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REFERENCES

1. **Agnello, V., G. Ábel, M. Elfahal, G. B. Knight, and Q. X. Zhang.** 1999. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. USA **96:**12766–12771.
2. **Barth, H., C. Schäfer, M. I. Adah, F. Zhang, R. J. Linhardt, H. Toyoda, A. Kinoshita-Toyoda, T. Toida, T. H. Van Kuppevelt, E. Depla, F. Von Weizsacker, H. E. Blum, and T. F. Baumert.** 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J. Biol. Chem. **278:**41003–41012.
3. **Bartosch, B., J. Dubuisson, and F.-L. Cosset.** 2003. Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes. J. Exp. Med. **197:**633–642.
4. **Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F.-L. Cosset.** 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J. Biol. Chem. **278:**41624–41630.
5. **Borrow, P., and M. B. Oldstone.** 1994. Mechanism of lymphocytic choriomeningitis virus entry into cells. Virology **198:**1–9.
6. **Carbone, R., S. Fre, G. Iannolo, F. Belleudi, P. Mancini, P. G. Pelicci, M. R. Torrisi, and P. P. Di Fiore.** 1997. eps15 and eps15R are essential components of the endocytic pathway. Cancer Res. **57:**5498–5504.
7. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science **244:**359–362.
8. **Chu, J. J., and M. L. Ng.** 2004. Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway. J. Virol. **78:**10543–10555.
9. **Clayton, R. F., A. Owsianka, J. Aitken, S. Graham, D. Bhella, and A. H. Patel.** 2002. Analysis of antigenicity and topology of E2 glycoprotein present on recombinant hepatitis C virus-like particles. J. Virol. **76:**7672–7682.
10. **Conner, S. D., and S. L. Schmid.** 2003. Regulated portals of entry into the cell. Nature **422:**37–44.
11. **Damke, H., T. Baba, A. M. van der Bliek, and S. L. Schmid.** 1995. Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. J. Cell Biol. **131:**69–80.
12. **Damm, E. M., L. Pelkmans, J. Kartenbeck, A. Mezzacasa, T. Kurzchalia, and A. Helenius.** 2005. Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. J. Cell Biol. **168:**477–488.
13. **Deleersnyder, V., A. Pillez, C. Wychowski, K. Blight, J. Xu, Y. S. Hahn, C. M. Rice, and J. Dubuisson.** 1997. Formation of native hepatitis C virus glycoprotein complexes. J. Virol. **71:**697–704.
14. **Drummer, H. E., A. Maerz, and P. Poumbourios.** 2003. Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins. FEBS Lett. **546:**385–390.
15. **Dubuisson, J., H. H. Hsu, R. C. Cheung, H. B. Greenberg, D. G. Russell, and C. M. Rice.** 1994. Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. J. Virol. **68:**6147–6160.
16. **Duvet, S., F. Chirat, A. M. Mir, A. Verbert, J. Dubuisson, and R. Cacan.** 2000. Reciprocal relationship between alpha1,2 mannosidase processing and reglucosylation in the rough endoplasmic reticulum of Man-P-Dol deficient cells. Eur. J. Biochem. **267:**1146–1152.
17. **Flint, M., C. Maidens, L. D. Loomis-Price, C. Shotton, J. Dubuisson, P. Monk, A. Higginbottom, S. Levy, and J. A. McKeating.** 1999. Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. J. Virol. **73:**6235–6244.
18. **Gardner, J. P., R. J. Durso, R. R. Arrigale, G. P. Donovan, P. J. Maddon, T. Dragic, and W. C. Olson.** 2003. L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. Proc. Natl. Acad. Sci. USA **100:**4498–4503.
19. **Gault, E., Y. Michel, A. Dehée, C. Belabani, J. C. Nicolas, and A. Garbarg-Chenon.** 2001. Quantification of human cytomegalovirus DNA by real-time PCR. J. Clin. Microbiol. **39:**772–775.
20. **Gianni, T., G. Campadelli-Fiume, and L. Menotti.** 2004. Entry of herpes simplex virus mediated by chimeric forms of nectin1 retargeted to endosomes or to lipid rafts occurs through acidic endosomes. J. Virol. **78:**12268–12276.
21. **Goff, S. P.** 2001. *Retroviridae*: The retroviruses and their replication, p. 1871–1940. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed., vol. 2. Lippincott, Williams & Wilkins, Philadelphia, Pa.
22. **Gollins, S. W., and J. S. Porterfield.** 1985. Flavivirus infection enhancement in macrophages: an electron microscopic study of viral cellular entry. J. Gen. Virol. **66:**1969–1982.
23. **Heinz, F. X., K. Stiasny, and S. L. Allison.** 2004. The entry machinery of flaviviruses. Arch. Virol. Suppl. **2004:**133–137.
24. **Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc. Natl. Acad. Sci. USA **100:**7271–7276.
25. **Kato, T., T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, and T. Wakita.** 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology **125:**1808–1817.
26. **Krey, T., H. J. Thiel, and T. Rümenapf.** 2005. Acid-resistant bovine pestivirus requires activation for pH-triggered fusion during entry. J. Virol. **79:**4191–4200.
27. **Lavillette, D., B. Bartosch, D. Nourrisson, G. Verney, F.-L. Cosset, F. Penin, and E. I. Pécheur.** 2006. Hepatitis C virus glycoproteins mediate low pH-dependent membrane fusion with liposomes. J. Biol. Chem. **281:**3909–3917.
28. **Le Mercier, P., D. Garcin, S. Hausmann, and D. Kolakofsky.** 2002. Ambisense Sendai viruses are inherently unstable but are useful to study viral RNA synthesis. J. Virol. **76:**5492–5502.
29. **Lecot, S., S. Belouzard, J. Dubuisson, and Y. Rouillé.** 2005. Bovine viral diarrhea virus entry is dependent on clathrin-mediated endocytosis. J. Virol. **79:**10826–10829.
30. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wölk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309:**623–626.
31. **Lindenbach, B. D., and C. M. Rice.** 2001. *Flaviviridae*: The viruses and their replication, p. 991–1042. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed., vol. 1. Lippincott, Williams & Wilkins, Philadelphia, Pa.
32. **Lozach, P. Y., H. Lortat-Jacob, A. de Lacroix de Lavalette, I. Staropoli, S. Foung, A. Amara, C. Houlès, F. Fieschi, O. Schwartz, J.-L. Virelizier, F. Arenzana-Seisdedos, and R. Altmeyer.** 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J. Biol. Chem. **278:**20358–20366.
33. **Mothes, W., A. L. Boerger, S. Narayan, J. M. Cunningham, and J. A. Young.** 2000. Retroviral entry mediated by receptor priming and low pH triggering of an envelope glycoprotein. Cell **103:**679–689.
34. **Motley, A., N. A. Bright, M. N. Seaman, and M. S. Robinson.** 2003. Clathrin-mediated endocytosis in AP-2-depleted cells. J. Cell Biol. **162:**909–918.
35. **Nichols, B.** 2003. Caveosomes and endocytosis of lipid rafts. J. Cell Sci. **116:**4707–4714.
36. **O'Doherty, U., W. J. Swiggard, and M. H. Malim.** 2000. Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. J. Virol. **74:**10074–10080.
37. **Op De Beeck, A., C. Voisset, B. Bartosch, Y. Ciczora, L. Cocquerel, Z. Keck, S. Foung, F.-L. Cosset, and J. Dubuisson.** 2004. Characterization of functional hepatitis C virus envelope glycoproteins. J. Virol. **78:**2994–3002.
38. **Pelkmans, L., E. Fava, H. Grabner, M. Hannus, B. Habermann, E. Krausz, and M. Zerial.** 2005. Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. Nature **436:**78–86.
39. **Pelkmans, L., and A. Helenius.** 2003. Insider information: what viruses tell us about endocytosis. Curr. Opin. Cell Biol. **15:**414–422.
40. **Pelkmans, L., J. Kartenbeck, and A. Helenius.** 2001. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat. Cell Biol. **3:**473–483.
41. **Pietiäinen, V., V. Marjomäki, P. Upla, L. Pelkmans, A. Helenius, and T. Hyypiä.** 2004. Echovirus 1 endocytosis into caveosomes requires lipid rafts, dynamin II, and signaling events. Mol. Biol. Cell **15:**4911–4925.
42. **Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani.** 1998. Binding of hepatitis C virus to CD81. Science **282:**938–941.
43. **Puri, A., J. Winick, R. J. Lowy, D. Covell, O. Eidelman, A. Walter, and R. Blumenthal.** 1988. Activation of vesicular stomatitis virus fusion with cells by pretreatment at low pH. J. Biol. Chem. **263:**4749–4753.
44. **Rouillé, 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. **Rozen, S., and H. J. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers, p. 365–386. *In* S. Krawetz and S. Misener (ed.), Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, N.J.
46. **Rust, M. J., M. Lakadamyali, F. Zhang, and X. Zhuang.** 2004. Assembly of endocytic machinery around individual influenza viruses during viral entry. Nat. Struct. Mol. Biol. **11:**567–573.
47. **Sánchez-San Martín, C., T. López, C. F. Arias, and S. López.** 2004. Characterization of rotavirus cell entry. J. Virol. **78:**2310–2318.
48. **Sandrin, V., and F. L. Cosset.** 2006. Intracellular versus cell surface assembly of retroviral pseudotypes is determined by the cellular localization of the

viral glycoprotein, its capacity to interact with Gag and the expression of the Nef protein. J. Biol. Chem. **281:**528–542.
49. **Saunier, B., M. Triyatni, L. Ulianich, P. Maruvada, P. Yen, and L. D. Kohn.** 2003. Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes. J. Virol. **77:**546–559.
50. **Scarselli, E., H. Ansuini, R. Cerino, R. M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, and A. Vitelli.** 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. **21:**5017–5025.
51. **Sieczkarski, S. B., and G. R. Whittaker.** 2002. Dissecting virus entry via endocytosis. J. Gen. Virol. **83:**1535–1545.
52. **Sieczkarski, S. B., and G. R. Whittaker.** 2002. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. J. Virol. **76:** 10455–10464.
53. **Sun, X., V. K. Yau, B. J. Briggs, and G. R. Whittaker.** 2005. Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells. Virology **338:**53–60.
54. **Tscherne, D. M., C. T. Jones, M. J. Evans, B. D. Lindenbach, J. A. McKeating, and C. M. Rice.** 2006. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. J. Virol. **80:**1734–1741.
55. **Voisset, C., N. Callens, E. Blanchard, A. Op De Beeck, J. Dubuisson, and N. Vu-Dac.** 2005. High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I. J. Biol. Chem. **280:**7793–7799.
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. **Wang, L. H., K. G. Rothberg, and R. G. Anderson.** 1993. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. J. Cell Biol. **123:**1107–1117.
58. **Wychowski, C., D. Benichou, and M. Girard.** 1986. A domain of SV40 capsid polypeptide VP1 that specifies migration into the cell nucleus. EMBO J. **5:**2569–2576.
59. **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.

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NOTE

Generation of Infectious Hepatitis C Virus in Immortalized Human Hepatocytes

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Progress in understanding hepatitis C virus (HCV) biology has remained a challenge due to the lack of an efficient cell culture system for virus growth. In this study, we examined HCV core protein-mediated immortalized human hepatocytes (IHH) for growth of HCV. In vitro-transcribed full-length RNA from HCV genotype 1a (clone H77) was introduced into IHH by electroporation. Reverse transcription-PCR of cellular RNA isolated from HCV genome-transfected IHH suggested that viral RNA replication occurred. IHH transfected with the full-length HCV genome also displayed viral protein expression by indirect immunofluorescence. In contrast, cells transfected with polymerase-defective HCV (H77/GND) RNA as a negative control did not exhibit expression of the viral genome. Immunogold labeling demonstrated localization of E1 protein in the rough endoplasmic reticulum of RNA-transfected IHH. Virus-like particles of ~50 nm were observed in the cytoplasm. After being inoculated with culture media of cells transfected with the full-length HCV genome, naïve IHH displayed NS5a protein expression in a dilution-dependent manner, but expression of NS5a was inhibited by prior incubation of culture medium with HCV-infected patient sera. NS5a-positive immunofluorescence of cell culture media of IHH transfected with full-length H77 RNA yielded ~4.5 × 10^4 to 1 × 10^5 focus-forming units/ml. A similar level of virus growth was observed upon transfection of RNA from HCV genotype 2a (JFH1) into IHH. Taken together, our results suggest that IHH support HCV genome replication and virus assembly.

