNA interference. A 40-pmol amount of silencing RNA (siRNA) duplex for CD81 (Santa Cruz, Inc., Santa Cruz, CA) was electroporated into 2.5×10^6 Huh7 clone 54 (Huh7-54) cells (260 V, 950 μF). Control irrelevant siRNA (siIRR) was designed as described previously (37) and transfected, as was the siRNA of CD81. Cells were then propagated and tested for CD81 expression and JFH-1 virus infection.

Flow cytometric analysis. Cells were seeded in 10-cm dishes (Corning) and cultured overnight. Then, subconfluent cells were harvested either by trypsinization or by treatment with 0.05% EDTA in PBS. Parental Huh7 cells and Huh7 cell clones (1×10^6) were incubated with or without 1 μg mouse anti-CD81 antibody (JS-81; Pharmingen, Franklin Lakes, NJ) for 30 min at 4°C and washed with PBS. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (Cappel, Durham, NC) at 1:100 for 30 min at 4°C, washed repeatedly, and resuspended in PBS containing 1% (vol/vol) formaldehyde. SR-BI expression of each cell was tested using rat anti-human SR-BI antiserum (1:50) and FITC-conjugated anti-rat IgG secondary antibody (Cappel) by the same method as for CD81 (2). Rat preimmune serum

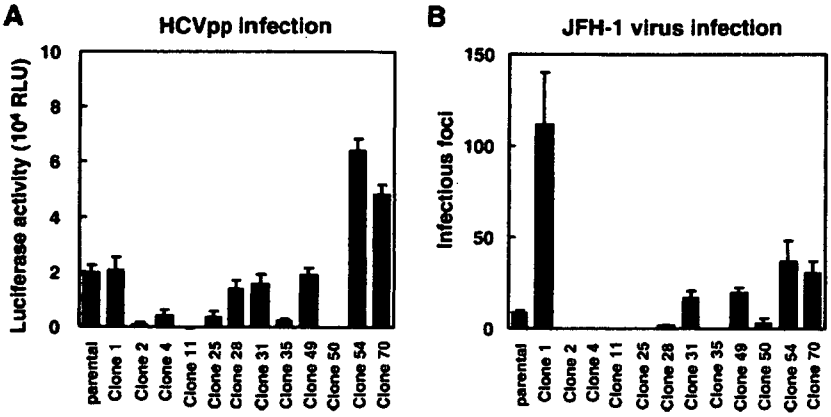

FIG. 2. Infectivity of HCVpp and JFH-1 virus in Huh7 cell clones. (A) Target cells (2×10^4 cells) were inoculated with supernatant containing HCVpp for 3 h in 48-well plates. After 72 h, cells were harvested and the luciferase activities quantified. All experiments were performed in triplicate, and infectivity is indicated as the RLU minus the activity of the nonenveloped negative control. (B) Target cells (1×10^4 cells) were infected with the same titer of JFH-1 virus for 48 h in 96-well plates. Infected cells were visualized with immunofluorescence using anticore antibody (2H9), and the foci were counted. All experiments were performed in triplicate, and the data are shown as means ± standard deviations.

was used as the negative-control primary antibody. LDL receptor expression was tested using rabbit anti-LDL receptor antibody (BP5014 at 1:10; Acris Antibodies GmbH, Hiddenhausen, Germany) and FITC-conjugated anti-rabbit IgG secondary antibody (Cappel). Analyses were performed using an EPICS ALTRA MultiCOMP unit (Beckman Coulter, Fullerton, CA) and FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). The expression levels are indicated in Table 1 as follows: −, mean fluorescence intensity (MFI) in relative units was below 1; +, MFI in relative units was between 1 and 3; ++, MFI in relative units was between 3 and 6; +++, MFI in relative units was over 6.

Analysis of cell surface CD81 expression levels and HCV infection. For EGFP virus production, in vitro-synthesized RNA was transfected into Huh7.5.1 cells by electroporation. Cell culture supernatants of transfected cells were collected and concentrated as described above. The infectious titer was also determined as described above. Huh7-70 and Huh7-25-CD81 cells were seeded in six-well plates at 1×10^5 cells/well 24 h before virus inoculation. Cells were inoculated with EGFP virus (multiplicity of infection, 2) for 4 h, followed by a PBS wash. The inoculated cells were cultured in complete medium and then harvested at 24, 48, 72, and 96 h after inoculation. CD81 expression and GFP-positive cells were analyzed by FACSCalibur as described above, using allophycocyanin-conjugated anti-mouse IgG (R&D Systems, Minneapolis, MN) as a secondary antibody at 1:10. All experiments were performed in triplicate, and analysis was performed using CellQuest Pro software (Becton Dickinson). CD81 expression on uninfected and infected cells was calculated from the geometric MFI of the each quadrant plot. The results are given as MFIs ± standard deviations.

RESULTS

Parental Huh7 cell shows limited permissiveness for JFH-1 virus. A parental Huh7 cell was infected with JFH-1 virus and infectivity assessed by counting the infected foci in anticore immunofluorescence. The number of infected foci increased linearly with lower doses of virus infection (<50 ffu/well). However, the number of infected foci did not increase with higher doses of infection (>200 ffu/well) (Fig. 1). Furthermore, when the parental Huh7 cell infected with JFH-1 was passaged, the infected cells did

TABLE 1. Permissiveness to infection and expression of candidate receptors[a]

Cell type	Replication efficiency (RLA)[b]	CD81 expression (MFI)[c]	SR-BI expression (MFI)[d]	LDLr expression (MFI)[e]	HCVpp infectivity[f]	JFH-1 infectivity[g]
Huh7 (parental)	+++	++	++	+	+	+
Clone 1	++	+	++	+	+	+++
Clone 2	+	−	+	+	±	−
Clone 4	−	−	+	+	±	−
Clone 11	+++	−	++	+	−	−
Clone 25	+++	−	++	+	±	−
Clone 28	±	+++	++	++	+	±
Clone 31	±	+	++	+	+	++
Clone 35	+++	−	++	++	±	−
Clone 49	±	++	+	++	+	++
Clone 50	−	++	+	+	+	±
Clone 54	+	+	++	++	++	++
Clone 70	±	++	++	+	++	++
Huh7-25-CD81	+++	+++	++	+	+++	+++

