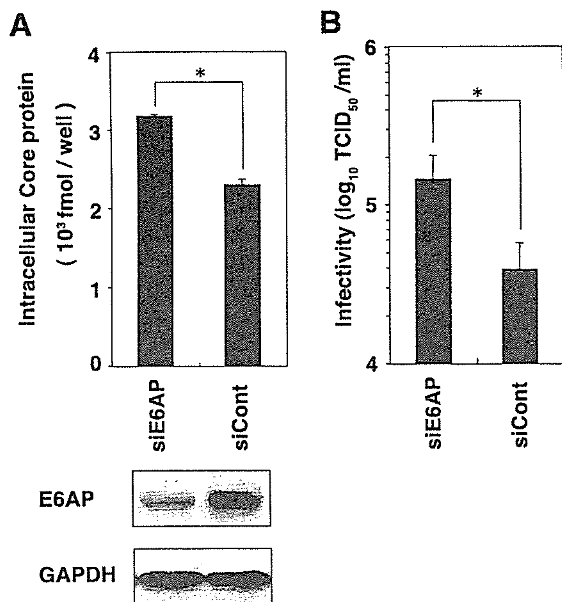

FIG. 10. E6AP silencing leads to an increase in the level of intracellular HCV core protein and supernatant infectivity titer in HCV-infected Huh-7 cells. (A) HCV JFH1-infected cells were replated in a six-well plate at 3×10^5 cells/well and transfected with 40 pmol of E6AP siRNA or control siRNA. The culture medium was changed at 24 h after transfection. The cells were harvested at day 2 after transfection, and the intracellular core protein levels were quantitated using the HCV core antigen ELISA. Equivalent amounts of the whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-E6AP MAb or anti-GAPDH MAb. (B) Culture supernatants were collected at day 2 after transfection and assayed for $TCID_{50}$ determinations. For both panels, the difference between E6AP siRNA and control siRNA was significant (*, $P < 0.05$, Student's t test).

in a ubiquitin-independent, ATP-independent, and 20S proteasome-dependent pathway (27). There have been reports that several cellular factors, such as p53 (2), p73 (2), and RPN4 (18), are degraded through two alternative pathways, the ubiquitin-dependent 26S proteasome-dependent pathway and the ubiquitin-independent 20S proteasome-dependent pathway. Here we provide evidence that E6AP mediates ubiquitylation of HCV core protein. Still unclear is whether the PA28γ-dependent pathway requires polyubiquitylation of HCV core protein. HCV core protein is predominantly localized in the cytoplasm, especially at the endoplasmic reticulum membrane, on the surface of lipid droplets, and on mitochondria and mitochondrion-associated membranes (51). In HCV JFH1-infected cells, HCV core was found to localize in the cytoplasm and frequently to accumulate in the perinuclear region and the lipid droplets (44). Our results indicated that E6AP colocalized with HCV core protein especially in the perinuclear region. PA28γ was found to colocalize with HCV core protein in the nucleus. Functional differences may exist between the E6AP-dependent pathway and the PA28γ-dependent pathway in the stability control of HCV core protein. The functional role of the E6AP-dependent pathway and the PA28γ-dependent pathway remains to be elucidated.

The HCV core-binding region of E6AP was mapped to the region between aa 418 and aa 517. The multicopy maintenance protein 7, Mcm7, interacts with E6AP through a short motif, termed the L2G box (aa 412 to 414), that lies within the E6 binding site of E6AP (23). Our data indicated that the E6 binding region containing the L2G motif is not required for interaction between HCV core protein and E6AP (Fig. 2C, lane M).

We propose here that E6AP may affect the production of HCV particles through controlling the amounts of HCV core protein. This mechanism may contribute to persistent infection. The E6AP binding domain of the core protein resides in the RNA-binding domain and binding domains for many host factors (40). These factors may affect the binding between E6AP and HCV core protein, resulting in control of E6AP-dependent core degradation. Another possibility is that HCV core protein may affect the normal function of E6AP, thereby contributing to pathogenesis. It will be intriguing to investigate whether HCV core protein has any effect on E6AP-dependent degradation of host factors. The other intriguing possibility is that HCV core-E6AP complex may function as an E3 ligase-like E6-E6AP complex to target host factors for proteasomal degradation and contribute to viral pathogenesis.

In conclusion, we have demonstrated that E6AP interacts with HCV core protein in vitro and in vivo and mediates ubiquitin-dependent degradation of the core protein, leading to downregulation of HCV particles. We propose that the E6AP-mediated ubiquitin-proteasome pathway may play a role in affecting the production of HCV particles through controlling the amounts of viral nucleocapsid protein. Identification of the specific E3 ubiquitin ligase may contribute to gaining a better understanding of the biology of the HCV life cycle as well as molecular details of the ubiquitin-dependent degradation of HCV core protein.

ACKNOWLEDGMENTS

We thank D. Bohmann (EMBL) for providing pMT123, K. Miyazono (University of Tokyo) for pcDEF3-6Myc-WWP1, and K. Iwai (Osaka City University) for recombinant baculovirus carrying His 6-mouse E1. Huh-7.5.1 cells and Huh-7 cells were kindly provided by F. V. Chisari (Scripps Research Institute). We also thank P. Zhou (Weill Medical College of Cornell University), S. I. Wells (Cincinnati Children's Hospital Medical Center), and A. W. Hudson (Medical College of Wisconsin) for critical readings of the manuscript; M. Matsuda, S. Yoshizaki, M. Ikeda, and M. Sasaki for technical assistance; Y. Sugiyama and S. Senzui for plasmid construction; and T. Mizoguchi for secretarial work.

This work was supported in part by a grant for Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation; by grants-in-aid from the Ministry of Health, Labor and Welfare; by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology; and by the program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), Japan. T.I. was supported in part by a grant from Novartis Foundation (Japan) for the Promotion of Science and by the Tokyo Metropolitan University President's Fund, Special Emphasis Research Project of Japan.

REFERENCES

1. **Aizaki, H., Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsuura, and T. Miyamura.** 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. Hepatology **27:** 621–627.
2. **Asher, G., P. Tsvetkov, C. Kahana, and Y. Shaul.** 2005. A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73. Genes Dev. **19:**316–321.
3. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1994. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc. Natl. Acad. Sci. USA **91:**8239–8243.

4. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7:2745–2752.
5. **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.
6. **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.
7. **Franck, N., J. Le Seyec, C. Guguen-Guillouzo, and L. Erdtmann.** 2005. Hepatitis C virus NS2 protein is phosphorylated by the protein kinase CK2 and targeted for degradation to the proteasome. J. Virol. 79:2700–2708.
8. **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.
9. **Giannini, C., and C. Brechot.** 2003. Hepatitis C virus biology. Cell Death Differ. 10(Suppl. 1):S27–S38.
10. **Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice.** 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. J. Virol. 67:2832–2843.
11. **Harris, K. F., I. Shoji, E. M. Cooper, S. Kumar, H. Oda, and P. M. Howley.** 1999. Ubiquitin-mediated degradation of active Src tyrosine kinase. Proc. Natl. Acad. Sci. USA 96:13738–13743.
12. **Hijikata, M., H. Mizushima, T. Akagi, S. Mori, N. Kakiuchi, N. Kato, T. Tanaka, K. Kimura, and K. Shimotohno.** 1993. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J. Virol. 67:4665–4675.
13. **Huibregtse, J. M., M. Scheffner, S. Beaudenon, and P. M. Howley.** 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc. Natl. Acad. Sci. USA 92:2563–2567.
14. **Huibregtse, J. M., M. Scheffner, and P. M. Howley.** 1993. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. Mol. Cell. Biol. 13:775–784.
15. **Hussy, P., H. Langen, J. Mous, and H. Jacobsen.** 1996. Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. Virology 224:93–104.
16. **Ichimura, T., H. Yamamura, K. Sasamoto, Y. Tominaga, M. Taoka, K. Kakiuchi, T. Shinkawa, N. Takahashi, S. Shimada, and T. Isobe.** 2005. 14-3-3 proteins modulate the expression of epithelial Na^+ channels by phosphorylation-dependent interaction with Nedd4-2 ubiquitin ligase. J. Biol. Chem. 280:13187–13194.
17. **Iwai, K., K. Yamanaka, T. Kamura, N. Minato, R. C. Conaway, J. W. Conaway, R. D. Klausner, and A. Pause.** 1999. Identification of the von Hippel-Lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. Proc. Natl. Acad. Sci. USA 96:12436–12441.
18. **Ju, D., and Y. Xie.** 2004. Proteasomal degradation of RPN4 via two distinct mechanisms, ubiquitin-dependent and -independent. J. Biol. Chem. 279: 23851–23854.
19. **Kao, W. H., S. L. Beaudenon, A. L. Talis, J. M. Huibregtse, and P. M. Howley.** 2000. Human papillomavirus type 16 E6 induces self-ubiquitination of the E6AP ubiquitin-protein ligase. J. Virol. 74:6408–6417.
20. **Kato, T., M. Miyamoto, A. Furusaka, T. Date, K. Yasui, J. Kato, S. Matsushima, T. Komatsu, and T. Wakita.** 2003. Processing of hepatitis C virus core protein is regulated by its C-terminal sequence. J. Med. Virol. 69:357–366.
21. **Kishino, T., M. Lalande, and J. Wagstaff.** 1997. UBE3A/E6-AP mutations cause Angelman syndrome. Nat. Genet. 15:70–73.
22. **Komuro, A., T. Imamura, M. Saitoh, Y. Yoshida, T. Yamori, K. Miyazono, and K. Miyazawa.** 2004. Negative regulation of transforming growth factor-beta (TGF-beta) signaling by WW domain-containing protein 1 (WWP1). Oncogene 23:6914–6923.
23. **Kuhne, C., and L. Banks.** 1998. E3-ubiquitin ligase/E6-AP links multicopy maintenance protein 7 to the ubiquitination pathway by a novel motif, the L2G box. J. Biol. Chem. 273:34302–34309.
24. **Kumar, S., A. L. Talis, and P. M. Howley.** 1999. Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination. J. Biol. Chem. 274:18785–18792.
25. **Kunkel, M., M. Lorinczi, R. Rijnbrand, S. M. Lemon, and S. J. Watowich.** 2001. Self-assembly of nucleocapsid-like particles from recombinant hepatitis C virus core protein. J. Virol. 75:2119–2129.
26. **Kuo, G., Q. L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, et al.** 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244:362–364.
27. **Li, X., D. M. Lonard, S. Y. Jung, A. Malovannaya, Q. Feng, J. Qin, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley.** 2006. The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REGγ proteasome. Cell 124:381–392.
28. **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.
29. **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.
30. **Moriishi, K., T. Okabayashi, K. Nakai, K. Moriya, K. Koike, S. Murata, T. Chiba, K. Tanaka, R. Suzuki, T. Suzuki, T. Miyamura, and Y. Matsuura.** 2003. Proteasome activator PA28γ-dependent nuclear retention and degradation of hepatitis C virus core protein. J. Virol. 77:10237–10249.
31. **Moriya, K., H. Fujie, Y. Shintani, H. Yotsuyanagi, T. Tsutsumi, K. Ishibashi, Y. Matsuura, S. Kimura, T. Miyamura, and K. Koike.** 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat. Med. 4:1065–1067.
32. **Moriya, K., H. Yotsuyanagi, Y. Shintani, H. Fujie, K. Ishibashi, Y. Matsuura, T. Miyamura, and K. Koike.** 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J. Gen. Virol. 78:1527–1531.
33. **Natsume, T., Y. Yamauchi, H. Nakayama, T. Shinkawa, M. Yanagida, N. Takahashi, and T. Isobe.** 2002. A direct nanoflow liquid chromatography-tandem mass spectrometry system for interaction proteomics. Anal. Chem. 74:4725–4733.
34. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–199.
35. **Oda, H., S. Kumar, and P. M. Howley.** 1999. Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination. Proc. Natl. Acad. Sci. USA 96:9557–9562.
36. **Ogino, T., H. Fukuda, S. Imajoh-Ohmi, M. Kohara, and A. Nomoto.** 2004. Membrane binding properties and terminal residues of the mature hepatitis C virus capsid protein in insect cells. J. Virol. 78:11766–11777.
37. **Okamoto, K., K. Moriishi, T. Miyamura, and Y. Matsuura.** 2004. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. J. Virol. 78:6370–6380.
38. **Owsianka, A. M., and A. H. Patel.** 1999. Hepatitis C virus core protein interacts with a human DEAD box protein DDX3. Virology 257:330–340.
39. **Pavio, N., D. R. Taylor, and M. M. Lai.** 2002. Detection of a novel unglycosylated form of hepatitis C virus E2 envelope protein that is located in the cytosol and interacts with PKR. J. Virol. 76:1265–1272.
40. **Polyak, S. J., K. C. Klein, I. Shoji, T. Miyamura, and J. R. Lingappa.** 2006. Assemble and interact: pleiotropic functions of the HCV core protein, p. 89–119. *In* S.-L. Tan (ed.), Hepatitis C viruses: genomes and molecular biology. Horizon Bioscience, Norwich, United Kingdom.
41. **Poynard, T., M. F. Yuen, V. Ratziu, and C. L. Lai.** 2003. Viral hepatitis C. Lancet 362:2095–2100.
42. **Ravaggi, A., G. Natoli, D. Primi, A. Albertini, M. Levrero, and E. Cariani.** 1994. Intracellular localization of full-length and truncated hepatitis C virus core protein expressed in mammalian cells. J. Hepatol. 20:833–836.
43. **Ray, R. B., L. M. Lagging, K. Meyer, and R. Ray.** 1996. Hepatitis C virus core protein cooperates with *ras* and transforms primary rat embryo fibroblasts to tumorigenic phenotype. J. Virol. 70:4438–4443.
44. **Rouille, Y., F. Helle, D. Delgrange, P. Roingeard, C. Voisset, E. Blanchard, S. Belouzard, J. McKeating, A. H. Patel, G. Maertens, T. Wakita, C. Wychowski, and J. Dubuisson.** 2006. Subcellular localization of hepatitis C virus structural proteins in a cell culture system that efficiently replicates the virus. J. Virol. 80:2832–2841.
45. **Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, et al.** 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA 87:6547–6549.
46. **Santolini, E., G. Migliaccio, and N. La Monica.** 1994. Biosynthesis and biochemical properties of the hepatitis C virus core protein. J. Virol. 68: 3631–3641.
47. **Sato, S., M. Fukasawa, Y. Yamakawa, T. Natsume, T. Suzuki, I. Shoji, H. Aizaki, T. Miyamura, and M. Nishijima.** 2006. Proteomic profiling of lipid droplet proteins in hepatoma cell lines expressing hepatitis C virus core protein. J. Biochem. (Tokyo) 139:921–930.
48. **Scheffner, M., J. M. Huibregtse, and P. M. Howley.** 1994. Identification of a human ubiquitin-conjugating enzyme that mediates the E6-AP-dependent ubiquitination of p53. Proc. Natl. Acad. Sci. USA 91:8797–8801.
49. **Scheffner, M., J. M. Huibregtse, R. D. Vierstra, and P. M. Howley.** 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75:495–505.
50. **Scheffner, M., U. Nuber, and J. M. Huibregtse.** 1995. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature 373:81–83.
51. **Suzuki, R., S. Sakamoto, T. Tsutsumi, A. Rikimaru, K. Tanaka, T. Shimoike, K. Moriishi, T. Iwasaki, K. Mizumoto, Y. Matsuura, T. Miyamura, and T. Suzuki.** 2005. Molecular determinants for subcellular localization of hepatitis C virus core protein. J. Virol. 79:1271–1281.
52. **Suzuki, R., K. Tamura, J. Li, K. Ishii, Y. Matsuura, T. Miyamura, and T. Suzuki.** 2001. Ubiquitin-mediated degradation of hepatitis C virus core pro-

tein is regulated by processing at its carboxyl terminus. Virology 280:301–309.

53. **Suzuki, T., K. Omata, T. Satoh, T. Miyasaka, C. Arai, M. Maeda, T. Matsuno, and T. Miyamura.** 2005. Quantitative detection of hepatitis C virus (HCV) RNA in saliva and gingival crevicular fluid of HCV-infected patients. J. Clin. Microbiol. **43:**4413–4417.
54. **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.
55. **Talis, A. L., J. M. Huibregtse, and P. M. Howley.** 1998. The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells. J. Biol. Chem. **273:**6439–6445.
56. **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.
57. **Wertz, I. E., K. M. O'Rourke, Z. Zhang, D. Dornan, D. Arnott, R. J. Deshaies, and V. M. Dixit.** 2004. Human de-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. Science **303:**1371–1374.
58. **Xu, Z., J. Choi, W. Lu, and J. Ou.** 2003. Hepatitis C virus F protein is a short-lived protein associated with the endoplasmic reticulum. J. Virol. **77:**1578–1583.
59. **Yamaguchi, R., S. Momosaki, G. Gao, C. C. Hsia, M. Kojiro, C. Scudamore, and E. Tabor.** 2004. Truncated hepatitis C virus core protein encoded in hepatocellular carcinomas. Int. J. Mol. Med. **14:**1097–1100.
60. **Yasui, K., T. Wakita, K. Tsukiyama-Kohara, S. I. Funahashi, M. Ichikawa, T. Kajita, D. Moradpour, J. R. Wands, and M. Kohara.** 1998. The native form and maturation process of hepatitis C virus core protein. J. Virol. **72:**6048–6055.
61. **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).

Published online 21 December 2006; doi:10.1038/nprot.2006.395

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 *XbaI* (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 á 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.

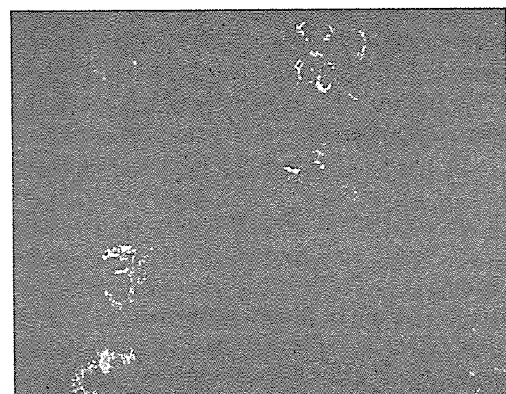

Figure 1 | Infected foci of cell culture–generated HCV-infected cells. Huh 7.5.1 cells were seeded at a density of 1×10^4 cells per well in 96-well plates. Each well was inoculated with serially diluted virus solution. At 3 d after infection, inoculated cells were fixed with 100% methanol and were then 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.