Hepatitis C virus (HCV) is an important cause of morbidity and mortality worldwide. The most important feature of HCV infection is the development of chronic hepatitis in a significant number of infected individuals, with the potential for disease progression to cirrhosis and hepatocellular carcinoma (6, 7, 11, 27). At present, the only approved therapies for chronic HCV infection are alpha interferon (IFN-α) with or without ribavirin (9, 21), but these fail to clear HCV from a significant number of patients (22). A number of HCV genomes have been cloned, and sequence divergence indicates that there are several genotypes and a series of subtypes of this virus (28). In the United States, HCV genotypes 1a and 1b are predominant in patients with chronic hepatitis C (32). Progress in understanding HCV biology has remained challenging due to the lack of an efficient cell culture system for virus growth. Establishment of self-replicating full-length HCV genomic replicons from genotypes 1a and 1b in human hepatoma (Huh-7) cells has provided an important tool for the study of HCV replication mechanisms (3, 10, 23). Recently, different groups have reported the generation of infectious virus from transfection of genomic RNA of HCV genotype 2a into Huh-7 cells or its derivatives (5, 15, 29, 33). However, generation of infectious HCV genotype 1a has not been successful to date.

We and others have shown that HCV core protein transcriptionally regulates a number of cellular genes (26). We previously described the generation of immortalized human hepatocytes (IHH) by transfection of the HCV core genomic region from genotype 1a (2, 25). IHH exhibited a weak level of HCV core protein expression, albumin secretion, glucose phosphatase activity, and absence of smooth-muscle actin. IHH also displayed focal cytoplasmic and membrane staining for carcinoembryonic antigen (CEA), biliary glycoprotein (BGP1/CEACAM1), and nonspecific cross-reacting antigen (NCA/CEACAM6) and expression of hepato-biliary transport marker genes (MRP, LST1, and NTCP) (unpublished observations). Together, these results suggested that IHH are well differentiated. HCV core protein selectively degrades STAT1, reduces phosphorylated STAT1 accumulation in the nucleus in a proteasome-dependent manner, and impairs IFN-α-induced signal transduction via expression of suppressor of cytokine signaling-3 (1, 4, 16, 18). HCV core protein is competent to partially rescue growth of a genetically engineered influenza A virus lacking its own IFN antagonist (4). The core protein can modulate interferon regulatory factor, Jak-STAT, and inducible nitric oxide synthetase pathways, which suggests that there are mechanisms by which the core could affect HCV persistence and pathogenesis (20). Since HCV core protein transcriptionally regulates several cellular genes involved in cell growth, apoptosis, and defense mech-

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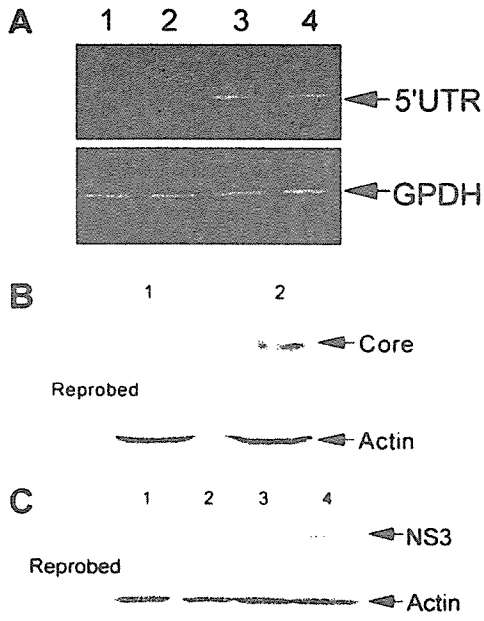

FIG. 1. HCV RNA and protein expression in IHH. (A) RT-PCR analysis was performed using 5′UTR-specific primers from RNA isolated at day 5 from two different sets of IHH transfected with H77/GND RNA as a negative control (lanes 1 and 2) or H77 RNA (lanes 3 and 4). GPDH was amplified as an internal control. The sizes of the amplified bands were verified from the migration of a φX174-HaeIII digested DNA marker (not shown). (B) Western blot analysis for core protein expression in H77/GND RNA-transfected (lane 1) and H77 RNA-transfected (lane 2) IHH, using a specific antiserum. The blot was reprobed with antibody to actin for similar protein loads in each lane. (C) Western blot analysis for NS3 protein expression in two different sets of IHH transfected with H77/GND RNA (lanes 1 and 2) or H77 RNA (lanes 3 and 4), using a specific monoclonal antibody. The blot was reprobed with antibody to actin for similar protein loads in each lane. The molecular weights of the protein bands were verified from the migration of protein molecular weight markers (Cambrex, Rockland, Maine).

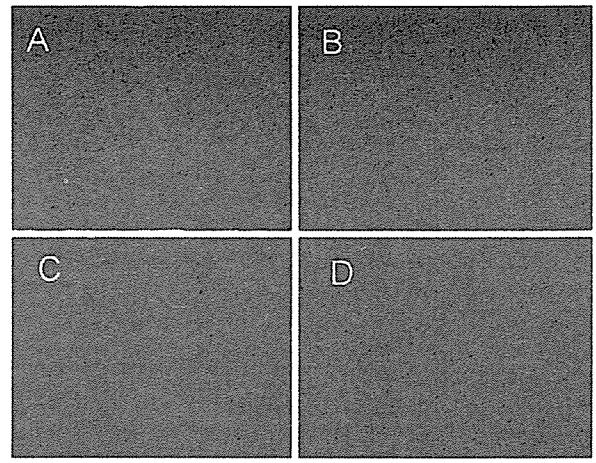

FIG. 2. Intracellular expression of HCV proteins. IHH transfected with RNA from the H77 clone (A) or the negative control, H77/GND (B), were treated with an NS5a-specific monoclonal antibody for detection of protein expression by intracellular immunofluorescence 5 days after transfection. IHH were similarly transfected with RNA from the JFH1 clone (C) or the negative control, JFH1/GND (D), and treated with a specific monoclonal antibody for intracellular localization of NS3. Green indicates NS5a staining, and red indicates NS3 staining.

anisms, we hypothesize that IHH may set the stage for HCV genome replication and assembly.

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Replication of the HCV genome and virus protein expression. We investigated whether IHH confer HCV genome replication and generation of infectious virus particles. For this purpose, full-length RNAs from HCV genotype 1a (clone H77) (13) were used. The clone H77 contains a 5′ untranslated region (5′UTR), a coding sequence, and a 3′UTR, which are suggested to be necessary for replication (14, 30). In vitro-transcribed full-length HCV RNA from clone H77 was used for transfection of IHH by electroporation. H77/GND (polymerase-defective) RNA was used similarly as a negative control. Briefly, H77 cDNA was linearized by digestion with XbaI, and gel-purified DNA was used for in vitro transcription by T7 RNA polymerase (Promega, Madison, Wis.). In vitro-transcribed RNA (1 to 2 μg) was introduced by electroporation (950 μF and 270 V) into 5×10^6 IHH, using a Bio-Rad Gene Pulser Xcell system (Hercules, Calif.). The transfected cells were plated on collagen-coated plastic dishes and maintained in culture for HCV replication. Total cellular RNA was extracted 5 days posttransfection. To detect the HCV genome, total cellular RNA and random hexamers were used for cDNA synthesis with a SuperScriptIII first-strand-synthesis system (Invitrogen), following the supplier's protocol. PCR amplification was performed with cDNA as a template, using sense (5′-CACTCCCCTGTGAGGAACTACTGTCT-3′) and antisense (5′-TGGTGCACGGTCTACGAGACCTCCC-3′) primers from 5′UTR at 94°C for 30 s, annealing at 55°C for 60 s, and extension at 72°C for 90 s. Glyceraldehyde-3-phosphate dehydrogenase (GPDH) was used as an internal control, using specific primers (17). Reverse transcription-PCR (RT-PCR) analyses suggested amplification of sequence from the 5′UTR (Fig. 1A). In contrast, cells transfected with H77/GND RNA did not exhibit the presence of the HCV genomic sequence. To rule out the integration of H77 plasmid DNA into IHH, genomic DNA from the cell lines was isolated and examined for the HCV genome by PCR. Our results suggested that the HCV sequence was absent, indicating HCV genomic RNA replication occurred in the cytoplasm of IHH (data not shown). Filtered culture supernatant was also treated with RNaseA prior to isolation of viral RNA. RT-PCR was performed for the NS5A region (17), and we observed amplification of a specific RNA sequence.

Western blot analysis using specific antibodies was performed to analyze the expression of core and NS3 proteins in control and experimental cells. Equal amounts of proteins from whole-cell lysates in sample buffer were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose, incubated with specific antibodies, and detected by chemiluminescence (Amersham, Piscataway, N.J.). HCV core protein was detected by a specific rabbit antiserum, and NS3 was detected by a

specific mouse monoclonal antibody (ViroGen, Watertown, Mass.). The blots were stripped and reprobed, using a mouse monoclonal antibody to actin (Oncogene Science, Cambridge, Mass.). IHH that supported HCV genome replication displayed the presence of core (~21 kDa) and NS3 (~63 kDa) proteins (Fig. 1B and C). On the other hand, IHH transfected with H77/GND RNA did not show a detectable level of core or NS3 proteins. A weak level of core protein was detected in this set of IHH for immortalization by HCV core protein (Fig. 1B). IHH transfected with HCV full-length RNA were passaged at 4- or 5-day intervals. HCV RNA and protein expression were detected in cell cultures for up to 12 days, and the cultures were discontinued for lack of growth after 2 weeks.

To further examine intracellular expression of HCV protein, IHH transfected with H77 RNA were fixed with 3.7% formaldehyde and incubated at room temperature for 1 h with monoclonal antibodies to NS5a (Biogenesis, Kingstone, N.H.). Cells were washed three times with phosphate-buffered saline (PBS), stained with anti-mouse immunoglobulin (Ig) conjugated with Alexa 568 (Molecular Probes, Eugene, Oreg.), and mounted for fluorescence microscopy. Primary antibodies and secondary antibody-fluorochrome conjugates were titrated for use of optimum dilutions where there was no background fluorescence. We observed cytoplasmic expression of NS5a (Fig. 2A) in 60% IHH after 5 days of transfection. HCV genotype 2a (clone JFH1) has been shown to grow in Huh-7 cells or its derivatives (5, 12, 15, 29, 33). In vitro-transcribed RNA from clone JFH1 was used for transfection of IHH to determine if the immortalized hepatocyte cell line supports HCV growth. Intracellular localization of NS3 protein from JFH1 RNA-transfected IHH was detected by immunofluorescence (Fig. 2C). We have also used Huh-7.5 cells transfected with JFH1 RNA as positive controls (29) and observed NS3 expression by indirect immunofluorescence (data not shown). On the other hand, IHH similarly transfected with RNA from H77/GND or the JFH1/GND clone did not display virus protein expression by immunofluorescence (Fig. 2B and D).

Immunogold localization of virus-like particles. Phase-contrast microscopy suggested that HCV genome-transfected IHH were swollen with large vacuoles in the cytoplasm, whereas the negative controls did not show any detectable changes. We also looked for cellular changes, using electron microscopy, and at the ultrastructural level some of these vacuoles appeared to be empty (Fig. 3A and E). Others contained lipid (Fig. 3E) or material isolated for degradation (Fig. 3D). Ultrastructural changes also included increased polymorphism of the nuclei (Fig. 3E). Immunogold labeling was performed for localization of HCV-like particles in transfected IHH. For this, transfected IHH (4 days in culture) were detached from collagen-coated petri dishes by a brief trypsin treatment, pelleted in a microcentrifuge, and fixed in 4% paraformaldehyde and 1% glutaraldehyde in PBS for 16 h at 4°C. After being washed with PBS, the cells were washed in distilled water, dehydrated in ethanol, and infiltrated with LR White resin (London Resin Company, Berkshire, United Kingdom). The cell pellets were polymerized in BEEM capsules (Ted Pella, Inc., Redding, Calif.) at −20°C under UV light. Thin sections were cut from blocks, collected on Formvar-coated nickel grids, and blocked with 1% fish gelatin and 1% bovine serum albumin (BSA) in PBS for 10 min. Sections were incubated for 2 h in a 1:100 dilution (titrated beforehand for best results) of monoclonal antibody to E1 glycoprotein (305/C3) or normal mouse IgG in PBS containing 0.1% BSA, washed in PBS containing 0.1% BSA, and incubated for 1 h in protein A-10-nm colloidal gold (CG) diluted at a ratio of 1:200 in PBS containing 0.1% BSA. After being washed with PBS, the grids were fixed for 3 min in glutaraldehyde, washed in distilled water, stained with uranyl acetate and lead citrate, and photographed with a JEOL 100 CX electron microscope. No clusters of CG particles were observed in the controls, which were stained with normal mouse IgG without the primary antibody or were mock-transfected and stained with the 305/C3 monoclonal antibody. Several hundred cells were evaluated in each case. Immunogold labeling with E1-specific monoclonal antibody demonstrated the presence of HCV-like particles and E1 protein in IHH. Numerous labeled virus-like particles were observed in the cytoplasm (Fig. 3A and E) and near the plasma membrane (Fig. 3C) of H77 RNA-transfected IHH. The labeled particles were ~50 nm in diameter. Extensive labeling was also associated with the rough endoplasmic reticulum, consistent with the synthesis of E1 viral protein (Fig. 3B). In addition, we observed cytoplasmic autophagic vacuoles which contained gold-labeled virus-like particles (Fig. 3D).