[a] See Materials and Methods for definitions of symbols.
[b] Detection of replication of the JFH-1 subgenomic replicon on Huh7 clones at 48 h after transfection of SGR-JFH1/Luc RNA.
[c] Detection of CD81 by flow cytometry using JS-81 antibodies on the surfaces of the indicated cells.
[d] Detection of SR-BI by flow cytometry using rat anti-SR-BI antiserum on the surfaces of the indicated cells.
[e] Detection of LDL receptor (LDLr) by flow cytometry using rabbit anti-LDLr antibody (BP5014) on the surfaces of the indicated cells.
[f] Detection of luciferase activities by subtraction from the activity of the nonenveloped control.
[g] Infected foci were detected by immunofluorescence using HCV anticore antibody (2H9).

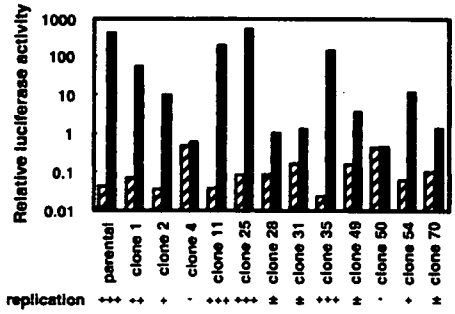

FIG. 3. Replication of JFH-1 subgenomic replicon in Huh7 cell clones. Reporter replicon RNA was transfected into Huh7 cell clones, and luciferase activities at 4 and 48 h after RNA transfection were then detected. The SGR-JFH1/Luc-GND RNA was used as the negative control. All data indicate the RLA compared to the luciferase activities at 4 h after transfection. The RLA of SGR-JFH1/Luc (solid bars) and SGR-JFH1/Luc-GND (hatched bars) are also shown. All experiments were performed in duplicate, and the data are shown as the means.

not expand (data not shown). Thus, the original parental Huh7 cell had limited permissiveness for JFH-1 infection.

Huh7 cell clones have different levels of permissiveness for HCVpp and JFH-1 virus infections. The parental Huh7 cell did not show dose-dependent permissiveness for JFH-1 infection. Thus, single-cell cloning of this Huh7 cell was performed by limiting dilution, and the efficiency of virus infection for each clone was then investigated. Seventy clones were obtained. Among the isolated cloned cells, 65 clones were first screened with JFH-1 virus infection, and the other 5 clones were not tested, because of their slower growth. We found different numbers of focus formation in the clones (46 positive and 19 negative). Among them, we selected seven positive (clones 1, 28, 31, 49, 50, 54, and 70) and five negative (clones 2, 4, 11, 25, and 35) clones for further analysis. Next, these 12 cell clones were infected with HCVpp and JFH-1 virus. Interestingly, the efficiencies of both virus infections of the Huh7 cell clones differed among the clones (Fig. 2; Table 1). Furthermore, some differences were observed between HCVpp and JFH-1 virus permissiveness. Four clones that were not permissive for JFH-1 virus showed slight permissiveness for HCVpp (clones 2, 4, 25, and 35). In addition, three clones were more permissive for both HCVpp and JFH-1 virus than for the parental Huh7 cell (clones 1, 54, and 70). However, clone 11 was not permissive at all for either virus infection. Thus, the parental Huh7 cell population was most likely heterogeneous and included cells with different characteristics.

Replication of JFH-1 subgenomic replicon in Huh7 cell clones does not correlate with the efficiency of virus infection. In a previous study, a subgenomic replicon of JFH-1 was shown to efficiently replicate in Huh7 cells (18). Thus, to investigate whether replication efficiency correlated with infectious efficiency, we transiently transfected SGR-JFH1/Luc RNA into each Huh7 cell clone and measured the resultant RLA. At 48 h posttransfection, levels of replication of the replicon in the Huh7 cell clones differed among the clones (Fig. 3), but the efficiency did not correlate with JFH-1 virus infectivity (Table 1).