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.

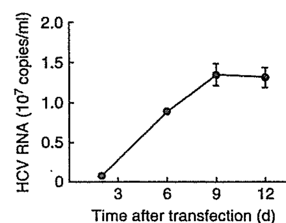

Figure 2 | Transient HCV RNA secretion by transfected Huh7 cells.

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).

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

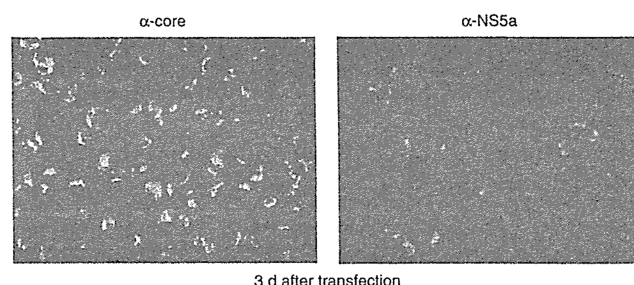

Figure 3 | Immunofluorescence microscopy of HCV proteins in Huh7 cells transfected with JFH-1 RNA. Transfected cells were seeded on coverslips 2 d after transfection, and HCV proteins were detected with antibody to core protein (α-core; 2H9; ref. 3) and to nonstructural protein 5A (α-NS5a; mouse polyclonal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

● TIMING
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

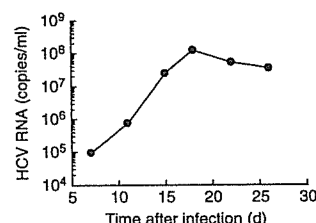

Figure 4 | HCV RNA titers in culture supernatants of inoculated Huh7.5.1 cells.

? 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**).

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

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

Published online at http://www.natureprotocols.com
Rights and permissions information is available online at http://npg.nature.com/reprintsandpermissions

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).

Virology Journal

BioMed Central

Research

Open Access

Replication of a hepatitis C virus replicon clone in mouse cells

Susan L Uprichard*[1,2], Josan Chung[1], Francis V Chisari[1] and Takaji Wakita[3]

Address: [1]The Scripps Research Institute, Department of Molecular and Experimental Medicine, La Jolla, CA 92037 USA, [2]The University of Illinois at Chicago, Department of Medicine and Department of Microbiology and Immunology, Chicago, IL 60612, USA and [3]Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo 183-8526, Japan

Email: Susan L Uprichard* - sluprich@uic.edu; Josan Chung - jchung@scripps.edu; Francis V Chisari - fchisari@scripps.edu; Takaji Wakita - wakita@tmin.ac.jp

* Corresponding author

Published: 28 October 2006

Virology Journal 2006, 3:89 doi:10.1186/1743-422X-3-89

Received: 23 October 2006
Accepted: 28 October 2006

This article is available from: http://www.virologyj.com/content/3/1/89

© 2006 Uprichard et al; licensee BioMed Central Ltd.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: Hepatitis C Virus (HCV) is a significant public health burden and small animal models are needed to study the pathology and immunobiology of the virus. In effort to develop experimental HCV mouse models, we screened a panel of HCV replicons to identify clones capable of replicating in mouse hepatocytes.

Results: We report the establishment of stable HCV replication in mouse hepatocyte and fibroblast cell lines using replicons derived from the JFH-1 genotype 2a consensus sequence. Viral RNA replication efficiency in mouse cells was comparable to that observed in human Huh-7 replicon cells, with negative-strand HCV RNA and the viral NS5A protein being readily detected by Northern and Western Blot analysis, respectively. Although HCV replication was established in the absence of adaptive mutations that might otherwise compromise the *in vitro* infectivity of the JFH-1 clone, no infectious virus was detected when the culture medium from full length HCV RNA replicating mouse cells was titrated on Huh-7 cells, suggesting that the mouse cells were unable to support production of infectious progeny viral particles. Consistent with an additional block in viral entry, infectious JFH-1 particles produced in Huh-7 cells were not able to establish detectable HCV RNA replication in naïve mouse cells.

Conclusion: Thus, this report expands the repertoire of HCV replication systems and possibly represents a step toward developing mouse models of HCV replication, but it also highlights that other species restrictions might continue to make the development of a purely murine HCV infectious model challenging.

Background

Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus that causes acute and chronic hepatitis [1]. Between 70–90% of those who become infected fail to clear the virus and remain chronically infected with the risk of developing liver cirrhosis and hepatocellular carcinoma [2]. Unfortunately, there is no vaccine available to prevent this infection, and the only approved treatment has toxic side effects and is only effective in a subset of patients [3,4].

Even though recent work has led to the development of *in vitro* HCV infection systems, which allow for molecular analysis of the entire viral life cycle [5-7], the study of the immunobiology and pathogenesis of HCV still requires the development of genetically defined small animal

models. One obstacle to the development of HCV mouse models has been the limited host range of the virus. The restrictions that block HCV infection in mice are not well defined, but appear to involve multiple steps such as viral entry and genome replication.

Notably however, HCV replicons based on engineered viral genomes into which the antibiotic resistant marker neomycin phosphotransferase (neo) has been inserted [8](Fig. 1A) provide a means of experimentally by-passing viral entry and actively selecting for HCV replication after transfection of RNA into cells. The ability to select for cells replicating the neo-expressing replicon RNA led to the discovery that efficient replication of most HCV replicons in cell culture requires adaptive mutations in the viral genome [9-13]. Although HCV replication initially could only be achieved in the human hepatoma cell line, Huh-7, the ability to select for replication enhancing mutations eventually led to the establishment of HCV replication in other hepatic (HepG2 and IMY-N9 [14]) and nonhepatic (HeLa [15,16] and HEK293 [15,17]) human cell lines.

Unlike other published studies that focus exclusively on HCV replication in human and/or primate cell lines, Zhu et al (2003) further demonstrated that replication of the HCV-N genotype 1b subgenomic replicon could be initiated in one of the several mouse cell lines tested. However, this replication could only be established in a single mouse cell line after transfection of total RNA extracted from HeLa cells that were already replicating the adapted replicon (i.e. total human cellular RNA presumably containing a quasispecies of HCV replicons)[16]. In contrast, HCV replication could not be initiated in these mouse cells by transfection of *in vitro* transcribed replicon RNA generated from either the parental replicon construct or from any of the "adapted" replicon clones isolated from their original mouse replicon cells. Hence, no "mouse-permissive" HCV replicon clone was identified.

Because the development of HCV mouse models would be greatly facilitated by the identification of defined HCV clone(s) capable of establishing and maintaining replication in mice, we assembled a panel of HCV replicons derived from different HCV genotypes and assessed their ability to replicate in mouse cells. We show that JFH-1 genotype 2a subgenomic and full length replicons are able to stably replicate in multiple mouse hepatocyte and fibroblast cell lines following transfection of the replicon RNA. Although the HCV replication achieved in mouse hepatocytes was not dependent on adaptive mutations that might compromise the infectivity of the viral clone, no infectious HCV was detected in the media of mouse cells replicating full length HCV RNAs, nor were infectious JFH-1 virus particles produced in Huh-7 cells able to infect naïve mouse cells. Thus, these mouse cells are permissive for JFH-1 HCV replication, but exhibit blocks in both viral entry and production of infectious particles.

Results

Establishment of G418-resistant mouse hepatocyte colonies after transfection of subgenomic replicons encoding the neomycin selection gene

Because different HCV replicon clones exhibit a range of replication efficiencies in Huh-7 cells, we hypothesized that different replicons might also display differences in their ability to replicate in mouse cells. Thus, we screened a panel of subgenomic HCV replicons derived from genotypes 1b, 1a, and 2a for their ability to confer G418 resistance to mouse hepatocytes *in vitro*. We initially screened for HCV replication in immortalized Met Mouse Hepatocytes (MMH) cells; however, because HCV replication efficiency within different Huh-7 cell lines can vary, we tested

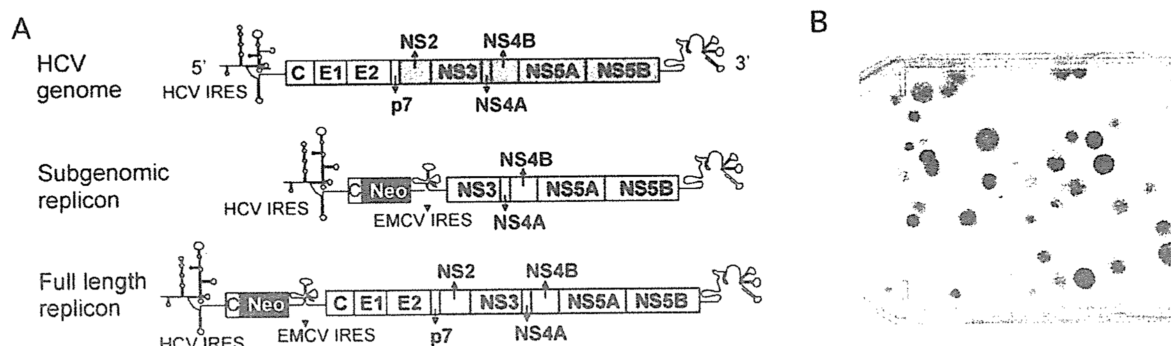

Figure 1
(A) Schematic diagram of HCV genomic and replicon RNA. (B) Representative crystal violet staining of G418-resistant colony formation in MMHD3 mouse hepatocytes after transfection with sgJFH-1 HCV RNA. Notably, although MMH cells exhibit relatively low transfection efficiency, numerous G418-resistant colonies form following transfection of HCV replicon RNA.