Processing of cells into LR White resin for immunogold localization omits the conventional osmium tetroxide fixation step to preserve antigenicity but results in reduced tissue contrast. In addition, the identification of virus particles by immunogold labeling at the ultrastructural level can be tricky. For this, we carried out a series of control experiments to ensure labeling specificity. First, we observed clusters of CG on virus-like particles and single CG particles in the endoplasmic reticulum in several independent anti-E1-labeling experiments. Second, H77/GND RNA-transfected IHH (negative controls) showed no such clusters of CG in the cytoplasm or single CG particles localized along the endoplasmic reticulum or membranes (Fig. 3F). Third, incubation of sections of HCV genome-transfected IHH with normal mouse IgG at IgG concentrations similar to those used for the anti-E1 antibody did not result in any specific immunogold labeling. Fourth, omitting anti-E1 antibody did not result in any specific immunogold labeling. Finally, CG particles in the anti-E1-labeling experiments were primarily confined to cells and were not observed to any degree in the spaces around cells, again suggesting the labeling was specific for E1 protein in cells. Thus, the appearance of virus-like particles in RNA-transfected IHH indicated that HCV 1a replicates and assembles as virus particles.

Infection of IHH by HCV from culture medium. We next examined the presence of HCV in IHH cell culture medium. On different days after transfection, culture medium was filtered through a 0.45-μm cellulose acetate membrane (Millipore, Bedford, Mass.), concentrated to ~10- to 20-fold by Millipore ultrafiltration (100-kDa cut off), and used for detection of the HCV genomic sequence by RT-PCR (Fig. 4A). The presence of HCV 5′UTR was detected in culture medium from HCV genome-transfected IHH but not from polymerase-defective HCV RNA-transfected IHH. We obtained ~1.1×10^8 genome copies/ml of culture medium using real-time RT-PCR, as described recently (33). Culture supernatant collected for up to 7 days suggested that the peak HCV genome copy number occurred between 4 and 5 days after transfection.

Next, we determined whether the culture medium contained infectious HCV. For this, culture media was serially twofold

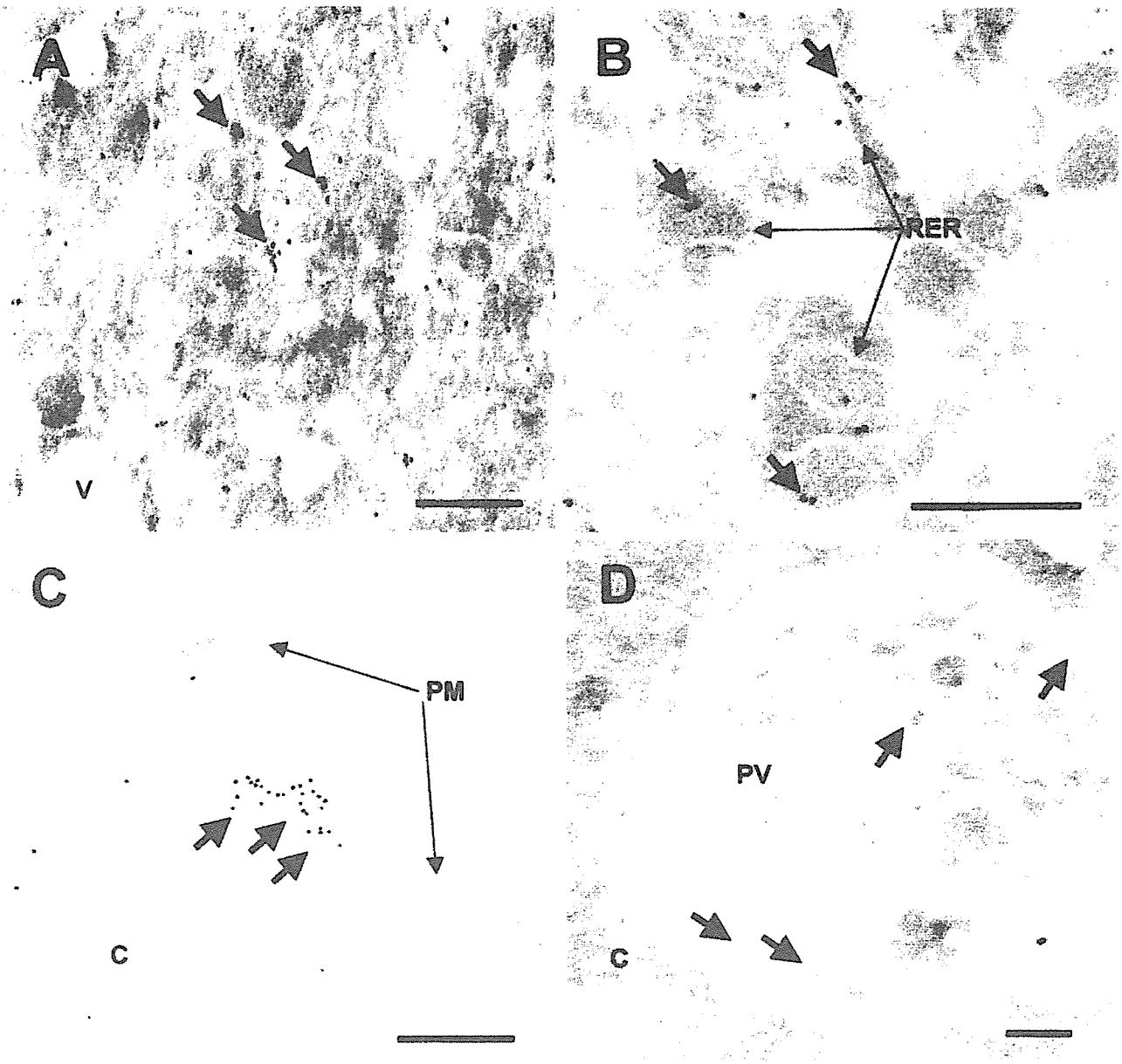

FIG. 3. Immunogold localization of HCV E1 protein and virus-like particles in IHH with a specific monoclonal antibody. (A) Localization of virus-like particles in the cytoplasm is indicated by arrows. (B) Localization of HCV E1 protein to the rough endoplasmic reticulum is marked by arrows. (C) Localization of virus-like particles in the cortical cytoplasm adjacent to the plasma membrane is indicated by arrows. (D) Localization of virus-like particles in a large vacuole in the cytoplasm of IHH is shown by arrows. (E) Clusters of CG indicated by arrows show virus-like particles in IHH. The labeled particle indicated by an arrow and an asterisk is shown at higher magnification in the inset. As observed by light microscopy, IHH contain cytoplasmic vacuoles and lipid droplets. (F) An H77/GND RNA-transfected control section of IHH incubated with monoclonal antibody to E1 glycoprotein did not exhibit immunogold labeling. Other negative controls were labeled with normal mouse IgG and were not incubated with the primary antibody (not shown). Abbreviations: C, cytoplasm; M, mitochondrion; PM, plasma membrane; RER, rough endoplasmic reticulum; V, vacuole; LD, lipid droplet; PV, autophagic vacuole. Magnification bars are 0.25 μm in panels A through F and 0.1 μm in the inset in panel E.

diluted and inoculated into naïve IHH. Cells were incubated for 4 h, washed, and incubated with fresh media for 3 days before indirect immunofluorescence was performed to determine the number of focus-forming units (FFU)/ml of NS5a (H77 clone) or NS3 (JFH1 clone), as recently described (33). Nuclear staining was performed using TO-PRO3-iodide (Molecular Probes), and cells were mounted for confocal laser scanning microscopy (model 1024; Bio-Rad). Figure 4B shows infection of IHH by H77 or JFH1 and is representative of our results. Fluorescent cells were counted, and the counts were correlated with the number of dilutions of cell culture media to determine FFU/ml of H77 and JFH1 clones. We observed $\sim 4.5 \times 10^4$ to 1×10^5 FFU/ml of H77 and JFH1 clones in the cell culture media 5 days after transfection.

We transfected in vitro-transcribed H77 or JFH1 RNA into IHH and isolated the RNA from the transfected cells. Culture

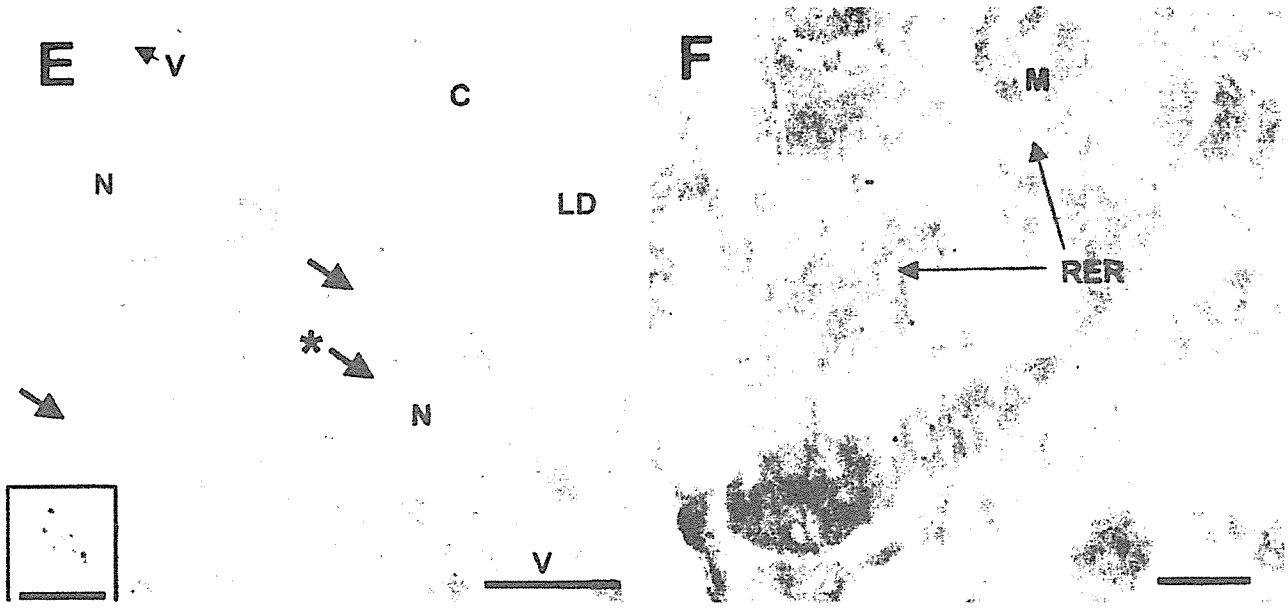

FIG. 3—*Continued.*

supernatant was also collected for isolation of RNA and determination of infectivity (FFU/ml). Real-time PCR suggested that maximal HCV RNA accumulation from H77 occurred at the intracellular level on day 2 and declined on day 5 (Fig. 4C). We observed a higher genome copy number and infectious virus titer on day 4. Similarly, JFH1 RNA-transfected IHH supernatant displayed a peak genome copy number per ml of 10^8 and infectivity of $\sim 7 \times 10^4$ FFU/ml on day 4.

HCV-infected patient serum (OP1843) displaying neutralizing activity against the vesicular stomatitis virus/HCV pseudotype (19) was used in determining neutralization of cell culture-grown HCV. Serum from a healthy volunteer was used as a negative control in HCV neutralization assays. A twofold serial dilution of heat-inactivated serum was incubated with ~100 FFU of HCV generated from the H77 clone at 37°C for 30 min. The virus-serum mixture was added to naïve IHH and incubated for 3 days. Neutralization was determined by measuring inhibition of NS5a protein expression by immunofluorescence. The results are shown as the percent inhibition based on focus-forming units per milliliter (Fig. 4D). Infectivity (~60%) was inhibited by prior incubation of HCV in culture medium with the patient serum at a 1/50 dilution. Inhibition was also observed at different dilutions of sera from three other HCV-infected patients. In contrast, sera from four healthy individuals did not inhibit infectivity at a 1/10 dilution. These results suggest that infectious HCV particles released in the culture medium are neutralized by specific antibodies.