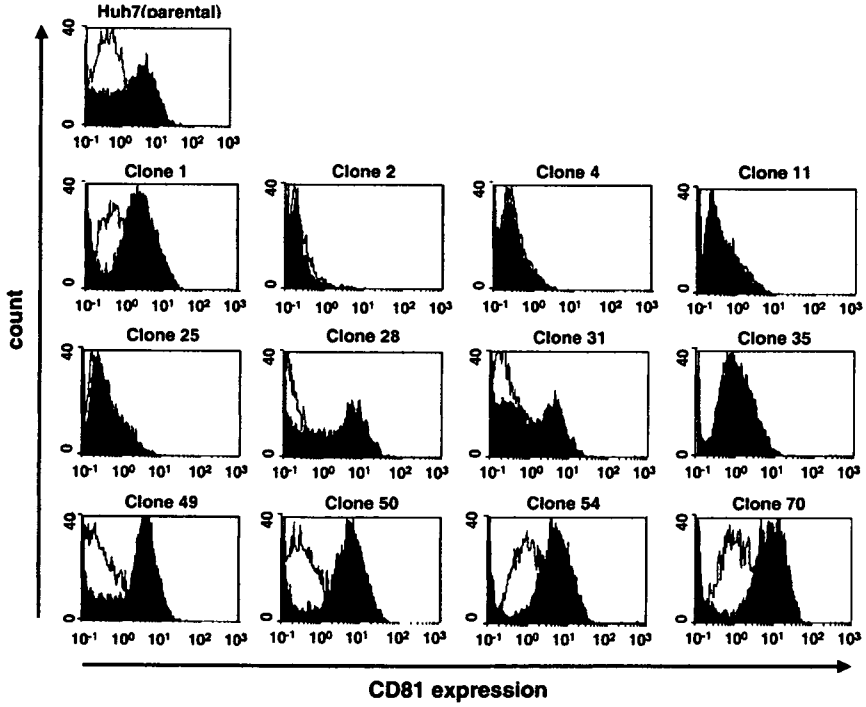

FIG. 4. CD81 expression on the surfaces of Huh7 cell clones. Huh7 cell clones were seeded in 10-cm dishes and cultured overnight. Then, subconfluent cells were harvested either by trypsinization or by treatment with 0.05% EDTA in PBS. Cells (1×10^6) were incubated with 1 μg of mouse anti-CD81 monoclonal antibody (JS-81) and subsequently stained with FITC-conjugated goat anti-mouse IgG. The negative control represents cells incubated with only secondary antibody. The analysis was performed by EPICS ALTRA MultiCOMP (Beckman Coulter). The *x* and *y* axes show fluorescence intensity and relative number of stained cells, respectively.

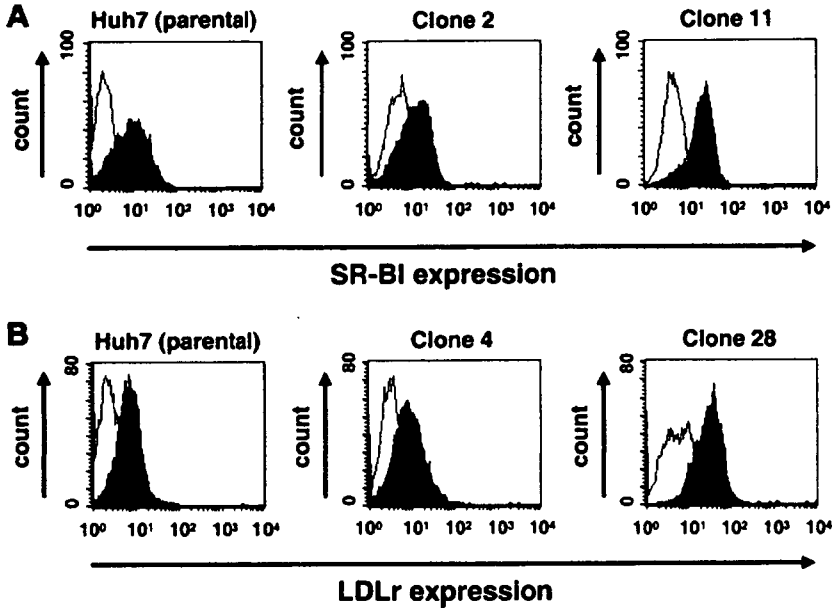

FIG. 5. SR-BI and LDL receptor on the surfaces of Huh7 cell clones. Huh7 cell clones (1×10^6 cells) were incubated with rat anti-SR-BI antiserum or rabbit anti-LDL receptor antibody and subsequently stained with FITC-conjugated secondary antibody. The negative control represents cells incubated with rat preimmune serum (SR-BI) or only secondary antibody (LDL receptor [LDLr]). The analysis was performed by FACSCalibur (Becton Dickinson). The *x* and *y* axes show fluorescence intensity and relative number of stained cells, respectively. (A) Expression of SR-BI on parental Huh7 cells, clone 2 (the cell clone with the lowest expression level), and clone 11 (the cell clone with the highest expression level). (B) Expression of LDL receptor on parental Huh7 cells, clone 4 (the cell clone with the lowest expression level), and clone 28 (the cell clone with the highest expression level).

Expression of CD81 on Huh7 cell clones is correlated with HCV permissiveness. The Huh7 cell clones displayed differing levels of permissiveness for HCVpp and JFH-1 virus infections, and replication of the replicon did not correlate with infectivity. Thus, the difference in infectivity was most likely due to host factors related to the initial phase of infection. Previous studies using pseudotype particles bearing envelope proteins of HCV have shown that CD81 is a candidate receptor of HCV (5, 6, 9, 15, 38). Therefore, we investigated CD81 expression on our Huh7 cell clones by flow cytometry using an anti-CD81 antibody. We found that the levels of CD81 expression on the Huh7 cells differed among the clones (Fig. 4). All of the CD81-negative clones (clones 2, 4, 11, 25, and 35) were also negative for JFH-1 virus infectivity. However, CD81-positive clones (clones 1, 28, 31, 49, 50, 54, and 70) showed HCV permissiveness at different levels (Table 1). Interestingly, clones 28 and 50 expressed relatively high levels of CD81 but low levels of permissiveness. This may have been due to a lower replication efficiency of these clones, although clones 49 and 70 also had low replication capacities but were permissive of HCV infection. Thus, the low permissiveness of clones 28 and 50 is most likely due to other, as yet unknown mechanisms (Table 1). To confirm that the different expression levels of CD81 among the clones were not due to the cell-harvesting conditions, we harvested using two different techniques, namely, trypsinization and EDTA treatment. Neither method affected the results. This finding suggests that CD81 expression is highly correlated with HCV infectivity, although the level of CD81 expression did not necessarily correlate with JFH-1 virus infectivity among these Huh7 cell clones (Table 1).

Expression levels of SR-BI and the LDL receptor on Huh7 cell clones do not correlate with permissiveness. CD81 expression correlated highly with infectivity of Huh7 cell clones. In a previous report, CD81 expression level determined permissiveness, as shown by a transient-transfection experiment (40). However, the levels of CD81 expression among the clones from this study did not correlate with virus infectivity (Table 1), suggesting that multiple factors determine the level of infectivity. As previous studies have suggested that SR-BI and the LDL receptor play important roles in HCV infection (4, 20, 33), we next investigated the expression of these molecules on the surfaces of our Huh7 cell clones. The expression of these molecules was also detected by flow cytometry. Small differences in the expression levels of SR-BI and the LDL receptor compared to that of CD81 were observed (Fig. 5; Table 1). In terms of virus permissiveness, SR-BI and LDL receptor expression did not display the high correlation seen with CD81 (Fig. 5; Table 1).