HCV replication in two independently derived MMH cell lines, MMHD3 [18] and MMH1-1 [19].

Replicon RNA from the clones listed in Table 1 was transcribed *in vitro* and electroporated into MMH cells. Transfected cells were then treated with 500 µg/ml G418, which was the minimal concentration required to effectively kill untransfected control cell cultures. Despite the fact that G418-resistent Huh-7 cell colonies survived the selection process after transfection with all of the replicon RNAs tested, RNA transcribed from the HCV genotype 1b clones (Con1 and HCV-N) and the HCV genotype 1a H77 clone did not generate G418-resistent mouse cell colonies (data not shown). In contrast, transfection of genotype 2a subgenomic (sg) JFH-1 RNA produce G418-resistant mouse hepatocyte colonies in both MMH cell lines in multiple independent transfection experiments (Fig. 1B).

Expression and replication of sgJFH-1 HCV replicons in immortalized mouse hepatocytes

To confirm the expression and replication of HCV in MMHD3 and MMH1-1 cells, G418-resistant cell colonies were expanded for further analysis. Western Blot analysis of total cell lysate verified that the viral NS5A protein was present in each of the MMHD3 and MMH1-1 replicon cell clones (Fig. 2A, lanes 5, and 6–11, respectively). Yet, constitutive HCV protein expression was not due the integration of an HCV transgene, as PCR analysis on cellular genomic DNA revealed no evidence of JFH-1 sequences (data not shown).

Clones were also examined for the presence of HCV RNA by strand-specific Northern Blot. Positive-strand (Fig. 2B, top panel) and negative-strand (Fig. 2B, middle panel) HCV RNA was detected not only in Huh-7 control cell clones (Fig. 2B, lanes 11–15), but also in the mouse clones derived from MMHD3 and MMH1-1 cells (Fig. 2B, lanes 2 and 4–9, respectively). Although the signal intensity observed with these independently labeled and hybridized riboprobes can not be compared to determine the ratio of positive-strand to negative-strand HCV RNA, both probes demonstrated stringent strand-specific hybridization when incubated with membranes containing serial dilutions (10^9, 10^8, and 10^7 copies) of *in vitro* transcribed positive-strand (Fig. 2B, lanes 16–18) and negative-strand (Fig. 2B, lanes 19–21) sgJFH-1 RNA controls, clearly demonstrating that negative-strand HCV RNA replication intermediates were being produced in the clones.

Table 1: HCV Replicons Screened

Genotype	Clone
1b	sgCon1 WT
1b	sgCon1 S1179I
1b	sgHCV-N
1a	sgH77
2a	sgJFH-1

sg = subgenomic

RT-QPCR analysis of the same RNA samples indicated that HCV RNA levels among the mouse sgJFH-1 replicon clones ranged from 2.9×10^6 – 7.0×10^7 copies per microgram (copies/µg) of total cellular RNA. This was similar to the 7.4×10^6 – 8.5×10^7 copies/µg observed in the human sgJFH-1 replicon clones. Thus, the average 2.0×10^7 HCV RNA copies/µg calculated in the mouse cells was comparable to the average 5.0×10^7 HCV RNA copies/µg detected in the Huh-7 clones indicating that while the level of the HCV RNA does vary between individual cells clones, no statistical difference in RNA level was detectable between mouse and human cells.

Adaptive mutations are not required for replication of sgJFH-1 replicons in mouse hepatocytes

Because adaptive mutations are required for efficient replication of many HCV replicons in cell culture [9-11,20-22], we sequenced replicon clones obtained from our mouse cell lines to determine whether the population of sgJFH-1 replicons present in the MMH cells had acquired any adaptive mutations. RNA was extracted from 3 of the MMH replicon cell lines (MMHD3-sgJFH#1 and MMH1-1sgJFH#4 and #9). After reverse transcription, 4 PCR primer sets were used to amplify overlapping segments spanning the sgJFH-1 cDNA (Fig. 3A). These PCR products were ligated into the pGEMT-Easy vector and 12 individual clones of each fragment were sequenced. Although random mutations were observed throughout the cloned genomes, none of the changes were detected in more than 1 of the 12 clones sequenced (Fig. 3B), supporting the conclusion that adaptive mutations had not become established in the mouse replicon population. Also consistent with the absence of replicon adaptive mutations, total RNA isolated from both MMH and Huh-7 replicon cells containing comparable copies of sgJFH RNA (as determined by RT-QPCR) formed equivalent numbers of G418-resistant colonies/µg of viral RNA when re-transfected into naïve MMH cells (data not shown).

To determine if the initial establishment of the mouse replicon clones had selected for adapted MMH cells that were more permissive for HCV replication, we also "cured" the sgJFH-1 replicon from our MMH replicon cells with a 3 week treatment of IFNα (1000 U/ml) and IFNγ (500 U/ml), but subsequent sgJFH-1 RNA transfections into these "cured" (e.g. G418-sensitive, HCV-negative) mouse cells did not produce higher numbers of G418-resistent colonies/µg of input RNA compared to parallel transfections into the parental MMH cell lines indicating that we had

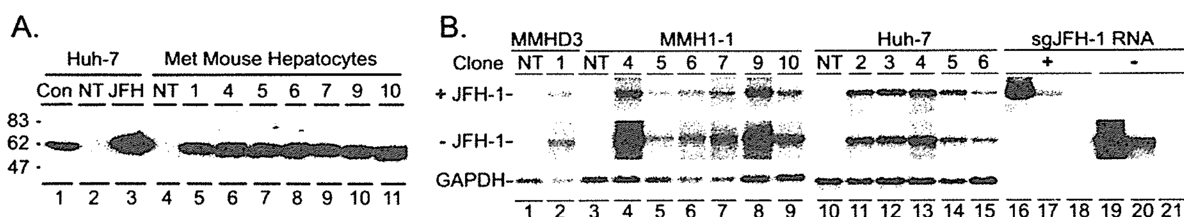

Figure 2
HCV protein and RNA detection in immortalized mouse hepatocytes. (A) Western Blot detection of HCV NS5A. Cell lysate was harvested from individual cell clones and resolved by SDS-PAGE. Samples include cell lysate from Huh-7 sgCon1 (lane 1); non-transfeted (NT) Huh-7 (lane 2); Huh-7 sgJFH-#2 (lane 3); NT MMHD3 cells (lane 4); MMHD3 sgJFH#1 (lane 5); and MMH1-1sgJFH#4, 5, 6, 7, 9, and 10 (lanes 6–11). (B) Northern Blot detection of sgJFH-1 RNA. Total RNA was isolated from non-transfected (NT) and individual MMHD3 (lanes 2), MMH1-1 (lanes 4–9), and Huh-7 (lanes 11–15) sgJFH-1 replicon clones. Positive-strand (top panel) and negative-strand (middle panel) HCV RNA was detected with ^{32}P-labeled strand-specific riboprobes. Cellular GAPDH was detected with a ^{32}P-labeled cDNA probe (bottom panel). Serial dilutions of *in vitro* transcribed positive-strand and negative-strand sgJFH-1 RNA (10^9, 10^8, 10^7) are shown in lanes 16–18 and lanes 19–21, respectively.

not selected MMH cells that more efficiently allow for establishment of HCV replication (data not shown).

Replication of sgJFH-1 HCV in other mouse cell lines

Because the sgJFH-1 replicon was able to replicate in MMH cells without any apparent viral or cellular adaptive mutations, we proceeded to determine if this ability was restricted to MMH cells or if the JFH-1 clone could replicate in other mouse cell lines. Hence, we transfected *in vitro* transcribed sgJFH-1 RNA into mouse AML12 hepatocytes as well as mouse NIH3T3 embryonic fibroblasts and selected for cells that were able to support replication of the neo-expressing replicon. Figure 4A shows strand-specific HCV Northern Blot analysis that verifies the presence of both positive-strand (Fig. 4A, top panel) and negative-strand (Fig. 4A, middle panel) HCV RNA in all the AML12 mouse hepatocyte cell clones. Similarly, negative strand-specific HCV Northern Blot analysis of total RNA from 9 NIH3T3 replicon cell clones also confirmed the presence of negative-strand HCV RNA in these non-hepatic mouse cells demonstrating that the sgJFH-1 HCV clone can replicate in a variety of mouse cell lines (Fig. 4B).