HCV RNA is directly translated, and the precursor viral polypeptide is cleaved proteolytically to form individual proteins. The replicase complex amplifies the RNA via a minus-strand intermediate. Plus-strand RNA progeny are packaged into virus particles and acquire their envelopes probably by budding into the lumen of the endoplasmic reticulum. HCV particles are likely to be exported via the constitutive secretory pathway. Based on this working principle, we have shown in this report that IHH support HCV genome replication and protein expression from genotype 1a. Immunogold labeling using a monoclonal antibody demonstrated localization of HCV E1 glycoprotein in the rough endoplasmic reticulum and the formation of virus-like particles. We transferred culture media of HCV-replicating cells into naïve IHH, and HCV infection was detected by RT-PCR and indirect immunofluorescence. We have also observed JFH1 replication and virus growth in IHH. The infectious units appeared to be similar for JFH1 grown in Huh-7 cells and in its derivatives. JFH1 may replicate with a higher efficiency than H77 at the RNA level in Huh-7 cells or its derivatives. However, we focused on determining the generation of infectious HCV from H77 and JFH1 in IHH. In our experimental system, we observed that virus genome copies of H77 and JFH1 were at similar levels in H77 and JFH1 RNA-transfected culture supernatant. The number of focus-forming units per milliliter of H77 and JFH1 was also similar. We did not purify virus particles for negative staining due to the relatively low number of infectious units in the culture media. Three different groups of investigators have reported different densities of HCV genotype 2a particles. Zhong et al. (33) observed peak infectivity at an apparent density of 1.105 gm/ml, and Wakita et al. (29) observed peak infectivity at a density of ~1.15 gm/ml. Lidenbach et al. (15) observed a broad distribution of virus infectivity over a range of 1.01 to 1.12 gm/ml. A similar finding suggesting variation of between 1.06 and 1.16 gm/ml in buoyant density of cell culture-grown HCV genotype 2a was reported by Cai et al. (5). HCV is known to associate with serum immunoglobulin and lipoproteins (24). We have observed HCV infectivity within a density range of 1.09 to 1.12 in sucrose gradients, which did not correlate with highest copy number of virus genomic RNA (data not shown).

Recently, HCV production from a HCV-ribozyme construct of genotype 1a (clone H77) in Huh-7 cells was reported, although the infectivity of the virus was not determined (8). Virus genome replication and assembly are multistep processes and are influenced by the intracellular milieu. Inhibition of host cell growth

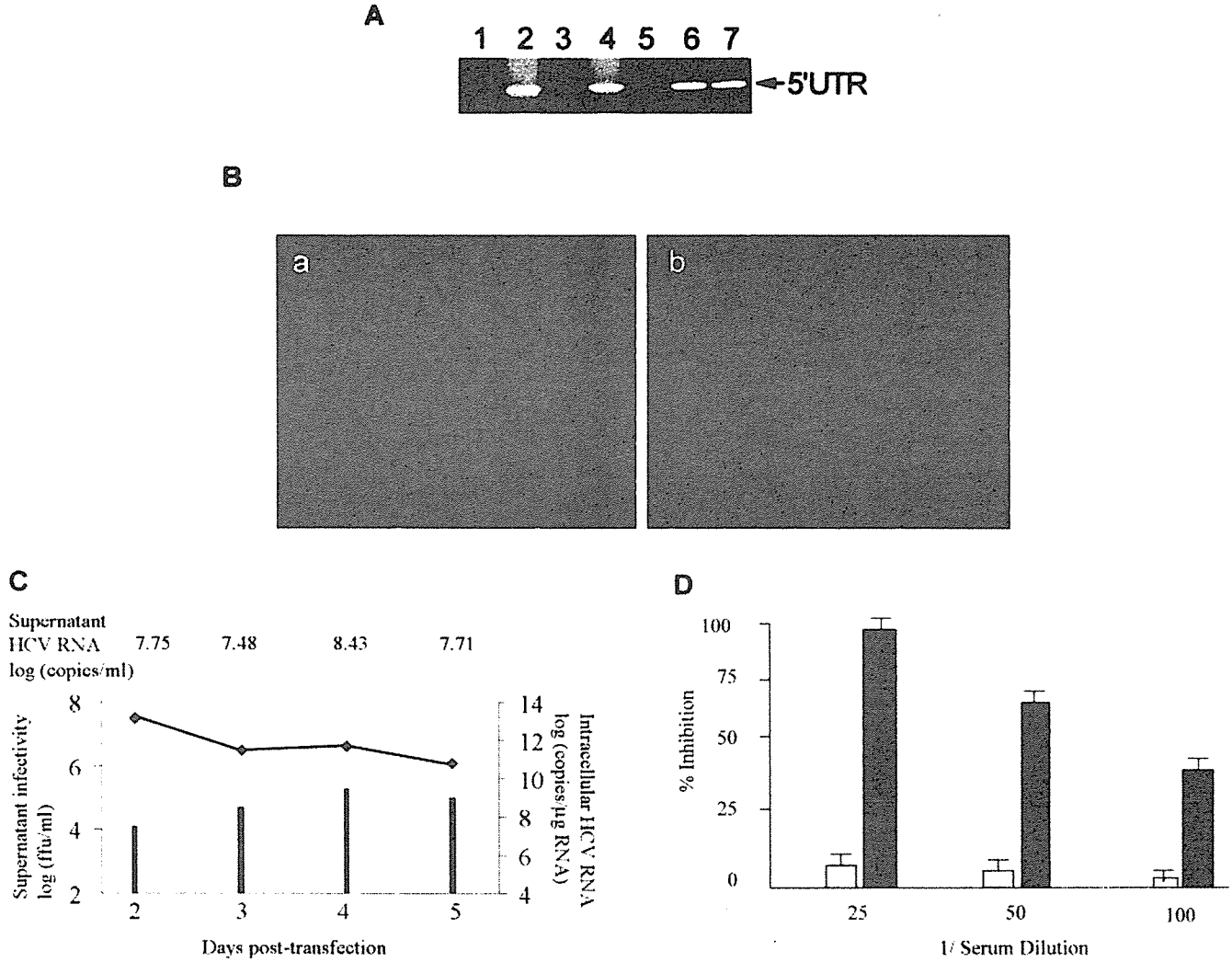

FIG. 4. Presence of HCV in culture medium and infectivity of naïve IHH. (A) RT-PCR analysis was performed using sequence-specific primers for detection of 5′UTR from culture medium of HCV RNA-transfected IHH. Filtered culture medium from IHH transfected with H77/GND RNA (lane 1), full-length H77 RNA (lane 2), JFH1/GND (lane 3), or full-length JFH1 RNA (lane 4) was analyzed for amplification of 5′UTR. The HCV genome was amplified similarly from Huh-7 cells transfected with JFH1/GND RNA (lane 5) or full-length JFH1 RNA (lane 6). Cloned H77 DNA was included as a positive control in PCR amplification (lane 7). (B) Immunofluorescence of IHH at day 3 after infection with filtered culture medium from H77 (a) or JFH1 (b) for detection of NS5a or NS3 protein expression, respectively. (C) Generation of infectious HCV after transfection of H77 genomic RNA into IHH. In vitro-transcribed H77 RNA (2 μg) was introduced into 1×10^6 IHH by electroporation. Copies of HCV RNA at the intracellular (◆) level and in the culture supernatant (numbers on top) were measured by real-time PCR on the indicated days. Infectivity of the virus in the supernatant of cultures of naïve IHH was determined and is expressed as FFU/ml (black bars). (D) Neutralization of virus infectivity by HCV-infected patient serum (black bars). Twofold serial dilutions of test serum were incubated with ~100 focus-forming units of virus generated from the H77 clone at 37°C for 30 min. The virus-serum mixture was added to naïve IHH and incubated for 3 days for determination of focus-forming units of NS5a protein by indirect immunofluorescence, using NS5a-specific antibody. A similar experiment was performed in parallel with serum from a healthy individual (hatched bars). The results are presented as the percent inhibition of virus infectivity measured in focus-forming units, and variations from triplicate assays are indicated by error bars.

and induction of cytokines, such as interferons, may have an impact on virus replication (3). Our study supports proof of the concept of HCV replication and assembly of genotype 1a in IHH. To our knowledge, this is the first report describing the generation of cell culture-grown HCV from genotype 1a. We speculate that cellular defense mechanisms against HCV infection are attenuated or compromised in IHH. Further studies may help to unravel the specific mechanisms for growth of HCV in IHH and to address important biological questions about the life cycle of HCV. Studies are in progress to determine the factors influencing virus growth, such as serial passage for adaptation in IHH, mutations at specific sites on the HCV genome, and selection of cell populations for attenuated protective mechanisms. We will also characterize the biophysical properties of cell culture-grown HCV and its infectivity in available animal models in the near future.

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ADDENDUM

While our manuscript was under revision, Yi et al. (31) reported the growth of H77-S in Huh-7.5 cells.

REFERENCES

1. **Basu, A., K. Meyer, R. B. Ray, and R. Ray.** 2001. Hepatitis C virus core protein modulates the interferon-induced transacting factors of JAK/Stat signaling pathway but does not affect the activation of IRF-1 or 561 genes. Virology **288**:379–390.
2. **Basu, A., K. Meyer, R. B. Ray, and R. Ray.** 2002. Hepatitis C virus core protein is necessary for the maintenance of immortalized human hepatocytes. Virology **298**:53–62.
3. **Blight, K. J., J. A. McKeating, J. Marcotrigiano, and C. M. Rice.** 2003. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J. Virol. **77**:3181–3190.
4. **Bode, J. G., S. Ludwig, C. Ehrhardt, U. Albrecht, A. Erhardt, F. Schaper, P. C. Heinrich, and D. Haussinger.** 2003. IFN-α antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J. **17**:488–490.
5. **Cai, Z., C. Zhang, K. S. Chang, J. Jiang, B. C. Ahn, T. Wakita, T. J. Liang, and G. Lou.** 2005. Robust production of infectious hepatitis C virus (HCV) from stably HCV cDNA-transfected human hepatoma cells. J. Virol. **79**: 13963–13973.
6. **Di Bisceglie, A. M., R. L. Carithers, and G. J. Gores.** 1998. Hepatocellular carcinoma. Hepatology **28**:1161–1165.
7. **Hayashi, J., H. Aoki, Y. Arakawa, and O. Hino.** 1999. Hepatitis C virus and hepatocarcinogenesis. Intervirology **42**:205–210.
8. **Heller, T., S. Saito, J. Auerbach, T. Williams, T. R. Moreen, A. Jazwinski, B. Cruz, N. Jeurkar, R. Sapp, G. Luo, and T. J. Liang.** 2005. An in vitro model of hepatitis C virion production. Proc. Natl. Acad. Sci. USA **102**:2579–2583.
9. **Hoofnagle, J. H., and A. M. Di Bisceglie.** 1997. The treatment of chronic viral hepatitis. N. Engl. J. Med. **336**:347–356.
10. **Ikeda, M., M. Yi, K. Li, and S. M. Lemon.** 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J. Virol. **76**:2997–3006.
11. **Jeffers, L.** 2000. Hepatocellular carcinoma: an emerging problem with hepatitis C. J. Natl. Med. Assoc. **92**:369–371.
12. **Kato, T., A. Furusaka, M. Miyamoto, T. Date, K. Yasui, J. Hiramoto, K. Nagayama, T. Tanaka, and T. Wakita.** 2001. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J. Med. Virol. **64**:334–339.
13. **Kolykhalov, A. A., E. V. Agapov, K. J. Blight, K. Mihalik, S. M. Feinstone, and C. M. Rice.** 1997. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science **277**:570–574.
14. **Kolykhalov, A. A., K. Mihalik, S. M. Feinstone, and C. M. Rice.** 2000. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3′ nontranslated region are essential for virus replication in vivo. J. Virol. **74**:2046–2051.
15. **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.
16. **Lin, W., W. H. Choe, Y. Hiasa, Y. Kamegaya, J. T. Blackard, E. V. Schmidt, and R. T. Chung.** 2005. Hepatitis C virus expression suppresses interferon signaling by degrading STAT1. Gastroenterology **128**:1034–1041.
17. **Majumder, M., A. K. Ghosh, R. Steele, X. Y. Zhou, N. J. Phillips, R. Ray, and R. B. Ray.** 2002. Hepatitis C virus NS5A protein impairs TNF-mediated hepatic apoptosis, but not by an anti-FAS antibody, in transgenic mice. Virology **294**:94–105.
18. **Melen, K., R. Fagerlund, M. Nyqvist, P. Keskinen, and I. Julkunen.** 2004. Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. J. Med. Virol. **73**:536–547.
19. **Meyer, K., A. Beyene, T. L. Bowlin, A. Basu, and R. Ray.** 2004. Coexpression of hepatitis C virus E1 and E2 chimeric envelope glycoproteins displays separable ligand sensitivity and increases pseudotype infectious titer. J. Virol. **78**:12838–12847.
20. **Miller, K., S. McArdle, M. J. Gale, Jr., D. A. Geller, B. Tenoever, J. Hiscott, D. R. Gretch, and S. J. Polyak.** 2004. Effects of the hepatitis C virus core protein on innate cellular defense pathways. J. Interferon Cytokine Res. **24**:391–402.
21. **Moradpour, D., and H. E. Blum.** 1999. Current and evolving therapies for hepatitis C. Eur. J. Gastroenterol. Hepatol. **11**:1199–1202.
22. **Pawlotsky, J. M.** 2005. Current and future concepts in hepatitis C therapy. Semin. Liver Dis. **25**:72–83.
23. **Pietschmann, T., V. Lohmann, A. Kaul, N. Krieger, G. Rinck, G. Rutter, D. Strand, and R. Bartenschlager.** 2002. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J. Virol. **76**:4008–4021.
24. **Prince, A. M., T. Huima-Byron, T. S. Parker, and D. M. Levine.** 1996. Visualization of hepatitis C virions and putative defective interfering particles isolated from low-density lipoproteins. J. Viral Hepat. **3**:11–17.
25. **Ray, R. B., K. Meyer, and R. Ray.** 2000. Hepatitis C virus core protein promotes immortalization of primary human hepatocytes. Virology **271**:197–204.
26. **Ray, R. B., and R. Ray** 2001. Hepatitis C virus core protein: intriguing properties and functional relevance. FEMS Microbiol. Lett. **202**:149–156.
27. **Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, Q. Choo, M. Houghton, and G. Kuo.** 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA **87**:6547–6549.
28. **Simmonds, P., E. C. Holmes, T. A. Cha, S. W. Chan, F. McOmish, B. Irvine, E. Beall, P. L. Yap, J. Kolberg, and M. S. Urdea.** 1993. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J. Gen. Virol. **74**:2391–2399.
29. **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. Nature Med. **11**:791–796.
30. **Yanagi, M., R. H. Purcell, S. U. Emerson, and J. Bukh.** 1997. Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. Proc. Natl. Acad. Sci. USA **94**:8738–8743.
31. **Yi, M., R. A. Villanueva, D. L. Thomas, T. Wakita, and S. M. Lemon.** 6 February 2006, posting date. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. USA **103**:2310–2315. [Online.]
32. **Zein, N. N., J. Rakela, E. L. Krawitt, K. R. Reddy, T. Tominaga, and D. H. Persing.** 1996. Hepatitis C virus genotypes in the United States: epidemiology, pathogenicity, and response to interferon therapy. Ann. Intern. Med. **124**:634–639.
33. **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.