CD81 expression restores HCV infection permissiveness in a nonpermissive Huh7 cell clone. To confirm the importance of CD81 expression for HCV permissiveness, CD81 was expressed on a nonpermissive, non-CD81-expressing Huh7 cell clone (Huh7-25) (Fig. 4; Table 1) and the cells were then infected with JFH-1 virus. When CD81 was transiently and stably expressed on Huh7-25 cells, these cells were restored to permissiveness. The CD81-positive cells of transiently and stably transfected cells were 45.1 and 80.6%, respectively, and infectivities (infected foci) were 58.0 ± 7.9 and 257.7 ± 14.6, respectively, with inoculation of the same titer of JFH-1 virus (Fig. 6A). A clone in which CD81 was stably expressed (Huh7-

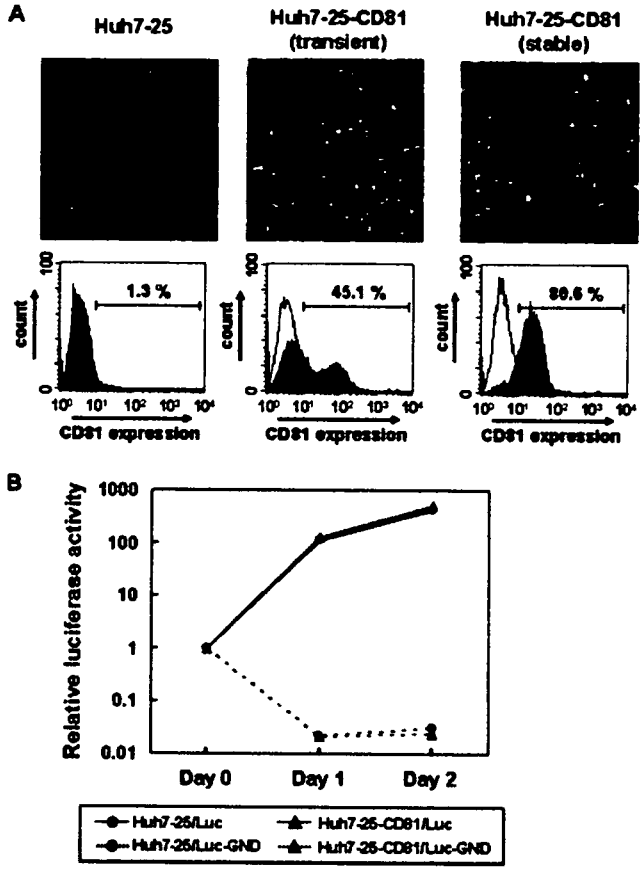

FIG. 6. Infectivity in CD81-transfected Huh7 cells. (A) CD81 was transiently and stably expressed on a Huh7 nonpermissive clone (Huh7-25). The expression of CD81 on the cell surface was examined by flow cytometry. Bars indicate the populations of the CD81-positive cells. Cells were inoculated with JFH-1 virus (1×10^6 copies of HCV RNA) for 3 h. Virus was then removed and cells incubated at 37°C for 48 h. Infected cells were detected by immunofluorescence using anti-core antibody. (B) Replication of SGR-JFH1 in Huh7-25 cells and Huh7-25 cells in which CD81 was stably expressed (Huh7-25-CD81 cells).

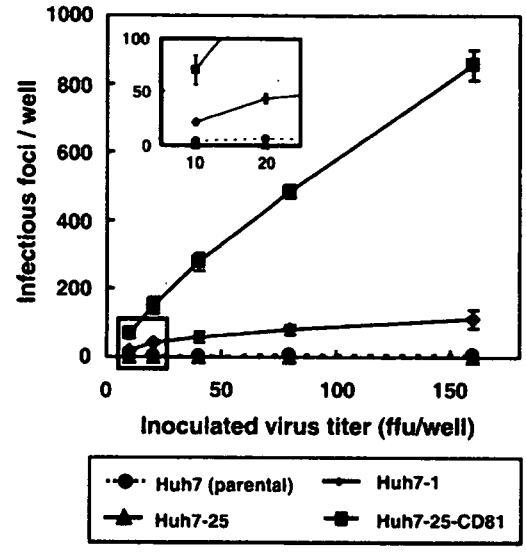

FIG. 7. Dependency of permissiveness on virus concentration in Huh7-25-CD81 cells. Huh7-25-CD81 cells (1×10^4) were infected with JFH-1 virus at 10 to 160 ffu/well in 96-well plates. Infected cells were visualized and assayed as described in the legend for Fig. 1. All experiments were performed in triplicate, and the data are shown as means ± standard deviations. The upper-left panel shows a magnified version of the area in the square at the lower left.

25-CD81) had high infectivity for JFH-1 virus (Fig. 6A), although the replication efficiency was equal to that of Huh7-25 cells (Fig. 6B). In addition, the infected foci indicated a linear, dose-dependent increase in virus dose (Fig. 7). Thus, ectopic expression of CD81 in a nonpermissive Huh7 cell clone restored HCV infection permissiveness. Furthermore, the Huh7-25-CD81 cell clone supported the highest permissiveness for HCV infection.

Knockdown of CD81 expression reduced HCV infection permissiveness. A permissive and CD81-expressing Huh7 cell clone (Huh7-54) was transfected with siRNA for CD81. At 48 h after transfection, the expression of CD81 on the cell surface declined by about 60% compared to that for the cell transfected with siIRR (Fig. 8A). The CD81 knockdown cell was then infected with JFH-1 virus, and infectivity was determined. Infectivity of the CD81 knockdown cell declined by about 80% compared to that for the siIRR-transfected cell or mock-transfected cells (Fig. 8B). Thus, CD81 expression was shown to be an important factor in HCV infection permissiveness. Taken together, the level of CD81 expression is also a determinant of the level of HCV permissiveness in a cell clone.