Replication of full length JFH-1 HCV replicons in mouse cells

To determine if full length HCV replication also could be established in mouse cells, *in vitro* transcribed full length (fl) JFH-1 replicon RNA was electroporated into MMH1-1 and Huh-7 cells, and cells supporting replicon replication were selected with 500 μg/ml G418. Northern Blot analysis of total RNA isolated from the resulting G418-resistant clones confirmed that negative-strand flJFH-1 RNA was present in both MMH1-1 (Fig. 5, lanes 1–4) and Huh-7 (Fig. 5, lanes 5–10) transfected cultures. However, unlike sgJFH replicon clones in which HCV RNA levels were equivalent between human and mouse cells clones, RT-QPCR analysis indicated that HCV RNA levels were approximately 3-fold lower in flJFH-1 MMH1-1 cell clones ($9.5 \times 10^4 - 7 \times 10^5$ copies/μg total RNA) compared to flJFH-1 Huh7 cell clones ($3.1 \times 10^5 - 1.9 \times 10^6$ copies/μg total RNA).

Lack of infectious HCV particle entry into and secretion from mouse hepatocytes

Because the flJFH-1 is capable of producing infectious HCV particles, we proceeded to determine whether the MMH1-1 mouse cells were able to secrete infectious HCV particles and/or were permissive for flJFH-1 entry. To determine if the flJFH-1 replicon clones were secreting infectious HCV particles that could transmit G418-resistance, the culture media from individual clones was collected and used to inoculate naive Huh-7 and MMH1-1 cells. Although G418-resistant colonies formed when Huh-7 cells were inoculated with the supernatant from Huh-7 flJFH-1 replicon cells, the culture supernatant from MMH1-1 flJFH-1 replicon cells did not confer G418-resistance to Huh-7 cells suggesting that the mouse cells were not able to secrete infectious HCV particles. Titration of the same media on Huh-7 cells further confirmed that all the Huh-7 flJFH-1 cell clones were secreting infectious HCV particles (expressed as foci forming units per ml), while no infectious HCV was detected in the media from MMH1-1 flJFH-1 cells, even after 50-fold concentration by centrifugal filtration (Fig. 5B).

Notably, inoculation of naïve MMH1-1 cells with media from either Huh-7 flJFH-1 or MMH1-1 flJFH-1 cell cultures did not confer G418-resistence to the mouse cells confirming that HCV particles are unable to enter mouse cells. Importantly, inoculation with high titer authentic

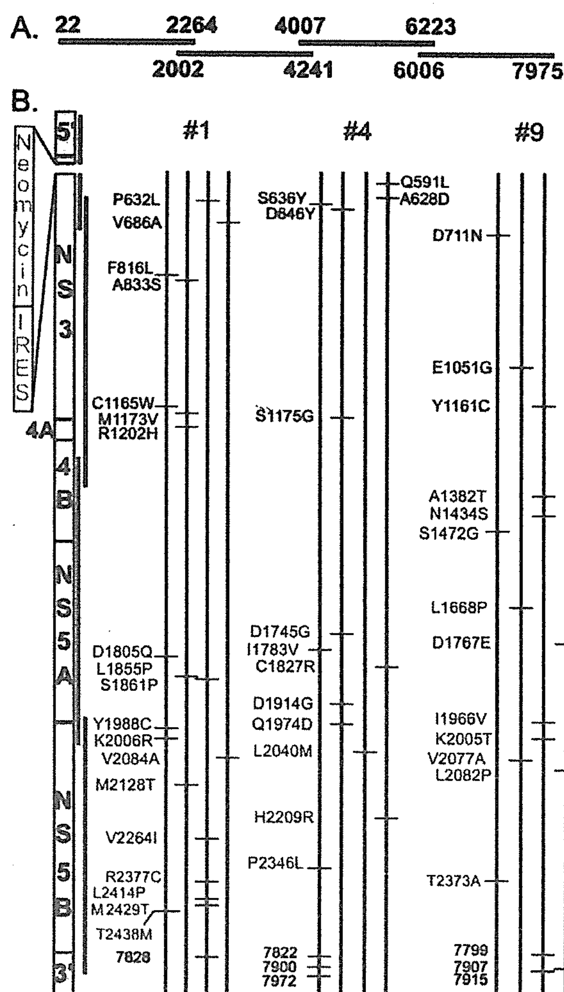

Figure 3

Sequence analysis of sgJFH-1 replicons in mouse hepatocytes. (A) PCR primer sets used to amplify overlapping HCV DNA segments. (B) Twelve clones of each fragment were sequenced with an ABI automatic DNA sequencer. Mutations within viral proteins are indicated by the amino acid position of the mutation within the context of the full length HCV open reading frame. Mutations in the 3' UTR are designated by their nucleotide position in the context of the full length HCV genome.

HCV virus produced in Huh-7 cells also failed to establish JFH-1 replication in MMH1-1, AML12, or NIH3T3 mouse cells (data not shown).

To determine whether the lack of detectable infectivity in the culture medium of flJFH-1 MMH1-1 replicon clones was due to an inability to secrete HCV particles or due to the secretion of non-infectious HCV particles, the culture supernatant from Huh-flJFH#7 and MMH1-1-flJFH#2 cultures were subjected to sucrose density gradient centrifugation. Fractions were collected, total RNA extracted, and RT-QPCR analysis performed to determine the amount of viral RNA in each fraction (Fig. 5C). HCV RNA in the supernatant of the Huh-flJFH#7 cells was detected over a broad density range of 1–1.15 g/ml, with a predominant peak at 1.07 g/ml, supporting the conclusion that the viral RNA was associated with enveloped particles (Fig. 5C). This is similar to the HCV RNA density profile reported for *in vitro*-derived infectious JFH-1 particles from which RNA was observed in density fractions from 1–1.15 g/ml with a peak at 1.09–1.10 g/ml [5,6]. In contrast, there was no specific low density peak of HCV RNA in the media of MMH1-1flJFH#2 cells and total viral RNA levels in the supernatant were significantly lower than that seen the in Huh-7 replicon cell supernatant. Hence, these results support the conclusion that mouse hepatocytes do not efficiently secrete enveloped, flJFH-1 RNA-containing particles.

Discussion

In this study, we have shown that replicon clones derived from the HCV JFH-1 consensus cDNA can establish and maintain efficient replication in both mouse hepatocytes (e.g. MMHD3, MMH1-1, AML12) and mouse embryonic fibroblasts (e.g. NIH3T3). This discovery reveals a previous unrealized breadth in the host range of the JFH-1 HCV clone, and expands our ability to develop experimental HCV model systems. In addition to observing stable expression of viral proteins and replication of viral RNA (Fig. 2), we found that HCV replication in mouse hepatocytes is not dependent on adaptive mutations in the viral genome (Fig. 3), but is sensitive to inhibition by interferons (data not shown) and the HCV-specific small molecule inhibitor, BILN2061 (data not shown). Taken together, these results demonstrate replication of non-adapted HCV replicon clones in mouse cells, and they potentially provide the basis for the production of a mouse model of HCV replication.

While Zhu et al., (2003) previously provided evidence that HCV replication in mouse hepatocytes was possible, the data presented here differs from that report in three fundamental ways. First, Zhu et al., (2003) could only initiate HCV replication in mouse cells by transfection with a HCV RNA quasispecies obtained from previously established HeLa replicon cells, but they were not able to establish replication in mouse cells with any individual replicon clone. In contrast, we have been able to identify a defined HCV replicon clone capable of replicating in various mouse cell lines. The identification of a specific mouse-permissive HCV clone is advantageous because it facilitates mouse model development and allows for

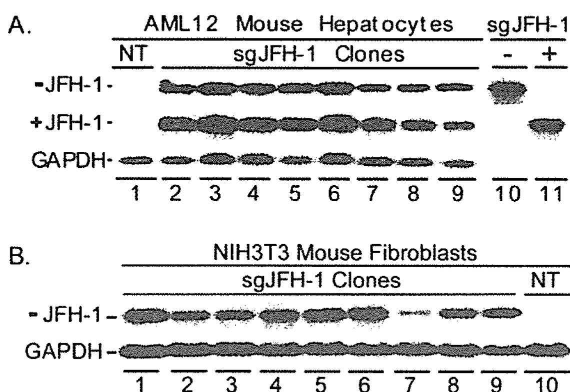

Figure 4
Northern Blot detection of sgJFH-1 RNA in AML12 mouse hepatocytes and NIH3T3 mouse fibroblasts. (A) Negative-strand HCV (top panel), positive-strand HCV (middle panel), and cellular GAPDH (bottom panel) was detected by Northern Blot analysis of total RNA isolated from non-transfected (NT) parental AML12 cells (lane 1) and 8 individual sgJFH-1 RNA transfected clones (lanes 2–9). *In vitro* transcribed positive-strand (lane 10) and negative-strand (lane 11) sgJFH-1 RNA was included as controls. (B) Northern Blot analysis for negative-strand HCV (top panel) and cellular GAPDH (bottom panel) was performed on total RNA isolated from non-transfected (NT) parental NIH3T3 cells (lane 10) and 9 randomly chosen sgJFH-1 RNA transfected clones (lanes 1–9).

reverse-genetics experimentation. Second, whereas replication of the subgenomic 1b HCV-N replicon reported by Zhu et al., (2003) was restricted to one specific mouse cell line, we have been able to establish JFH-1 HCV replication in multiple mouse cell lines significantly enhancing the likelihood that future *in vitro* and *in vivo* experimental HCV mouse models based on the JFH-1 clone might be possible. Finally, while the previous success was dependent on cell culture adaptive mutations that prevent HCV infectivity [23], we have demonstrated that replicons derived from the JFH-1 consensus sequence can replicate in mouse hepatocytes, in the absence of adaptive mutations that might otherwise alter the infectivity of the clone.