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Diverse Effects of Cyclosporine on Hepatitis C Virus Strain Replication

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Recently, a production system for infectious particles of hepatitis C virus (HCV) utilizing the genotype 2a JFH1 strain has been developed. This strain has a high capacity for replication in the cells. Cyclosporine (CsA) has a suppressive effect on HCV replication. In this report, we characterize the anti-HCV effect of CsA. We observe that the presence of viral structural proteins does not influence the anti-HCV activity of CsA. Among HCV strains, the replication of genotype 1b replicons was strongly suppressed by treatment with CsA. In contrast, JFH1 replication was less sensitive to CsA and its analog, NIM811. Replication of JFH1 did not require the cellular replication cofactor, cyclophilin B (CyPB). CyPB stimulated the RNA binding activity of NS5B in the genotype 1b replicon but not the genotype 2a JFH1 strain. These findings provide an insight into the mechanisms of diversity governing virus-cell interactions and in the sensitivity of these strains to antiviral agents.

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, has a positive-strand RNA genome (1, 26). The genome encodes a large precursor polyprotein, which is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (6, 8). NS5B is an RNA-dependent RNA polymerase that is crucial for viral genome replication (1, 26). There is genetic heterogeneity within the HCV genome. Currently, these differences are classified into six genotypes that are further segregated into a series of subtypes (4, 23). In Japan, genotype 1b is predominant; roughly 65% of cases of HCV-related chronic hepatitis involve genotype 1b. By comparison, genotype 2a is present in 17% of these patients (13, 23).

Sustained infection of HCV is the major cause of chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (16). Rarely, HCV causes fulminant hepatitis (13). The predominant treatment for HCV-infected patients is interferon (IFN) or polyethylene glycol-conjugated IFN alone or in combination with ribavirin (19, 20). However, alternative anti-HCV therapies are needed because virus is not eliminated in about half of the treated patients (19, 20). Lohmann et al. have developed the HCV subgenomic replicon system, in which an HCV subgenomic replicon autonomously replicates in Huh-7 cells (HCV replicon cells) (18). This replicon comprises the HCV 5′ untranslated region (5′UTR) containing an internal ribosomal entry site (IRES), the neomycin phosphotransferase gene, the encephalomyocarditis virus (EMCV) IRES, the coding region for HCV NS3 through NS5B, and the HCV 3′UTR (subgenomic replicon), but it lacks the coding region for the core and envelope proteins, as well as p7 and NS2 (Fig. 1). Subsequently, a genome-length (full-genome) replicon has been developed. This construct contains a full-genome length of HCV, including the coding regions for the core protein through NS2 (Fig. 1) (5, 10). We can evaluate HCV replication using these subgenomic or genome-length replicon systems. Previously, we established HCV subgenomic replicon cells carrying HCV genotype 1b NN strain (15, 29). We demonstrated that an immunosuppressant, cyclosporine (CsA), has anti-HCV activity in these cells (29). In addition, we determined the molecular mechanism of the anti-HCV effect of CsA on this replicon; cyclophilin B (CyPB), one of the cellular targets of CsA, is a cellular replication cofactor of the HCV genome (31). CyPB interacts with NS5B to promote its RNA binding activity (for a detailed description, see reference 31). CsA is suggested to suppress HCV genome replication by inhibiting the functional association of CyPB with NS5B. Another group also reported anti-HCV function of CsA using a subgenomic replicon of other genotype 1b strain, HCV-N (22). In this study, we demonstrate that CsA also has a strong anti-HCV activity in other available genotype 1b replicons carrying the Con1 and O strains (12, 18).

Recently, Wakita and colleagues reported that a replicon of HCV genotype 2a JFH-1 strain, which was isolated from a case of type-C fulminant hepatitis, has a much stronger level of replication activity than genotype 1b replicons in Huh-7 cells (13, 27). A production system of infectious viral particles was recently established with this high-replication-competent strain (17, 27, 34). This viral strain may acquire a growth advantage compared with many other strains, although the underlying mechanism is unknown. In this study, we described a characteristic difference in the replication of JFH1 compared to that of genotype 1b replicons.

Here, we report that JFH1 replication is less sensitive to CsA than genotype 1b strains, although the interaction of

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† N.I. and K.W. contributed equally to this work.

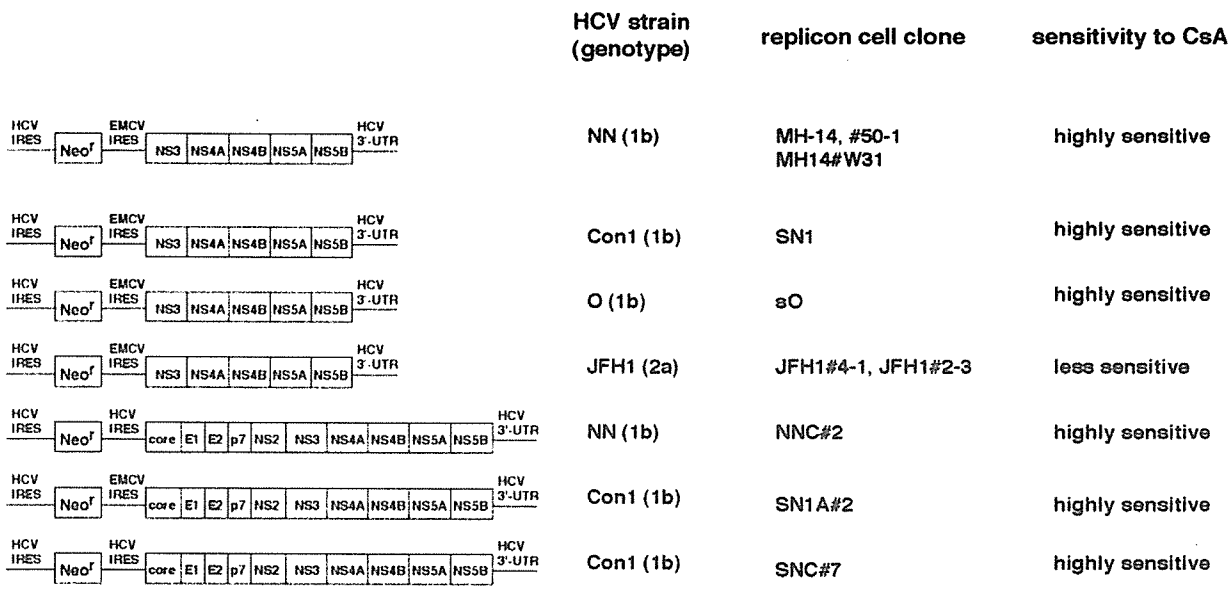

FIG. 1. Schematic representation of the constructs of HCV subgenomic and genome-length replicon RNA. On the left, the constructs of each replicon RNA are shown. HCV strains, as well as genotypes from which the replicon RNA sequences are derived, are indicated in the second column. The names of replicon cell clones established with each replicon RNA are in the third column. The sensitivity to CsA of each replicon RNA revealed in this study is summarized in the fourth column. The replicon RNAs comprise the HCV 5'UTR, including HCV IRES, the neomycin phosphotransferase gene (Neo^r), EMCV IRES, or HCV IRES, the coding region for HCV proteins NS3 to NS5B (subgenomic) or core to NS5B (genome length or full genome), and HCV 3'UTR. MH-14 (NN/1b/SG), #50-1 (NN/1b/SG), MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), sO (O/1b/SG), JFH1#4-1 (JFH1/2a/SG), and JFH1#2-3 (JFH1/2a/SG) cells carry subgenomic replicons, while NNC#2 (NN/1b/FL), SN1A#2 (Con1/1b/FL), and SNC#7 (Con1/1b/FL) cells have genome-length replicons. NNC#2 (NN/1b/FL) and SNC#7 (Con1/1b/FL) cells contain the replicon RNA without EMCV IRES.

CyPB with NS5B is observed with this replicon. However, genome replication and RNA binding activity of NS5B are independent of CyPB. We have exploited a chemical compound to demonstrate how strain diversity can be generated by underlying differences in the mechanisms of the virus-cell interaction. These findings provide important insight into the mechanisms that mediate the efficacy of antiviral agents.

MATERIALS AND METHODS

Cell culture. Huh-7 cells were cultured in Dulbecco's modified Eagle medium (Invitrogen) with 10% fetal bovine serum, nonessential amino acids (Invitrogen), and L-glutamine (Invitrogen). MH-14, #50-1, MH14#W31, SN1, sO (formerly named 1B2R1), JFH1#4-1, and JFH1#2-3 cells (12, 13, 15, 18, 29), carrying subgenomic replicons, and NNC#2, SN1A#2, and SNC#7 cells, carrying full-genome replicons, were cultured in the above medium supplemented with 300- to 500-μg/ml G418 (Invitrogen). In the assay measuring the response to CsA, NIM811, or PSC833 (Fig. 2, 3, and 4), we seeded small numbers of each replicon cells (7×10^3 to 15×10^3 cells/12-well plate) and treated with each drug. Culture medium was changed every 3 days (CsA, NIM811, or PSC833 was supplemented in the fresh medium for the treatment groups). We did not perform any passages in the assay period. At day 7, the cells were 70 to 90% confluent. A schematic representation of the constructs of HCV replicon RNAs, the name of HCV strains from which the replicon RNA sequences are derived, and the name of replicon cell clones used in this study are summarized in Fig. 1. Since many replicon clones were used in this study, we list "strain/genotype/length of the replicon construct" in parentheses after the names of each cell clone in Results and in the figure legends to avoid confusion between names: for example, MH-14 (NN/1b/SG), JFH1#4-1 (JFH1/2a/SG), and SN1A#2 (Con1/1b/FL) cells. The designations SG and FL indicate subgenomic and full-genome replicons, respectively.

Establishment of replicon cells. MH-14, #50-1, sO, JFH1#4-1, and JFH1#2-3 cells were described previously (12, 13, 15, 29). The replicon RNAs were produced using a MEGAscript T7 kit (Ambion) from pMH14, pSN1, pNNC, pSN1A, and pSNC plasmids for the establishment of the MH14#W31, SN1, NNC#2, SN1A#2, and SNC#7 replicon cells, respectively. For the establishment of MH14#W31, we transfected RNA into the Huh-7 cell strain which was identical to the parental cells of JFH1#4-1 and JFH1#2-3. Each replicon RNA was transfected into Huh-7 cells, following the selection with the medium in the presence of 500- to 1,000-μg/ml G418 for around 4 weeks. The resultant cell colonies were isolated and expanded. The HCV RNA titers in cell clones carrying JFH1 replicons were not significantly different from those in established cell clones carrying genotype 1b replicons.

Plasmid construction. pSN1, the sequence of which is derived from I377NS3-3' (18), was prepared essentially as described previously (15). pSN1A was generated by inserting the region from the core to NS2 of pM1LE (15) into the upstream coding region for NS3 in pSN1. To obtain pSNC, the EMCV IRES of pSN1A was replaced by the HCV IRES. pNNC was produced by inserting the coding region from NS3 to NS5B of pM1LE into pSNC.

Real-time reverse transcription-PCR (RT-PCR) analysis. The 5'UTR of HCV genome RNA was quantified using the ABI PRISM 7700 sequence detector (Applied Biosystems) as described previously (29).