CD81 expression levels followed by HCV infection. CD81 expression on the cell surface may alter with culture conditions or HCV infections. To test this, we produced infectious HCV particles harboring the EGFP gene. J6/JFH1 EGFP virus was derived from pFGR-J6/N2X-JFH1/EGFP, and JFH-1 EGFP virus was from pFGR-JFH1/EGFP. J6/JFH1 EGFP virus was produced more efficiently from synthetic RNA-transfected cells than wild-type JFH-1 EGFP virus (data not shown). We thus used J6/JFH1 EGFP virus to inoculate Huh7-25-CD81 and Huh7-70 cells. Cell surface CD81 expression and HCV infection were detected simultaneously by flow cytometry. In Fig. 9A, results of the fluorescence-activated cell sorting analysis are given as quadrant plots of Huh7-25-CD81 cells inoculated with J6/JFH1 EGFP virus at 96 h after inoculation. The infected cells were observed by a shift to positive GFP fluorescence, and CD81 expression was detected by an anti-CD81 antibody. Under this experimental condition, HCV infections were detected in both cell types at 24 h after inoculation (Fig. 9B) (0.5% and 0.6% for Huh7-25-CD81 and Huh7-70 cells, respectively). The ratio of infected cells increased substantially in Huh7-25-CD81 cells (Fig. 9B) (27.7% at 96 h postinoculation); however, the ratio of infected cells was not increased in Huh7-70 cells (Fig. 9B) (0.9% at 96 h). On the other hand, the CD81 expression level of Huh7 cells increased until 2 days after cell passages and then declined gradually (data not shown). This kinetic property was also confirmed with mock-infected Huh7-25-CD81 and Huh7-70 cells (Fig. 9C) (incubation was started 24 h after passage). After J6/JFH1 EGFP virus inoculations, CD81 expression levels on the cell surfaces of uninfected and infected cells were also analyzed (Fig. 9C).

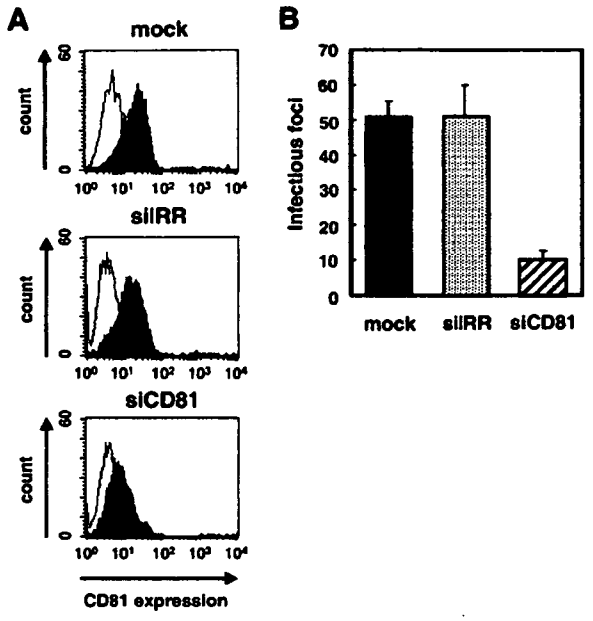

FIG. 8. siRNA silencing of CD81 expression on Huh7-54 cells and JFH-1 virus infection. (A) CD81-positive Huh7-54 cells transfected with either siIRR or siRNA of CD81 (siCD81) were stained with anti-CD81 antibody (JS-81) at 48 h posttransfection and analyzed by flow cytometry. (B) siRNA-transfected Huh-54 cells were inoculated with the same titer of JFH-1 virus (1×10^6 copies HCV RNA) for 3 h. Virus was then removed, and cells were incubated at 37°C for 48 h. Infected cells were visualized and assayed as described in the legend for Fig. 1. All experiments were performed in triplicate, and the data are shown as means ± standard deviations.

CD81 expression levels of uninfected cells were not different from those of mock-infected cells. Interestingly, CD81 expression levels of infected Huh7-25-CD81 cells were higher than those of uninfected cells at 48 and 72 h after virus infection (Fig. 9C, left panel). However, CD81 expression of infected cells also decreased gradually, as observed for uninfected cells. The CD81 expression level of Huh7-70 cells was lower than that of Huh7-25-CD81 cells, and the difference in CD81 expression level between uninfected and infected cells was not clear for Huh7-70 cells (Fig. 9C, right panel).

DISCUSSION

CD81 is a candidate receptor for HCV and plays an important, as yet unknown role in HCV infection. Pileri et al. were the first to demonstrate a relationship between CD81 and HCV when they found that the envelope protein E2 of HCV bound to the large extracellular loop of CD81 (30). However, experiments that use recombinant proteins are limited in that they can only provide information regarding molecular interactions. The development of pseudoparticles with HCV envelope proteins (HCVpp) made possible the investigation of HCV infection and cell entry, and some candidate receptors for HCV have been proposed previously (2, 3, 12, 25, 30, 31). The involvement of CD81 in HCV entry was also ascertained by the HCVpp system. The HCVpp system has shed some light on the role of cell surface molecules involved in the early steps of the HCV life cycle. On the other hand, a cell culture system to produce HCV bearing the genotype 2a genome has recently been established (22, 34, 41). This system enables investigation of the whole HCV life cycle.