The ability of the JFH-1 clone to replicate efficiently in tissue culture in the absence of adaptive mutations has recently led to the development of *in vitro* HCV infection models in Huh-7 cells [5-7] and the demonstration that full length HCV replicons are capable of producing infectious viral particles (Fig. 5B–C). Likewise it provided us the opportunity to study the ability of mouse cells that support JFH-1 replication to uptake and secrete infectious HCV JFH-1 particles. Consistent with previous HCV pseudoparticles data, we observed no evidence that HCV particles were able to productively enter mouse cells, even when we used G418 treatment to select for cells that might have become HCV positive following inoculation. More unexpectedly, we also observed that mouse hepatocytes replicating infectious JFH-1 RNA were not able to secrete infectious HCV RNA containing particles. Though it is possible there were low levels of infectious particles present that were below the detection limits of our assays, sucrose density gradient analysis and RT-QPCR analysis of HCV RNA isolated from the mouse culture supernatants supports the conclusion that very few, if any, enveloped, HCV RNA-containing particles were being secreted from these mouse cells (Fig. 5C). This may reflect an inability to assemble infectious particles, secrete viral particles, or both. Hence, in addition to productive viral entry, infec-

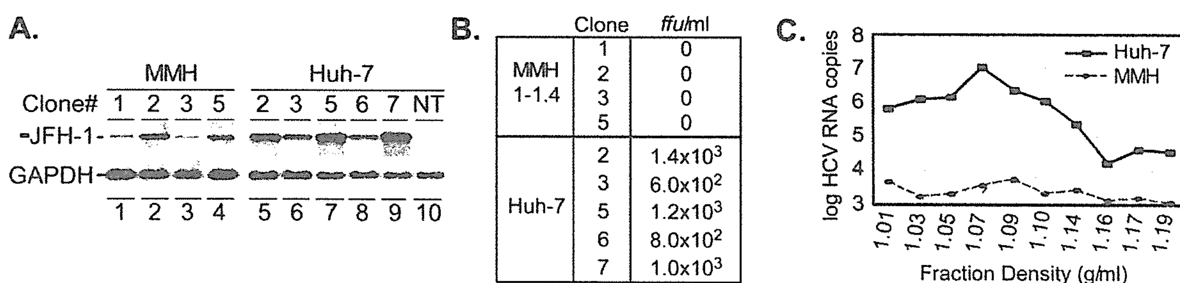

Figure 5
Intracellular and extracellular HCV RNA produced by flJFH-1 mouse replicon clones. (A) Northern Blot analysis for negative-strand HCV (top panel) and cellular GAPDH (bottom panel) was performed on total RNA isolated from MMH1-1 replicon clones (lanes 1–4), Huh-7 replicon clones (lanes 5–9), and non-transfected (NT) Huh-7 cells (lane 10). (B) HCV infectivity titer in the culture medium of the same MMH and Huh-7 clones was determined by incubating serial dilutions on naïve Huh-7 cells and performing immunofluorescence for HCV 3 days post-inoculation, as previously described [5]. The titer is expressed as NS5A-positive focus-forming units (*ffu*) per ml of medium. (C) RT-QPCR detection of extracellular HCV RNA following sucrose density gradient centrifugation.

tious particle production appears to be another aspect of the HCV life cycle that may not be readily recapitulated in mouse hepatocytes. Clearly, elucidating the basis of this phenomenon may help us understand the key steps required for infectious HCV particle secretion in human cells.

Conclusion

The data presented in this report demonstrate that JFH-1 derived HCV replicon clones can efficiently replicate in mouse cells. While this discovery certainly represents an advance in our ability to develop mouse models of HCV replication, further analysis of infectious JFH-1 HCV entry into and egress from mouse cells also identified at least 2 other aspects of the HCV life cycle that are not supported by the mouse hepatocytes tested. Such species restrictions will likely continue to make the development of a purely murine HCV infectious model challenging.

Methods

HCV constructs

Constructs containing the non-adapted wild type (pHCVrep1b BartMan_AvaII) and Huh-7 cell adapted [pHCVrep1bBB7 (S1179I)] HCV Con1 genotype 1b subgenomic replicons [9] and the genotype 1a H77 subgenomic replicon [H/SG-Neo(L+I)] [22] were provided by Dr. Charles Rice (Rockefeller University, NY). The pNNeo/3-5B(SI) construct encoding the genotype 1b HCV-N subgenomic replicon was provided by Dr. Stanley Lemon (University of Texas Medical Branch, Galveston)[13]. The constructs pSGR-JFH1, pFGR-JFH1, and pJFH-1 containing the sgJFH-1 HCV genotype 2a JFH-1 replicon, the full length (fl)JFH-1 replicon, and the genomic JFH-1 viral clone have been described [7,24,25]. In all constructs, the HCV cDNA is located at the 1+ position 5' of the T7 promoter. Plasmids were linearized at the 3' end of the HCV cDNA and used as a template for *in vitro* transcription by T7 RNA polymerase (MEGAscript; Ambion, Austin, TX). To generate strand-specific RNA probes, a 1 kb fragment of the JFH-1 NS5B coding region (HindIII-EcoRV) was cloned into the pBSKII+ vector to allow for T7 and SP6-driven transcription of JFH-1 negative and positive strand probes, respectively.

Cell culture

Huh-7 human hepatocytes and NIH3T3 mouse fibroblasts (CRL-1658; ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% fetal calf serum and Penicillin-Streptomycin-Glutamine (100×, liquid)(Gibco Invitrogen Corporation, Carlsbad, CA). AML12 mouse hepatocytes (ATCC; CRL-2254) were maintained in DMEM/F12 supplemented with 10% fetal calf serum, Penicillin-Streptomycin-Glutamine (100×, liquid), insulin-transferrin-selenium (100×, liquid)(Gibco Invitrogen), and 40 ng/ml dexamethasone (Sigma, St. Louis, MO). MMHD3 (Met Murine Hepatocyte) cells were obtained from Marco Tripodi (Università La Sapienza, Italy)[18]. This immortalized mouse hepatocyte cell line was derived from the liver of transgenic mice, which express the constitutively active cytoplasmic domain of the human hepatocyte growth factor receptor (cMet) in their livers. The MMH1-1 cells were independently derived from double transgenic cMet transgenic mice that also have a hepatitis B virus (HBV) transgene [19]. Although these cells contain a HBV transgene, expression and replication of HBV in these cells only occurs after the cells become confluent and are further differentiated in the presence of 2% DMSO for 8 days [19]. All of the experiments presented here were performed on subconfluent cell cultures in the absence of DMSO; therefore, no HBV replication was occurring in these cells (data not shown). Both MMHD3 and MMH1-1, were plated on collagen I Biocoat dishes (Becton Dickinson, Franklin Lakes, NJ) in RPMI 1640 (Gibco Invitrogen) supplemented with 10% fetal calf serum (Gibco Invitrogen), 55 ng/ml EGF (Becton Dickinson), 16 ng/ml IGF-II (Calbiochem, San Diego, CA), 10 μg/ml insulin (Sigma) and Penicillin-Streptomycin-Glutamine (100×, liquid)(Gibco Invitrogen)[19]. G418 was added to culture media as indicated (Invitrogen).

HCV RNA transfection and G418-resistant colony formation

In vitro transcribed HCV RNA or total cell RNA was transfected into cells using a modified electroporation protocol [10]. Trypsinized cells were washed twice with serum-free medium and resuspended to a final concentration of 1 × 10^7 cells/ml. One to ten micrograms of HCV RNA was then mixed with 0.4 ml of the cells in a 4 mm cuvette. A Gene Pulser system (BioRad Laboratories, Hercules, CA) was used to deliver the following single pulses: Huh-7 (0.27 kV, 100 OHMS, 960 μF); Met-based and AML12 cells (0.45 kV, 100 OHMS, 960 μF); NIH3T3 (0.27 kV, 100 OHMS, 960 μF). For the generation of replicon clones, transfected cultures were maintained in the presence of G418 at a concentration of 500 μg/ml until all cells died or distinct G418-resistant cell colonies formed. To visualize colony formation, cells were fixed and stained with crystal violet.