Immunoblot analysis. Immunoblot analysis was performed as described previously (30). The primary antibodies used in this study were anti-core, anti-E2 (kindly provided by M. Kohara, Tokyo Metropolitan Institute of Medical Science), anti-NS3, anti-NS5A (a generous gift from A. Takamizawa, Osaka University), anti-NS5B (NS5B-6; kindly provided by I. Fukuya, Osaka University), anti-CyPA (Upstate Cell Signaling), anti-CyPB (Affinity BioReagents), and anti-tubulin (Oncogene).

Immunoprecipitation assay and RNA-protein binding precipitation assay. Immunoprecipitation and RNA-protein binding precipitation were performed as described previously (30, 31).

RNA interference technique. The condition of small interfering RNA (siRNA) used in this study was described previously (31). Transfection was performed using siLentFect (Bio-Rad), according to the manufacturer's protocol.

Isolation of replication complex. The HCV replication complex was isolated from cells by treatment with 50-μg/ml digitonin at 27°C for 5 min, following treatment with 0.3-μg/ml proteinase K at 37°C for 5 min as described previously (31).

Purification of recombinant GST-fused CyPB protein. Glutathione *S*-transferase (GST) and GST-fused CyPB (GST-CyPB) protein expression was induced

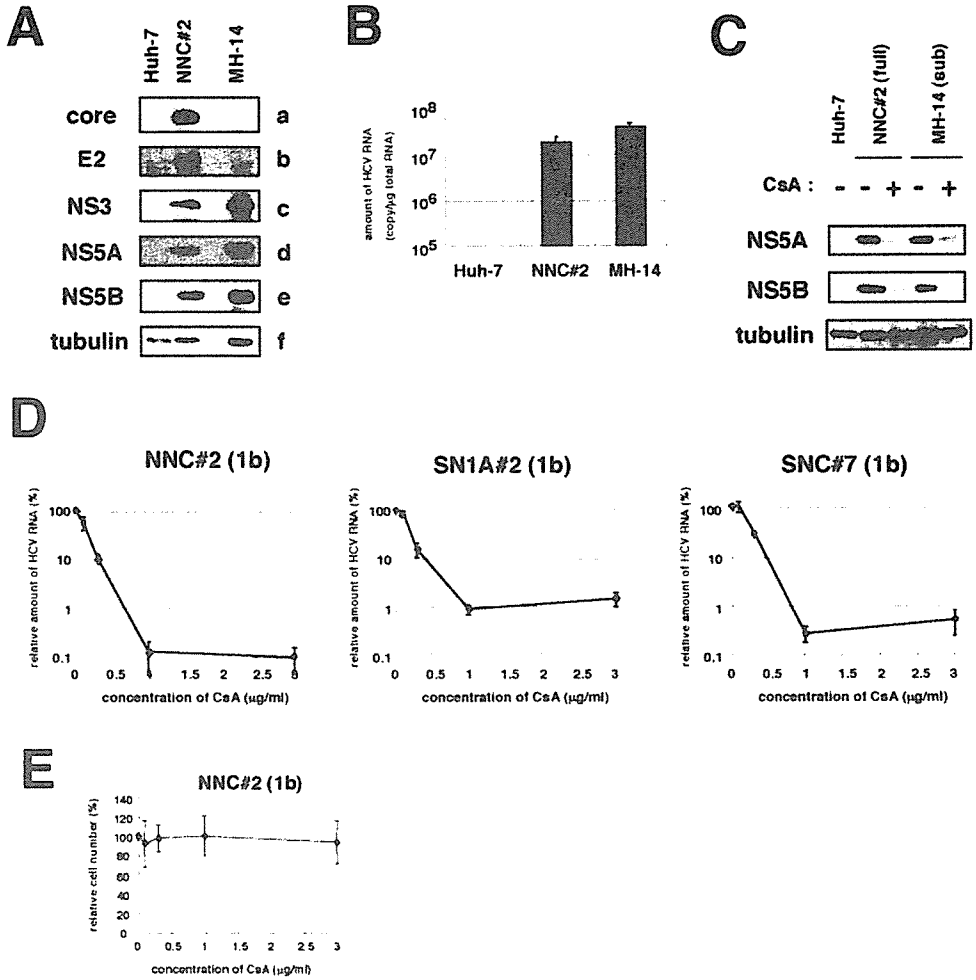

FIG. 2. CsA suppressed the replication of HCV genome, irrespective of the presence of the structural proteins. (A) Detection of HCV proteins from NNC#2 (NN/1b/FL) genome-length replicon. Core (a), E2 (b), NS3 (c), NS5A (d), NS5B (e), and tubulin (f) in Huh-7, NNC#2 (NN/1b/FL), and MH-14 (NN/1b/SG) cells analyzed by immunoblot analysis are shown. (B) HCV RNA in Huh-7, NNC#2 (NN/1b/FL), and MH-14 (NN/1b/SG) cells quantified by real-time RT-PCR analysis. The data represent the means of three independent experiments. (C) CsA decreased the production of HCV proteins in NNC#2 (NN/1b/FL), as well as in MH-14 (NN/1b/SG) cells. After treatment with 1-μg/ml CsA (+) for 5 days or without treatment (−), total-cell lysates of NNC#2 (NN/1b/FL) and MH-14 (NN/1b/SG) cells, together with Huh-7 cells as a negative control, were recovered to examine the production of HCV NS5A (top), NS5B (middle), and tubulin as an internal control (bottom) by immunoblot analysis. The same result was obtained at day 7 after treatment. (D) The sensitivity to CsA of HCV genome-length replicon was almost the same as that of the subgenomic replicon. HCV RNA was quantified by real-time RT-PCR analysis using total RNA from NNC#2 (NN/1b/FL), SN1A#2 (Con1/1b/FL), and SNC#7 (Con1/1b/FL) cells treated with various concentrations of CsA for 7 days. The relative amount of HCV RNA was plotted against the concentration of CsA (in micrograms per milliliter). (E) Effect of CsA on cell proliferation. NNC#2 (NN/1b/FL) cells were treated with various amount of CsA for 7 days. Cell numbers were counted, and cell numbers relative to those of cells without treatment were plotted against the concentration of CsA.

in transformed BL21 cells (Amersham) with 1 mM isopropyl-β-thiogalactopyranoside (IPTG). The cell lysate was incubated with glutathione-Sepharose resin (Amersham) and washed extensively. The recombinant protein was eluted by glutathione (pH 8.0) and subsequently dialyzed.

In vitro RNA binding assay. In vitro-translated ^{35}S-labeled NS5B proteins and poly(U)-Sepharose (Amersham) or protein G-Sepharose (Amersham) resin as a negative control were incubated in the presence of recombinant GST-CyPB protein at 4°C for 1 h. After being washed, precipitates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by imaging analyzer.

RESULTS

CsA suppressed the replication of HCV full-genome replicon. We and another group have reported an anti-HCV activity of CsA using subgenomic replicons (22, 29). HCV structural proteins, especially the core protein, have multiple functions. These proteins interact with many cellular factors and modulate a variety of cellular functions (32). Potentially, these viral proteins could diminish or circumvent the suppression of HCV genome replication by CsA. Core protein and E2 reportedly modulate the activity of IFN signaling (9, 25). To test this possibility, we established a full-genome HCV replicon system with cells transfected with the NN strain (NNC#2 cells [NN/1b/FL]) (Fig. 1). HCV RNA and protein productions were confirmed by real-time RT-PCR and immunoblot analysis (Fig. 2A and B). In addition, we confirmed that this replication was not due to the integration of the replicon construct into the

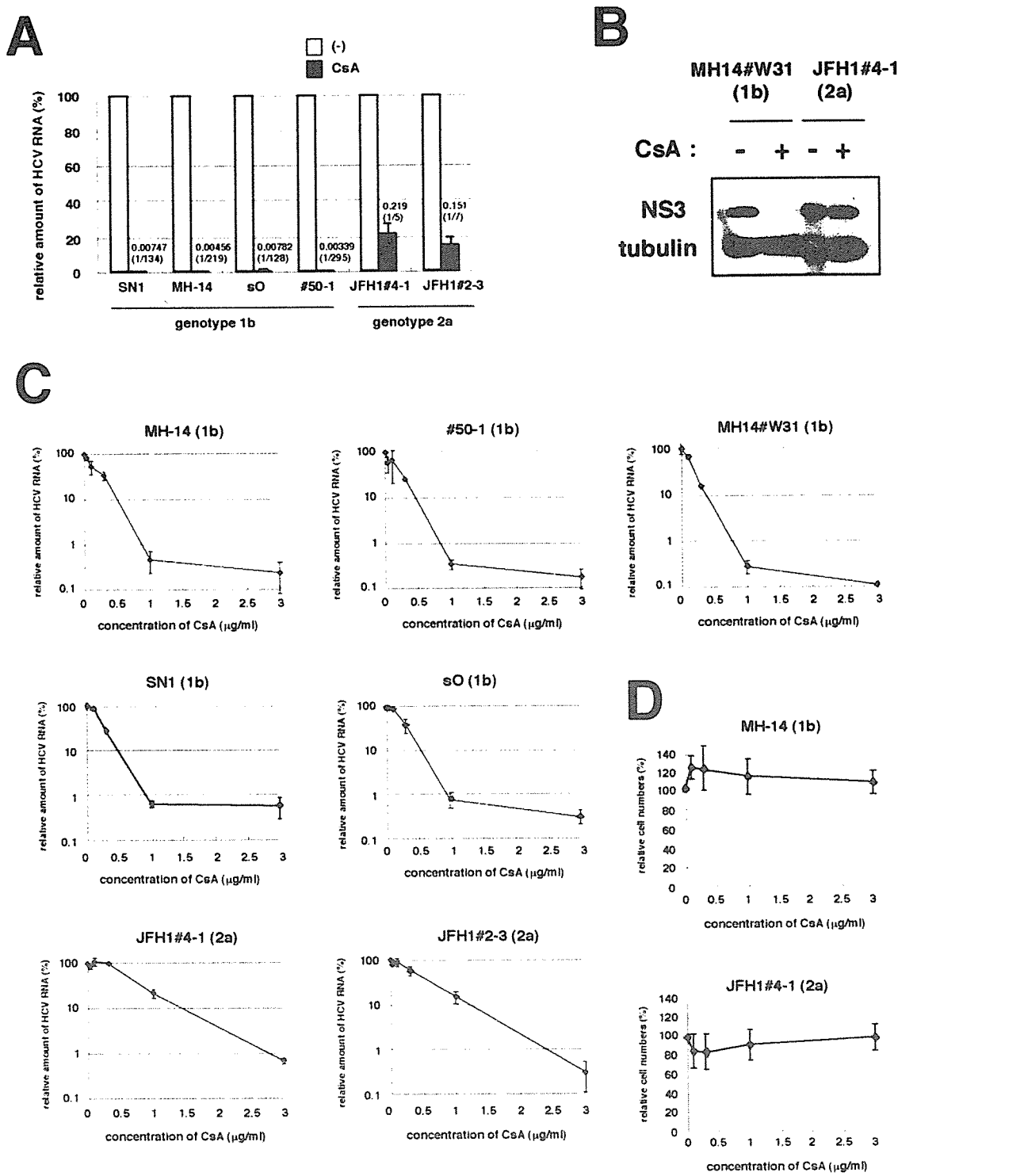

FIG. 3. Replication of a genotype 2a strain, JFH1, was less sensitive to CsA. (A) Sensitivity to CsA of HCV genotype 1b and JFH1 replicons. SN1 (Con1/1b/SG), MH-14 (NN/1b/SG), sO (O/1b/SG), #50-1 (NN/1b/SG), JFH1#4-1 (JFH1/2a/SG), and JFH1#2-3 (JFH1/2a/SG) cells, carrying HCV subgenomic replicon, were treated with 1-μg/ml CsA for 7 days. HCV RNA titers were quantified by real-time RT-PCR analysis, and the relative amounts are shown. The bars represent the means of three independent experiments. White bars, no treatment; black bars, 1-μg/ml CsA. The numbers above the black bars indicate fold difference of the titer with 1-μg/ml CsA treatment compared to no treatment. (B) Levels of NS3 and tubulin as an internal control in MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells without (−) or with (+) 1-μg/ml CsA treatment for 5 days were detected by immunoblot analysis. (C) HCV RNA was quantified and plotted as described in the legend to Fig. 2D with genotype 1b replicon cells such as MH-14 (NN/1b/SG), #50-1 (NN/1b/SG), MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), and sO (O/1b/SG) cells and JFH1-carrying replicon cells such as JFH1#4-1 (JFH1/2a/SG) and JFH1#2-3 (JFH1/2a/SG) cells. (D) Effect of CsA on cell proliferation. The growth of MH-14 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells were examined as described in the legend for Fig. 2E.