The Huh7 cell line is a human hepatoma cell line, established in 1982 (29), that is now recognized as being permissive for HCV particles (34). Since our Huh7 cell showed limited permissiveness, in this study we performed single-cell cloning of a Huh7 cell and found that the parental Huh7 cell produced a heterogeneous population upon culture (Table 1). The heterogeneity of subsequent infectivity may have been due to the limited permissiveness of the parental Huh7 cells. Further analysis of these Huh7 cell clones revealed that CD81 expression determined permissiveness with high correlation (Fig. 4; Table 1). Moreover, given that HCV replication efficiency was not changed by CD81 expression (Fig. 6B), this molecule must be important in the early steps of HCV infection. However, the level of CD81 expression on the Huh7 cell clones did not necessarily correlate with HCVpp and JFH-1 virus infectivity, with some clones displaying high permissiveness but relatively low CD81 expression and vice versa (Table 1). It is thus likely that replication efficiency is related to the appearance of infected foci, since the translation of HCV core protein is affected by HCV RNA replication. For example, Huh7 clone 1 indicated relatively low permissiveness for HCVpp but high permissiveness for JFH-1 virus, while clone 28 indicated the opposite. These differences may have arisen from the postinfection steps of virus infection, as the JFH-1 system depends on HCV infection and replication and the HCVpp system depends on HCV infection and pseudotype gene expression. Nonetheless, multiple factors are predicted to play a role in HCV infection, in addition to CD81.

When the siRNA for CD81 was transfected into a CD81-positive cell clone and expression subsequently down-regulated, the permissiveness for HCVpp (38) and JFH-1 virus was also down-regulated (Fig. 8). On the other hand, when a Huh7 cell line was transfected with serial doses of CD81 expression vector, the transfected cells indicated permissiveness according to the level of CD81 expression (40). Although the MFIs differed between transiently and stably transfected cells, more clonal CD81 expression was observed for stably transfected cells than for transiently transfected cells (Fig. 6A). This may account for the difference in infectivity between transiently and stably transfected cells.

SR-BI and the LDL receptor are other putative molecules thought to be involved in HCV infection (1, 31), and their relationships to HCV have been investigated using recombinant proteins and HCVpp (5, 6, 15). The expression levels of SR-BI and the LDL receptor on our Huh7 cell clones differed slightly among the clones (Fig. 4); however, their expression did not appear to determine HCV permissiveness, unlike with CD81. On the other hand, it was recently reported that CD81 and SR-BI function cooperatively and cholesterol dependently to initiate HCV entry (16). In the present study, JFH-1 virus infection levels varied among the Huh7 cell clones, and thus each molecule may have a threshold expression level that determines HCV permissiveness.

CD81 expression level on the cell surface may be changed with cell culture condition and HCV infection. Therefore, it is important to analyze a dynamic expression of CD81. In fact, higher CD81 expression was observed for infected Huh7-25-

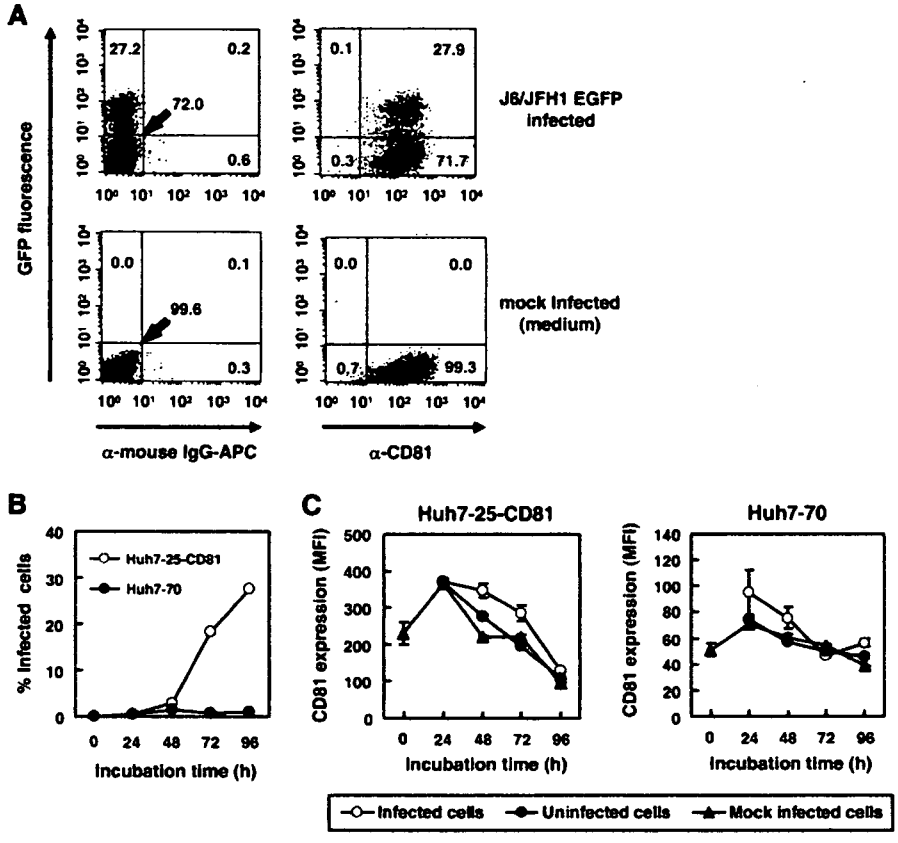

FIG. 9. Kinetics of cell surface CD81 expression and HCV infection. Huh7-25-CD81 and Huh7-70 cells were seeded into six-well plates at a density of 1×10^5 cells/well. Twenty-four hours later, J6/JFH1 EGFP virus (multiplicity of infection, 2) was inoculated. Cells were harvested at 24, 48, 72, and 96 h after inoculation and analyzed for infection and cell surface CD81 expression by FACSCalibur. The experiments were performed as described in the legend for Fig. 4, using anti (α)-mouse IgG–allophycocyanin (APC) as a secondary antibody. (A) Huh7-25-CD81 cells were harvested 96 h after inoculation and analyzed by fluorescence-activated cell sorting. Relative numbers of cells in the respective quadrants are given. (B) The proportion of infected cells was determined at each time point postinoculation and plotted for Huh7-25-CD81 and Huh7-70 cells. Mean values from triplicate experiments are given. (C) The cell surface CD81 expression (MFI) of infected cells, uninfected cells, and mock-infected cells is plotted at each time point after inoculation for Huh7-25-CD81 and Huh7-70 cells. Mean values and standard deviations are given.