RNA analysis

Total cell RNA was isolated by the guanidine thiocyanate method using standard protocols [26]. RNA was resolved in 1% agarose, 2.2 M formaldehyde gels and transferred to nylon membrane (Schleicher & Schuell, Keene, NH). Membranes were cut across the 28S ribosomal band so that the top of the blot could be hybridized with ^{32}P-labeled strand-specific riboprobes (MAXIscript; Ambion), while the bottom was hybridized with ^{32}P-labeled cellular GAPDH cDNA probe (Random Prime Synthesis; Invitro-

gen, Carlsbad, CA). Hybridized probe was visualized using a storage phosphor system (Cyclone; Packard Instrument Co.). Alternatively, 1 μg of RNA was DNAse treated (DNA-*free* reagent; Ambion) for reverse transcription quantitative polymerase chain reaction (RT-QPCR). RNA was used for cDNA synthesis using the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA), followed by real-time PCR quantification using a BioRad iCycler (BioRad Laboratories). HCV and GAPDH transcript levels were determined relative to a standard curve comprised of serial dilutions of plasmid containing the HCV cDNA or the mouse GAPDH gene. The PCR primers used to detect JFH-1 (GenBank AB047639) were 5'-TCTGCGGAACCGGTGAGTA-3' (sense) and 5'-TCAGGCAGTACCACAAGGC-3' (antisense). The PCR primers used to detect H77C (GenBank AF011751) were 5'-GTCTGCGGAACCGGTGAG-3' (sense) and 5'-GGCATTGAGCGGGTTTATC-3' (antisense). The PCR primers used to detect both genotype 1b HCV clones (Genbank AJ242652 and AF139594) were 5'-ATGGCGTTAGTATGAGTGTC-3' (sense) and 5'-GGCATTGAGCGGGTTGATC-3' (antisense). The PCR primers used to detect mouse GAPDH (GenBank M32599) were 5'-TCTGGAAAGCTGTGGCGTG-3' (sense) and 5'-CCAGTGAGCTTCCCGTTCAG-3' (antisense).

Western Blot analysis

Cells were harvested in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate,150 mM NaCl, 1 mM EDTA) supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis). Fifty micrograms of protein was resolved by SDS-PAGE and transferred to Hybond nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ). Membranes were sequentially blocked with 5% Nonfat Milk, incubated with a 1:500 dilution of the polyclonal rabbit NS5A antibody, M15 (provided by Dr. Michael Houghton, Chiron, Emeryville, CA), washed 3 times with PBS/0.05% Tween20, incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce, Rockford, Illinois), and washed again. Bound antibody complexes were detected with SuperSignal chemiluminescent substrate (Pierce).

Sequencing HCV replicons

Total cell RNA was extracted from 3 mouse replicon cell lines (MMHD3sgJFH#1, MMH1-1sgJFH#4, and MMH1-1sgJFH#9). Reverse transcription was carried out with random hexamers using 1 μg of RNA (Transcriptor Reverse Transcriptase; RocheApplied Sciences, Indianapolis, IN). HCV DNA was subsequently amplified with high fidelity Tgo polymerase (Roche). Four PCR primer sets were used to amplify overlapping segments spanning positions 22 to 2264, 2002 to 4241, 4007 to 6223, and 6006 to 7975 of the sgJFH-1 replicon (Fig. 3A). The PCR products were cloned into the pGEM-T Easy plasmid (Promega, Madison WI), and 12 clones of each fragment (4 from each cells line) were sequenced with an ABI automatic DNA sequencer using the M13 forward and reverse primers in the vector as well as 20–21 bp sequencing primers located within the HCV subgenomic replicon at nucleotide positions: 1252, 1790, 2053, 2290, 2571, 2820, 3269, 3836, 4256, 4634, 4813, 5215, 5425, 6148, 6555, and 7416.

Titration of infectious HCV

Cell supernatants were serially diluted in complete medium and used to infect naïve MMH and Huh-7 cells in 96-well plates. The level of HCV infection was determined 3 days post-infection by immunofluorescence staining for HCV NS5A as previously described [5]. The viral titer is expressed as focus-forming units per milliliter of supernatant (*ffu*/ml), determined by the number of NS5A-positive foci detected at the highest HCV-positive dilution.

Sucrose density gradient analysis

Sucrose density-gradient ultracentrifugation was performed as described [5]. Supernatant from flJFH-1 replicon cultures was collected, centrifuged at 4,000 rpm for 5 min to remove cellular debris, and concentrated by centrifugal filteration when indicated (Amicon Ultra; Millipore, Billerica, MA). Samples were loaded onto TNE buffer (50 mM Tris·HCl, pH 8;100 mM NaCl;1 mM EDTA)-based 20–60% sucrose gradient and centrifuged at 120,000 × *g* for 16 h at 4°C using a SW60 rotor in a Beckman Coulter L8-80 Ultracentrifuge (Fullerton, CA). Fractions were collected from the bottom of the gradient, and analyzed for HCV RNA by RT-QPCR.

Note

While this manuscript was being prepared, Chang et al., (JVirol. 2006 Aug;80:7364-74) published a report consistent with these data showing that JFH-1 replicons can replicate in mouse embryonic fibroblasts (MEFs).

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

SLU conceived and designed the study, obtained funding, and then performed the experiments. JC did the sequence analysis of sgJFH-1 from mouse cells and cloned the JFH-1 RNA probe template plasmid. FVC participated in data analysis, revising of the manuscript, and provided funding for JC. TW provided the critical unpublished reagents, participated in data analysis, and revising of the manuscript. All authors read and approved the manuscript.

Acknowledgements

We thank our colleagues, Drs. Charles Rice, Stanley Lemon, Marco Tripodi, and Michael Houghton for providing valuable reagents for these studies. We additionally thank Alana Althage for technical assistance and Drs. Bruno Sainz and Stefan Wieland for critical review of the manuscript.

This work was supported by the NIH Public Health Service grant AI-060391 (SLU) and the Sam and Rose Stein Endowment Fund. JC and other laboratory support staff were funded by CA-108304 (FVC) and CA-40489 (FVC). TW was supported by a grant from the Ministry of Health, Labor and Welfare of Japan, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, and Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation. This is manuscript #17950-MEM from The Scripps Research Institute.

References

1. Di Bisceglie AM: **Hepatitis C.** *Lancet* 1988, **351(9099)**:351-355.
2. Alter MJ, Margolis HS, Krawczynski K, Judson FN, Mares A, Alexander WJ, Hu PY, Miller JK, Gerber MA, Sampliner RE, Meeks EL, Beach MJ, The Sentinel Counties Chronic Non-A NBHST: **The natural history of community-acquired hepatitis C in the United States.** *NEnglJMed* 1992, **327**:1899-1905.
3. Zeuzem S, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, O'Grady J, Reichen J, Diago M, Lin A, Hoffman J, Brunda MJ: **Peginterferon alfa-2a in patients with chronic hepatitis C.** *N Engl J Med* 2000, **343(23)**:1666-1672.
4. Heathcote EJ, Shiffman ML, Cooksley WG, Dusheiko GM, Lee SS, Balart L, Reindollar R, Reddy RK, Wright TL, Lin A, Hoffman J, De Pamphilis J: **Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis.** *N Engl J Med 2000* 2000, **343(23)**:1673-1680.
5. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV: **Robust hepatitis C virus infection in vitro.** *Proc Natl Acad Sci USA* 2005, **102(3)**:773-778.
6. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM: **Complete replication of hepatitis C virus in cell culture.** *Science* 2005, **309(5734)**:623-626.
7. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ: **Production of infectious hepatitis C virus in tissue culture from a cloned viral genome.** *Nat Med* 2005, **11(7)**:791-796.
8. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R: **Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line.** *Science* 1999, **285(5424)**:110-113.
9. Blight KJ, Kolykhalov AA, Rice CM: **Efficient initiation of HCV RNA replication in cell culture.** *Science* 2000, **290(5498)**:1972-1975.
10. Krieger N, Lohmann V, Bartenschlager R: **Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations.** *J Virol 2001 May;75(10):4614-24* 2001, **75(10)**:4614-4624.
11. Lohmann V, Korner F, Dobierzewska A, Bartenschlager R: **Mutations in Hepatitis C Virus RNAs Conferring Cell Culture Adaptation.** *J Virol* 2001, **75(3)**:1437-1449.
12. Guo JT, Bichko VV, Seeger C: **Effect of alpha interferon on the hepatitis C virus replicon.** *J Virol* 2001, **75(18)**:8516-8523.
13. Ikeda M, Yi M, Li K, Lemon SM: **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* 2002, **76(6)**:2997-3006.
14. Date T, Kato T, Miyamoto M, Zhao Z, Yasui K, Mizokami M, Wakita T: **Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells.** *J Biol Chem* 2004, **279(21)**:22371-22376.
15. Kato T, Date T, Miyamoto M, Zhao Z, Mizokami M, Wakita T: **Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon.** *J Virol* 2005, **79(1)**:592-596.
16. Zhu Q, Guo JT, Seeger C: **Replication of hepatitis C virus subgenomes in nonhepatic epithelial and mouse hepatoma cells.** *J Virol* 2003, **77(17)**:9204-9210.
17. Ali S, Pellerin C, Lamarre D, Kukolj G: **Hepatitis C virus subgenomic replicons in the human embryonic kidney 293 cell line.** *J Virol* 2004, **78(1)**:491-501.
18. Amicone L, Spagnoli FM, Spath G, Giordano S, Tommasini C, Bernardini S, De Luca V, Della Rocca C, Weiss MC, Comoglio PM, Tripodi M: **Transgenic expression in the liver of truncated Met blocks apoptosis and permits immortalization of hepatocytes.** *Embo J* 1997, **16(3)**:495-503.
19. Pasquetto V, Wieland SF, Uprichard SL, Tripodi M, Chisari FV: **Cytokine-sensitive replication of hepatitis B virus in immortalized mouse hepatocyte cultures.** *J Virol* 2002, **76(11)**:5646-5653.
20. Maekawa S, Enomoto N, Sakamoto N, Kurosaki M, Ueda E, Kohashi T, Watanabe H, Chen CH, Yamashiro T, Tanabe Y, Kanazawa N, Nakagawa M, Sato C, Watanabe M: **Introduction of NS5A mutations enables subgenomic HCV replicon derived from chimpanzee-infectious HC-J4 isolate to replicate efficiently in Huh-7 cells.** *J Viral Hepat* 2004, **11(5)**:394-403.
21. Yi M, Lemon SM: **Adaptive mutations producing efficient replication of genotype 1a hepatitis C virus RNA in normal Huh7 cells.** *J Virol* 2004, **78(15)**:7904-7915.
22. Blight KJ, McKeating JA, Marcotrigiano J, Rice CM: **Efficient Replication of Hepatitis C Virus Genotype 1a RNAs in Cell Culture.** *J Virol* 2003, **77(5)**:3181-3190.
23. Bukh J, Pietschmann T, Lohmann V, Krieger N, Faulk K, Engle RE, Govindarajan S, Shapiro M, St Claire M, Bartenschlager R: **Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees.** *Proc Natl Acad Sci U S A* 2002, **99(22)**:14416-14421.
24. Kato T, Furusaka A, Miyamoto M, Date T, Yasui K, Hiramoto J, Nagayama K, Tanaka T, Wakita T: **Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient.** *J Med Virol* 2001, **64(3)**:334-339.
25. Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, Wakita T: **Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon.** *Gastroenterology* 2003, **125(6)**:1808-1817.
26. Chomczynski P, Sacchi N: **Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.** *AnalBiochem* 1987, **162**:156-159.