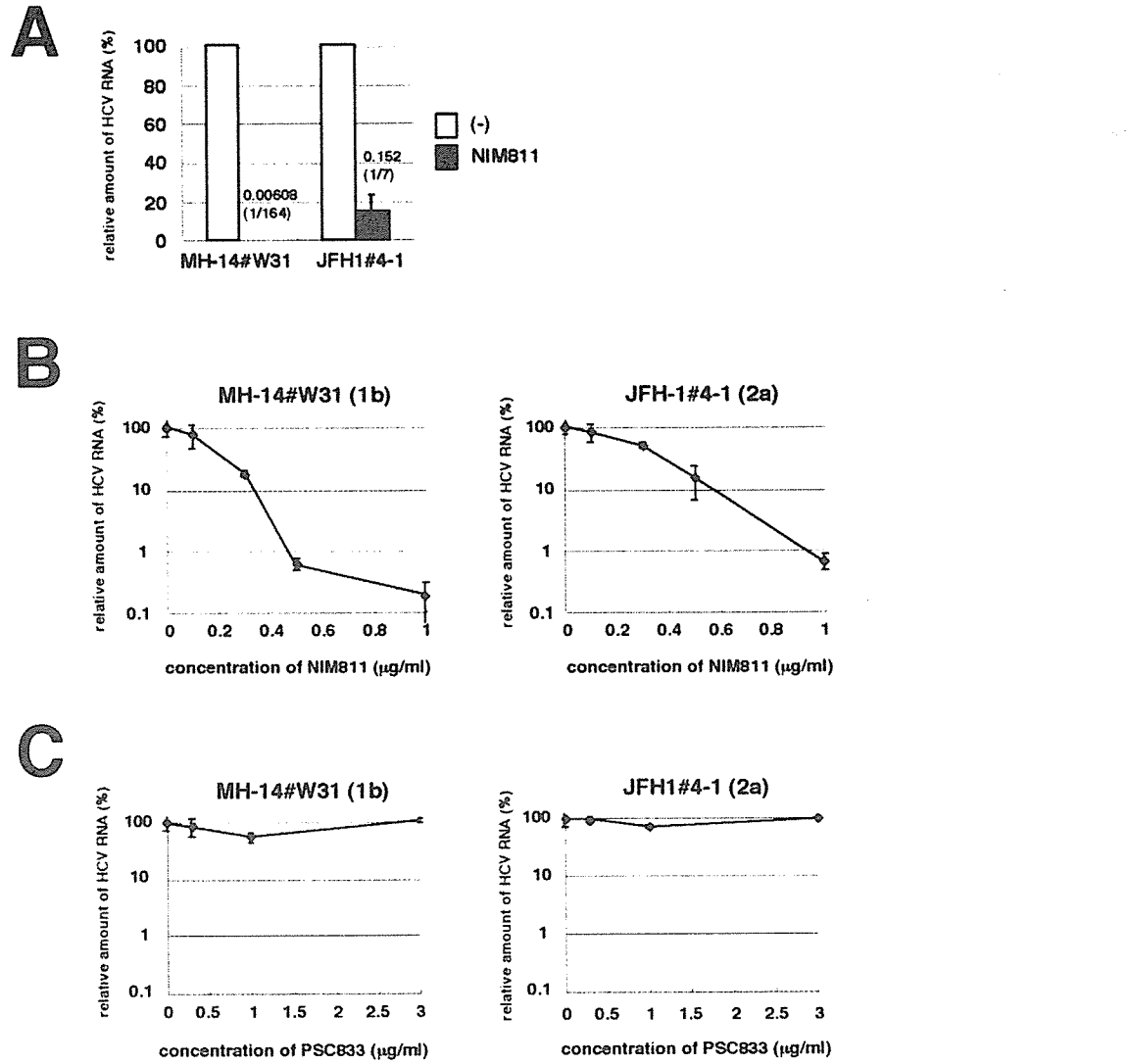

FIG. 4. JFH1 replication was less sensitive to a CsA derivative, NIM811. (A) MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells were treated with 0.5-μg/ml NIM811 for 7 days. HCV RNA titers were quantified as described in the legend to Fig. 3A. White bars, no treatment; black bars, 0.5-μg/ml NIM811. (B and C) HCV RNA in replicon cells treated with various concentrations of NIM811 (B) or PSC833 (C) for 7 days was quantified and plotted against the concentration of NIM811 (B) or PSC833 (C) (in micrograms per milliliter) as described in the legend to Fig. 3C.

cellular genome (data not shown). Similarly, we generated other full-genome replicon cells carrying sequences from the Con1 strain at the nonstructural coding region of the replicon RNA (SN1A#2 [Con1/1b/FL]) and SNC#7 (Con1/1b/FL) cells (Fig. 1). The replicon of SN1A#2 (Con1/1b/FL) cells possessed the EMCV IRES upstream of the open reading frame for HCV proteins, while that of SNC#7 (Con1/1b/FL) cells contained the HCV IRES (Fig. 1). SNC#7 (Con1/1b/FL) cells exhibited almost the same response as that of SN1A#2 (Con1/1b/FL) cells to CsA treatment (Fig. 2D). Consistent with a previous report (22), the EMCV IRES was not responsible for the anti-HCV activity of CsA. We compared the sensitivity to CsA of full-genome replicons with that of subgenomic replicons. CsA strongly decreased the production of HCV proteins in both the full-genome replicon, NNC#2 (NN/1b/FL) cells and the subgenomic replicon, MH-14 (NN/1b/SG) cells (Fig. 2C). Real-time RT-PCR analysis also revealed a dramatic reduction of the RNA level of full-genome replicons in NNC#2 (NN/1b/FL), SN1A#2 (Con1/1b/FL), and SNC#7 (Con1/1b/FL) cells (Fig. 2D). The 50% inhibitory concentrations (IC_{50}) of CsA in NNC#2 (NN/1b/FL), SN1A#2 (Con1/1b/FL), and SNC#7 (Con1/1b/FL) cells awere estimated to be 0.13, 0.19, and 0.24 μg/ml, respectively. The 90% inhibitory concentrations (IC_{90}) of CsA in these cells were 0.68, 0.94, and 0.81 μg/ml, respectively. The CsA dose-response curves of full-genome replicons and subgenomic replicons were similar (i.e., compare SN1A#2 or SNC#7 [Con1/1b/FL] versus SN1 [Con1/1b/SG], NNC#2 [NN/1b/FL] versus MH-14, #50-1, or MH14#W31 [NN/1b/SG]) (Fig. 3C). These results demonstrate that CsA suppresses the replication of full-genome replicons and subgenomic replicons to almost the same extent. Since CsA concentrations of up to 3 μg/ml did not affect the

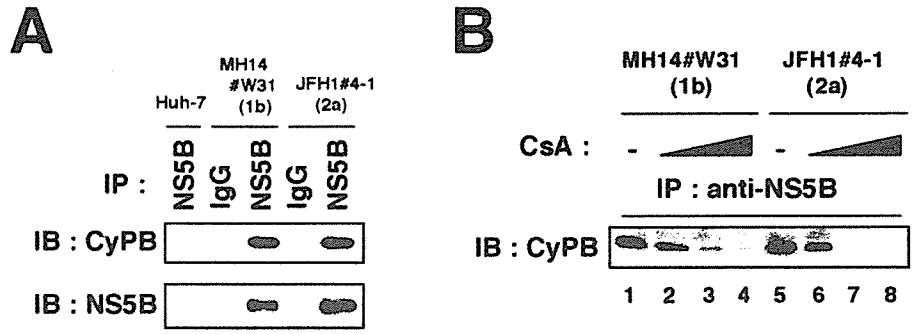

FIG. 5. Interaction of HCV NS5B with CyPB in the JFH1 replicon. (A) Coimmunoprecipitation of endogenous CyPB with NS5B. Lysates from MH14#W31 (NN/1b/SG), JFH1#4-1 (JFH1/2a/SG), and Huh-7 cells as a negative control were used for immunoprecipitation with normal mouse immunoglobulin G (IgG) or anti-NS5B antibody (NS5B), followed by immunoblot analysis with either anti-CyPB (top) or anti-NS5B antibodies (bottom). IP, antibodies used for immunoprecipitation. (B) The interaction of CyPB with NS5B in JFH1 replicon was disrupted by CsA treatment. Coimmunoprecipitation between CyPB and NS5B was analyzed with MH14#W31 (NN/1b/SG) or JFH1#4-1 (JFH1/2a/SG) cells treated without CsA (lanes 1 and 5) or with CsA (0.3 μg/ml in lanes 2 and 6, 1 μg/ml in lanes 3 and 7, and 3 μg/ml in lanes 4 and 8).

proliferation of any replicon cells (Fig. 2E and data not shown), the effect of CsA on replication is not due to the cytotoxic effect. In addition, we observed the reduction of production of infectious viral particles in the presence of 3-μg/ml CsA (data not shown) using the viral production system with full-genome JFH1 RNA (27).

The JFH1 replicon was less sensitive to CsA than were genotype 1b replicons. We compared the sensitivity of HCV replication to CsA in several subgenomic replicon cells. We used MH-14 (NN/1b/SG) and #50-1 (NN/1b/SG) cells carrying subgenomic replicons with HCV NN strain (15, 29), SN1 (Con1/1b/SG) cells carrying the Con1 subgenomic replicon (18), and sO (O/1b/SG) cells bearing the subgenomic O strain (12) as genotype 1b replicon-containing cells. We also employed JFH1#4-1 (JFH1/2a/SG) and JFH1#2-3 (JFH1/2a/SG) cell clones carrying the JFH1 subgenomic replicon (13). Treatment of CsA (1 μg/ml; 7 days) drastically decreased HCV RNA in all the subgenomic replicon cells carrying the HCV genotype 1b strain. HCV RNA levels in SN1 (Con1/1b/SG), MH-14 (NN/1b/SG), sO (O/1b/SG), and #50-1 (NN/1b/SG) cells decreased to 1/134, 1/219, 1/128, and 1/295, respectively (Fig. 3A). Genotype 1b replicon cells appeared highly sensitive to CsA. In contrast, the effect of CsA on HCV RNA levels in replicon cells containing sequences from the JFH1 strain was limited to 1/5 to 1/7 (Fig. 3A). These results of the response to CsA were reproduced in further additional cell clones.

The cellular characteristics of Huh-7 cell strains differ among laboratories. To exclude the possibility that differences between Huh-7 cell strains influence the sensitivity to CsA, we established genotype 1b replicon cells based on the identical Huh-7 cell strain, which were used as parental cells of JFH1#4-1 (JFH1/2a/SG) and JFH1#2-3 (JFH1/2a/SG) cells. The response of the corresponding replicon cells, MH14#W31 (NN/1b/SG), to CsA was almost the same as that of SN1 (Con1/1b/SG), MH-14 (NN/1b/SG), sO (O/1b/SG), and #50-1 (NN/1b/SG) cells (Fig. 3C). Thus, the difference in sensitivity of JFH1 and genotype 1b strains to CsA can be attributed to the characteristic differences of the HCV strains, not to the parental Huh-7 cell strain. In addition, the reduction of NS3 protein in JFH1#4-1 (JFH1/2a/SG) cells following treatment with CsA was less prominent than that in MH14#W31 (NN/1b/SG) cells (Fig. 3B).

We examined the dose-response curve of HCV RNA against the concentration of CsA (Fig. 3C). The effect of CsA in genotype 1b replicons plateaued at around 1 μg/ml, while in the dose-response curve in JFH1 replicon, the inhibition was not yet saturated (Fig. 3C). As concentrations of CsA up to 3 μg/ml did not affect the proliferation rate of any replicon cells (Fig. 3D and data not shown), the effect of CsA on replication was not due to the cytotoxic effect. The IC_{50} of CsA in MH-14 (NN/1b/SG), #50-1 (NN/1b/SG), MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), sO (O/1b/SG), JFH1#4-1 (JFH1/2a/SG), and JFH1#2-3 (JFH1/2a/SG) cells were estimated to be 0.15, 0.18, 0.16, 0.20, 0.25, 0.67, and 0.43 μg/ml, respectively. The IC_{90} was 0.86, 0.82, 0.76, 0.88, 0.92, 2.77, and 2.39 μg/ml, respectively. A similar dose-response curve in the JFH1 replicon was obtained by a transient replication assay with the luciferase reporter driven from a JFH1 replicon construct (data not shown) (14).