CD81 cells than for uninfected cells (Fig. 9C, left panel). However, this difference was not clear with Huh7-70 cells (Fig. 9C, right panel). This discrepancy may be due to the different CD81 expression levels between the cells. Huh7-25-CD81 cells express higher levels of CD81 on the cell surface (Fig. 6 and 9; Table 1), and when such cells are infected with HCV, CD81 molecules might be stabilized to keep higher expression levels on the cell surface. Alternatively, CD81 expression in Huh7-25-CD81 cells may be controlled differently than in other Huh7 cell clones because CD81 is expressed from the transfected vector in Huh7-25-CD81 cells. Taken together, a more detailed analysis will be necessary for a dynamic expression of CD81 and HCV infection.

CD81 is a member of the tetraspanin family, and its functions are unclear. It is known that CD81 is a component of the tetraspanin web on the plasma membrane (21) and that the homologous region shared with CD9 is involved in egg-sperm fusion (42). Thus, CD81 may play an important role in cell-virus fusion through the tetraspanin web. Having said that, it is unclear what kinds of molecules associate with CD81 on human hepatic cells. Since CD81 and other tetraspanins are thought to interact with various molecules (13), including integrins (36), GPR56 (24), 14-3-3 (8), and signaling enzymes (7, 37, 39), various signal transductions through CD81 and multiprotein complexes may be involved in the level of HCV permissiveness. Thus, the tetraspanin-enriched microdomain on permissive cell lines may be necessary for virus-host interaction (14, 26).

On the other hand, the level of CD81 expression on the Huh7 cell clones did not correlate with the level of permissiveness, indicating that CD81-independent molecules were also involved in permissiveness. Recently, our laboratory and others have indicated that heparan sulfate proteoglycan (HSPG) may play an important role in the initial cell surface binding of HCV particles (19, 28). Since HCV particles are thought to be concentrated by HSPG on the surfaces of cells, the differences in infectivity among Huh7 cell clones may be due to differences in the expression levels or types of HSPG. Furthermore, other unknown molecules that harbor affinity with HCV particles may also be important. A more detailed analysis is clearly required.

In conclusion, we investigated HCV permissiveness and host factors by use of cell-cultured infectious particles and a hetero-

geneous population of Huh7 cells derived from a single cell. We discovered that HCV particle permissiveness is determined by CD81 expression with high correlation. However, the level of permissiveness of each Huh7 cell clone is not explained by only CD81 expression levels, suggesting that another host factor(s) is involved.