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

Arch Virol (2007) 152: 457–461
DOI 10.1007/s00705-006-0883-z
Printed in The Netherlands

Archives of Virology

Brief Review

Binding activity of norovirus and sapovirus to histo-blood group antigens

H. Shirato-Horikoshi, S. Ogawa, T. Wakita, N. Takeda, and G. S. Hansman

Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

Received August 22, 2006; accepted October 26, 2006; published online November 27, 2006
© Springer-Verlag 2006

Summary

Noroviruses (NoVs) and sapoviruses (SaVs) are causative agents of human gastroenteritis. There is increasing evidence that certain human NoV strains bind to histo-blood group antigens (HBGAs). We found that several NoV virus-like particles (VLPs) showed binding activity to HBGAs, while neither SaV genogroup I (GI) VLP nor SaV GV VLP showed such activity.

*

Human noroviruses (NoVs) and human sapoviruses (SaVs) are etiological agents of human gastroenteritis. Human NoV strains can be grouped into two genogroups (GI and GII), and at least 14 GI and 17 GII genotypes (GI/1–14 and GII/1–17) [11]. SaV strains can be divided into five genogroups (GI–GV), of which the GI, GII, GIV and GV strains infect humans, while the GIII strains infect porcine species [1]. Human NoV and SaV strains are noncultivable, but the expression of the recombinant capsid protein VP1 (rVP1) in insect cells results in the self-assembly of virus-like particles (VLPs) that are antigenically similar to native viruses [2, 9]. In the past several years, increasing evidence has emerged that human NoVs bind to histo-blood group antigens (HBGAs) [8, 12]. These carbohydrate epitopes are present in mucosal secretions and throughout many tissues of the human body, including the small intestine, which may be specifically targeted by certain NoV strains. To the best of our knowledge, the relationship between human SaVs and HBGAs has not yet been reported.

In the present study, we examined the binding activities of human NoV and SaV VLPs to HBGAs present in human saliva and to synthetic carbohydrates. Four NoV strains belonging to different genotypes were examined: the GI/1 124 strain (accession number AB031013), the GI/2 258 strain (AB078335), the GII/4 104 strain (AB078336), and the GII/1 Hawaii strain (U07611). Hawaii VLPs were used as a negative control [5]. Two SaV strains belonging to two different genogroups were also examined: the SaV GI Mc114 strain (AY237422) and the SaV GV NK24 strain (AY646856). Saliva samples were collected from 29 healthy donors. The amounts of Lewis a (Le^a), Lewis b (Le^b), H, A and B antigens in the saliva samples were determined semi-quantitatively by hemagglutination inhibition, and 12 saliva samples with relatively high amounts

Author's address: Dr. Grant Hansman, Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-murayama, Tokyo 208-0011, Japan. e-mail: ghansman@nih.go.jp

Table 1. Semiquantitation of soluble ABH and Lewis antigens

Donor no.	Interpretation of saliva testing	Grouping	Hemagglutination inhibition titer				
			H	A	B	Lewis-a	Lewis-b
1	Secretor/O/Lewis-positive	H^high^/Le-b^high^	>256	0	0	32	32
2	Secretor/O/Lewis-positive		>256	0	0	32	32
3	Secretor/O/Lewis-positive		128	0	0	32	64
4	Secretor/AB/Lewis-positive	A^high^	16	>256	32	8	2
5	Secretor/AB/Lewis-positive		8	>256	8	8	4
6	Secretor/AB/Lewis-positive		16	128	8	4	4
7	Secretor/B/Lewis-negative	B^high^	16	0	>256	0	0
8	Secretor/B/Lewis-positive		16	0	128	16	4
9	Secretor/B/Lewis-positive		16	0	128	2	4
10	Nonsecretor	Le-a^high^	4	8	0	>256	8
11	Nonsecretor		4	1	0	>256	4
12	Nonsecretor		0	1	0	>256	4

of antigens were selected for saliva-VLP binding assay (Table 1). We then used 2 enzyme-linked immunosorbent assay (ELISA)-based assays, a saliva-VLP binding assay and a carbohydrate-VLP binding assay to examine the binding activities of the NoV and SaV VLPs to HBGAs.

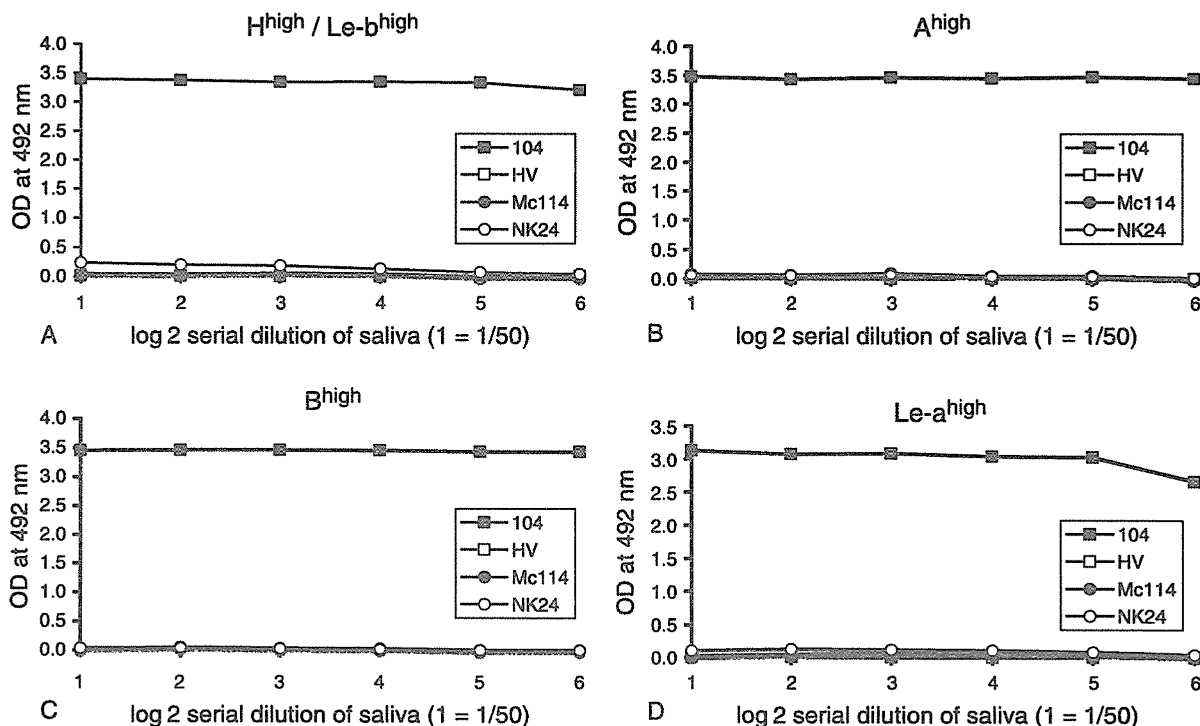

Fig. 1. NoV and SaV VLP binding activity to the saliva samples. Optical densities were measured at 492 nm and were plotted against the serial diluted saliva samples. Each experiment was performed with three donors from each HBGA group (Table 1) and repeated twice. Three samples from the same group produced similar results (data not shown). The results from the donors 1 (**A**), 4 (**B**), 7 (**C**) and 10 (**D**) are shown