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REFERENCES

1. **Agnello, V., G. Ábel, M. Elfahal, G. B. Knight, and Q. X. Zhang.** 1999. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. USA **96**:12766–12771.
2. **Barth, H., R. Cerino, M. Arcuri, M. Hoffmann, P. Schürmann, M. I. Adah, B. Gissler, X. Zhao, V. Ghisetti, B. Lavezzo, H. E. Blum, F. von Weizsäcker, A. Vitelli, E. Scarselli, and T. F. Baumert.** 2005. Scavenger receptor class B type I and hepatitis C virus infection of primary *Tupaia* hepatocytes. J. Virol. **79**:5774–5785.
3. **Barth, H., C. Schäfer, M. I. Adah, F. Zhang, R. J. Linhardt, H. Toyoda, A. Kinoshita-Toyoda, T. Toida, T. H. Van Kuppevelt, E. Depla, F. Von Weizsacker, H. E. Blum, and T. F. Baumert.** 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J. Biol. Chem. **278**:41003–41012.
4. **Bartosch, B., G. Verney, M. Dreux, P. Donot, Y. Morice, F. Penin, J. M. Pawlotsky, D. Lavillette, and F. L. Cosset.** 2005. An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. J. Virol. **79**:8217–8229.
5. **Bartosch, B., J. Dubuisson, and F. L. Cosset.** 2003. Infectious hepatitis C virus pseudoparticles containing functional E1–E2 envelope protein complexes. J. Exp. Med. **197**:633–642.
6. **Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F. L. Cosset.** 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J. Biol. Chem. **278**:41624–41630.
7. **Berditchevski, F., K. F. Tolias, K. Wong, C. L. Carpenter, and M. E. Hemler.** 1997. A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. J. Biol. Chem. **272**:2595–2598.
8. **Clark, K. L., A. Oelke, M. E. Johnson, K. D. Eilert, P. C. Simpson, and S. C. Todd.** 2004. CD81 associates with 14-3-3 in a redox-regulated palmitoylation-dependent manner. J. Biol. Chem. **279**:19401–19406.
9. **Cormier, E. G., F. Tsamis, F. Kajumo, R. J. Durso, J. P. Gardner, and T. Dragic.** 2004. CD81 is an entry coreceptor for hepatitis C virus. Proc. Natl. Acad. Sci. USA **101**:7270–7274.
10. **Date, T., M. Miyamoto, T. Kato, K. Morikawa, A. Murayama, D. Akazawa, J. Tanabe, S. Sone, M. Mizokami, and T. Wakita.** An infectious and selectable full-length replicon system with hepatitis C virus JFH-1 strain. Hepatol. Res., in press.
11. **Drummer, H. E., A. Maerz, and P. Poumbourios.** 2003. Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins. FEBS Lett. **546**:385–390.
12. **Gardner, J. P., R. J. Durso, R. R. Arrigale, G. P. Donovan, P. J. Maddon, T. Dragic, and W. C. Olson.** 2003. L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. Proc. Natl. Acad. Sci. USA **100**:4498–4503.
13. **Hemler, M. E.** 2005. Tetraspanin functions and associated microdomains. Nat. Rev. Mol. Cell Biol. **6**:801–811.
14. **Hemler, M. E.** 2003. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. Annu. Rev. Cell Dev. Biol. **19**:397–422.
15. **Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc. Natl. Acad. Sci. USA **100**:7271–7276.
16. **Kapadia, S. B., H. Barth, T. Baumert, J. A. McKeating, and F. V. Chisari.** 2007. Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. J. Virol. **81**:374–383.
17. **Kato, T., T. Date, M. Miyamoto, M. Sugiyama, Y. Tanaka, E. Orito, T. Ohno, K. Sugihara, I. Hasegawa, K. Fujiwara, K. Ito, A. Ozasa, M. Mizokami, and T. Wakita.** 2005. Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. J. Clin. Microbiol. **43**:5679–5684.
18. **Kato, T., T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, and T. Wakita.** 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology **125**:1808–1817.
19. **Koutsoudakis, G., A. Kaul, E. Steinmann, S. Kallis, V. Lohmann, T. Pietschmann, and R. Bartenschlager.** 2006. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. J. Virol. **80**:5308–5320.
20. **Lavillette, D., A. W. Tarr, C. Voisset, P. Donot, B. Bartosch, C. Bain, A. H. Patel, J. Dubuisson, J. K. Ball, and F. L. Cosset.** 2005. Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. Hepatology **41**:265–274.
21. **Levy, S., S. C. Todd, and H. T. Maecker.** 1998. CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. Annu. Rev. Immunol. **16**:89–109.
22. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wölk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309**:623–626.
23. **Lindenbach, B. D., and C. M. Rice.** 2001. *Flaviviridae*: the viruses and their replication, p. 991–1042. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, PA.
24. **Little, K. D., M. E. Hemler, and C. S. Stipp.** 2004. Dynamic regulation of a GPCR-tetraspanin-G protein complex on intact cells: central role of CD81 in facilitating GPR56-G_q/11 association. Mol. Biol. Cell **15**:2375–2387.
25. **Lozach, P. Y., H. Lortat-Jacob, A. de Lacroix de Lavalette, I. Staropoli, S. Foung, A. Amara, C. Houlès, F. Fieschi, O. Schwartz, J. L. Virelizier, F. Arenzana-Seisdedos, and R. Altmeyer.** 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J. Biol. Chem. **278**:20358–20366.
26. **Martin, F., D. M. Roth, D. A. Jans, C. W. Pouton, L. J. Partridge, P. N. Monk, and G. W. Moseley.** 2005. Tetraspanins in viral infections: a fundamental role in viral biology? J. Virol. **79**:10839–10851.
27. **Matsuura, Y., H. Tani, K. Suzuki, T. Kimura-Someya, R. Suzuki, H. Aizaki, K. Ishii, K. Moriishi, C. S. Robison, M. A. Whitt, and T. Miyamura.** 2001. Characterization of pseudotype VSV possessing HCV envelope proteins. Virology **286**:263–275.
28. **Morikawa, K., Z. Zhao, T. Date, M. Miyamoto, A. Murayama, D. Akazawa, J. Tanabe, S. Sone, and T. Wakita.** Infectious hepatitis C virus particle binding to the Huh7 cell surface is mediated by glycosaminoglycans and its internalization by CD81. J. Med. Virol., in press.
29. **Nakabayashi, H., K. Taketa, K. Miyano, T. Yamane, and J. Sato.** 1982. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res. **42**:3858–3863.
30. **Piteri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani.** 1998. Binding of hepatitis C virus to CD81. Science **282**:938–941.
31. **Scarselli, E., H. Ansuini, R. Cerino, R. M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, and A. Vitelli.** 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. **21**:5017–5025.
32. **Takeuchi, T., A. Katsume, T. Tanaka, A. Abe, K. Inoue, K. Tsukiyama-Kohara, R. Kawaguchi, S. Tanaka, and M. Kohara.** 1999. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology **116**:636–642.
33. **Voisset, C., N. Callens, E. Blanchard, A. Op De Beeck, J. Dubuisson, and N. Vu-Dac.** 2005. High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I. J. Biol. Chem. **280**:7793–7799.
34. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11**:791–796.
35. **Yanagi, M., R. H. Purcell, S. U. Emerson, and J. Bukh.** 1999. Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. Virology **262**:250–263.
36. **Yang, X., O. V. Kovalenko, W. Tang, C. Claas, C. S. Stipp, and M. E. Hemler.** 2004. Palmitoylation supports assembly and function of integrin–tetraspanin complexes. J. Cell Biol. **167**:1231–1240.
37. **Yauch, R. L., F. Berditchevski, M. B. Harler, J. Reichner, and M. E. Hemler.** 1998. Highly stoichiometric, stable, and specific association of integrin alpha3beta1 with CD151 provides a major link to phosphatidylinositol 4-kinase, and may regulate cell migration. Mol. Biol. Cell **9**:2751–2765.
38. **Zhang, J., G. Randall, A. Higginbottom, P. Monk, C. M. Rice, and J. A. McKeating.** 2004. CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. J. Virol. **78**:1448–1455.

39. **Zhang, X. A., A. L. Bontrager, and M. E. Hemler.** 2001. Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific beta(1) integrins. J. Biol. Chem. **276**:25005–25013.
40. **Zhong, J., P. Gastaminza, J. Chung, Z. Stamataki, M. Isogawa, G. Cheng, J. A. McKeating, and F. V. Chisari.** 2006. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. J. Virol. **80**:11082–11093.
41. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA **102**:9294–9299.
42. **Zhu, G. Z., B. J. Miller, C. Boucheix, E. Rubinstein, C. C. Liu, R. O. Hynes, D. G. Myles, and P. Primakoff.** 2002. Residues SFQ (173–175) in the large extracellular loop of CD9 are required for gamete fusion. Development **129**:1995–2002.