JFH1 replicon was less sensitive to a CsA derivative, NIM811. Analysis of several CsA derivatives has revealed that the anti-HCV effect of CsA on the genotype 1b replicon is mediated by the inhibition of CyP (31). We examined the sensitivity of JFH1 replicon to CsA derivatives. CsA is known to have three major cellular targets: CyP, calcineurin (CN)/NF-AT, and P glycoprotein (P-gp) (28, 31). A CsA derivative, NIM811, inhibits CyP and P-gp but not CN/NF-AT, while another derivative, PSC833, inhibits P-gp but neither CyP nor CN/NF-AT (31). The decrease of HCV RNA in MH14#W31 (NN/1b/SG) cells with NIM811 treatment (0.5 μg/ml; 7 days) was more than an order of magnitude greater than that in JFH1#4-1 (JFH1/2a/SG) cells (Fig. 4A). The slope of the dose-response curve of NIM811 treatment of the JFH1 replicon was gentler than that of genotype 1b (Fig. 4B). The IC_{50} of NIM811 in MH14W#31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells were 0.17 and 0.30 μg/ml, respectively. The IC_{90} were 0.46 and 0.93 μg/ml, respectively. In contrast, PSC833, which does not inhibit CyP, did not alter HCV RNA level in either genotype 1b or the JFH1 replicon (Fig. 4C). Thus, a CyP

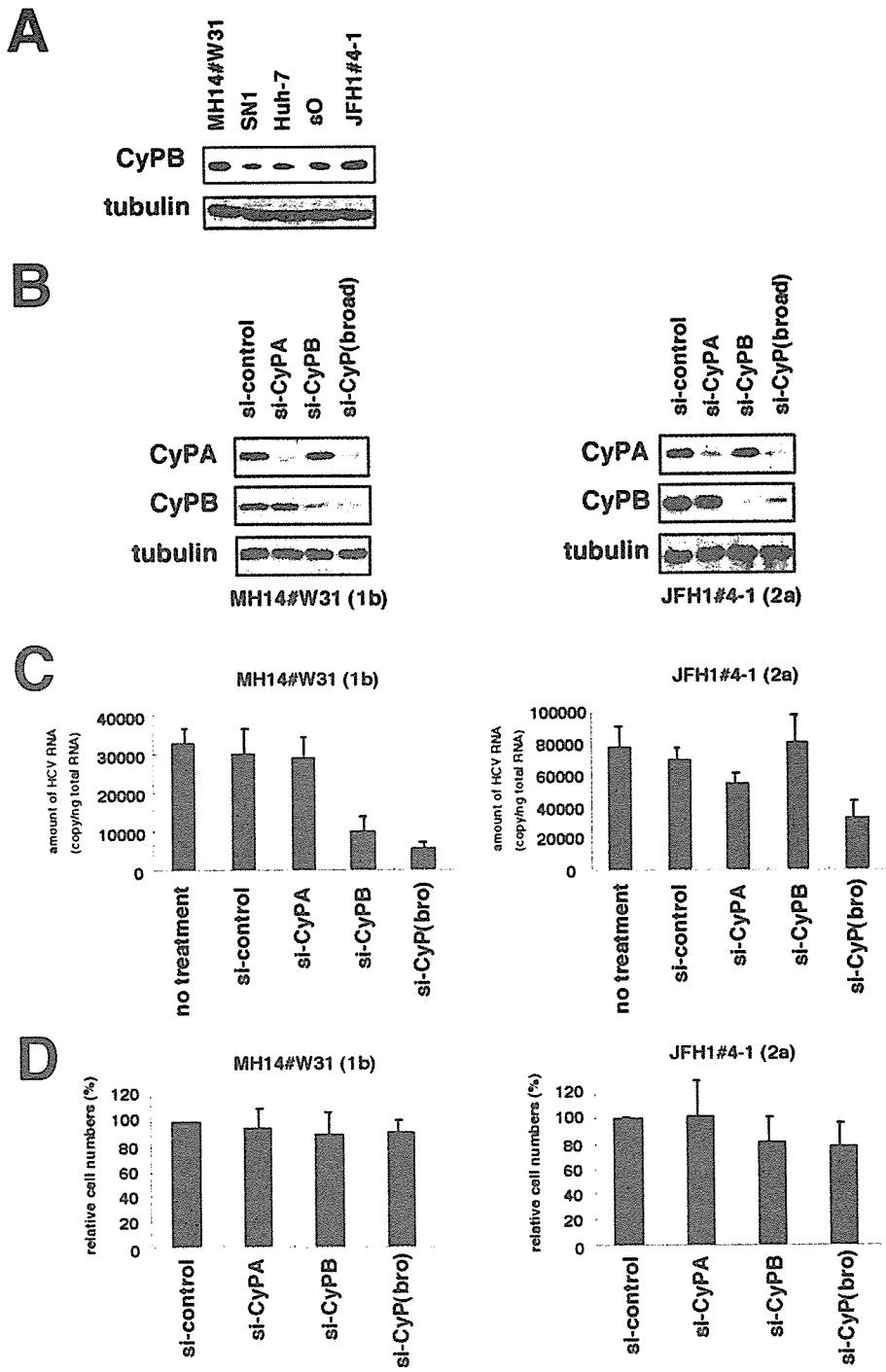

FIG. 6. CyPB in HCV replication of genotype 1b and JFH1. (A) Expression level of endogenous CyPB protein (top) and tubulin as an internal control (bottom) in MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), sO (O/1b/SG), JFH1#4-1 (JFH1/2a/SG), and Huh-7 cells. (B) Knockdown of endogenous CyP proteins. MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells were transfected with siRNA specific for CyPA (si-CyPA), CyPB (si-CyP), a broad range of CyP subtypes [si-CyP(broad)], or a randomized siRNA (si-control). At 72 h posttransfection, CyPA (top), CyPB (middle) and tubulin as an internal control (bottom) were detected in total cell lysates of MH14#W31 (NN/1b/SG) (left) and JFH1#4-1 (JFH1/2a/SG) (right) cells by immunoblot analysis. (C) Depletion of CyPB did not affect HCV replication of JFH1 replicon. At 5 days posttransfection, HCV RNA titers in MH14#W31 (NN/1b/SG) (left) and JFH1#4-1 (JFH1/2a/SG) (right) cells were quantified by real-time RT-PCR analysis. no treatment, treatment with only the transfection reagent in the absence of siRNA. (D) Effect of siRNA on cell proliferation. Cell numbers of MH14W#31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells treated with siRNA for 5 days were counted. Relative cell numbers were indicated.

inhibitor was less effective at suppressing the replication of the JFH1 replicon than genotype 1b replicons.

Interactions between CyPB and JFH1 NS5B. Previously, we have shown that CyPB interacts with NS5B to promote HCV genome replication and that CsA inhibits this binding in a genotype 1b replicon (31). Here, we examined the association between CyPB and NS5B in a JFH1 replicon. Immunoprecipitation analysis revealed an interaction of CyPB with NS5B in JFH1#4-1 (JFH1/2a/SG) cells (Fig. 5A). This interaction was dissociated following the treatment of CsA, as observed with the genotype 1b replicon (Fig. 5B).

Role of CyPB in replication of the JFH1 replicon. Although we observed some differences of expression levels of endogenous CyPB among the replicon cells in the immunoblot analysis (Fig. 6A), there was no particular correlation between endogenous CyPB expression levels and replication sensitivity to CsA among cells. CyPB reportedly regulates HCV genome replication of the genotype 1b replicon (31). We then explored the requirement of CyPB for the replication of JFH1 replicon with RNA interference. Transfecting siRNAs designed to recognize several CyP subtypes [si-CyP(broad)] (Fig. 6B) reduced HCV RNA to <1/5 in MH14#W31 (NN/1b/SG) cells (Fig. 6C). Specific knockdown of CyPB but not CyPA (Fig. 6B) decreased HCV RNA in MH14#W31 (NN/1b/SG) cells, consistent with a previous report (Fig. 6C) (31). In contrast, HCV RNA in JFH1#4-1 (JFH1/2a/SG) cells was not altered following the suppression of either endogenous CyPA or CyPB (Fig. 6B and C). We observed a weak decrease of HCV RNA levels (around one-half) with si-CyP(broad) (Fig. 6C). These data suggests the possibility that the replication of the JFH1 replicon is independent of CyPB, in contrast to the genotype 1b replicon. In the previous study, it was reported that the doubling time, saturation density, and response to cell confluence of the replicon cells carrying JFH1 were different from those in cells carrying a genotype 1b replicon, suggesting the possibility that the coupling relationship between the replication and cell growth was different between genotype 1b and the JFH1 replicon (21). The introduction of either si-CyPB or si-CyP(broad), however, had little effect on cell growth in MH14#W31 (NN/1b/SG) or JFH1#4-1 (JFH1/2a/SG) cells (Fig. 6D). And we did not observe cells being confluent in the experiment period. The above results suggest that the different response to si-CyPB in the two lines is independent of the conditions of cell growth.

The role of CyPB in the RNA binding activity of JFH1 NS5B. CyPB regulates HCV genome replication of a genotype 1b replicon by promoting the RNA binding activity of NS5B (31). We examined the effect of CyPB on the RNA binding activity of NS5B in JFH1. NS5B in the replication complex was isolated from cells by treatment with digitonin-proteinase K, as described previously (31). This fraction was incubated with poly(U) RNA-Sepharose or protein G-Sepharose as a negative control for the detection of RNA binding NS5B in the replication complex. RNA-bound NS5B in this fraction from MH14#W31 (NN/1b/SG) cells was decreased drastically following treatment with CsA (Fig. 7A, lanes 5 and 6). However, the reduction of RNA binding of NS5B in the replication complex of JFH1#4-1 (JFH1/2a/SG) cells was not as prominent (Fig. 7A, lanes 11 and 12). We confirmed this result by an in vitro RNA binding assay, in which in vitro-synthesized NS5B was incubated with poly(U) RNA-Sepharose, together with

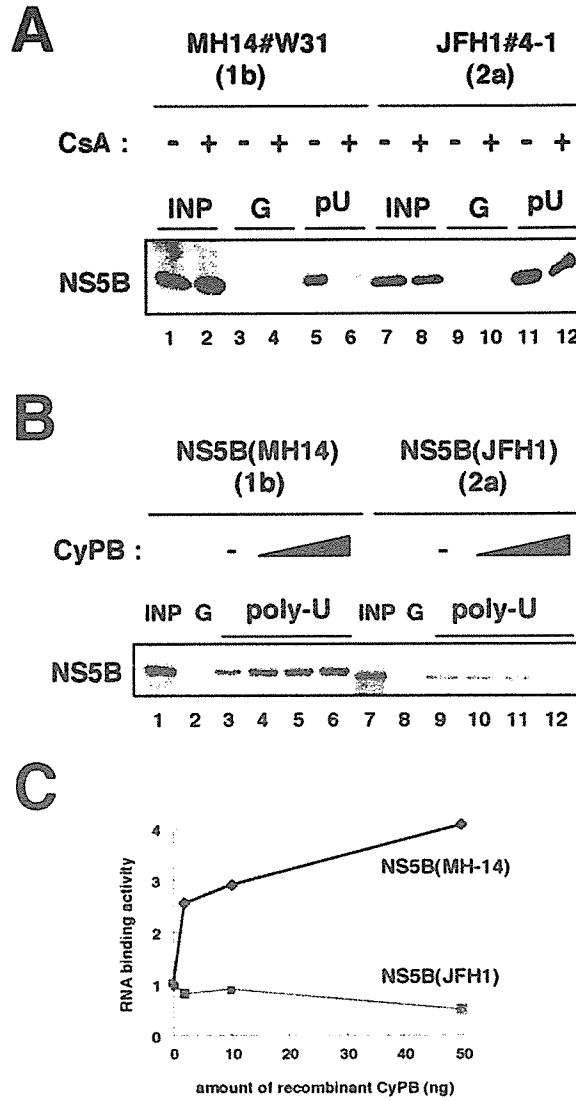

FIG. 7. RNA binding capacity of JFH1 NS5B was independent of CyPB. (A) An RNA-protein binding precipitation assay was performed using MH14#W31 (NN/1b/SG) cells (lanes 1 to 6) and JFH1#4-1 (JFH1/2a/SG) cells (lanes 7 to 12) as described in Materials and Methods. MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells preincubated without (lanes 1, 3, 5, 7, 9, and 11) or with (lanes 2, 4, 6, 8, 10, and 12) CsA were treated with digitonin, followed by digestion with proteinase K to isolate the replication complex. This fraction was then incubated with poly(U) RNA-Sepharose (lanes 5, 6, 11, and 12) or protein G-Sepharose as a negative control (lanes 3, 4, 9, and 10). Precipitates were detected by immunoblot analysis with anti-NS5B antibody. INP, one-sixth of the amount of cell lysate used in the precipitation assay; G and pU, samples with protein G-Sepharose and poly(U)-Sepharose, respectively. (B) An in vitro RNA binding assay was performed as described in Materials and Methods. In vitro-synthesized NS5B of MH-14 (lanes 1 to 6) or JFH1 (lanes 7 to 12) with the rabbit reticulocyte lysate in the presence of [^{35}S]methionine was incubated with protein G-Sepharose (lanes 2 and 8) or poly(U)-Sepharose in the absence (lanes 3 and 9) or presence of various amounts of purified recombinant GST-CyPB (2 ng in panels 4 and 10, 10 ng in panels 5 and 11, and 50 ng in panels 6 and 12). The resultant precipitates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by the detection of radiolabeled protein. (C) The density of the bands of NS5B in the RNA binding fraction was quantified and plotted against the amount of the recombinant GST-CyPB (in nanograms). Solid line, NS5B of MH-14; faint line, NS5B of JFH1.

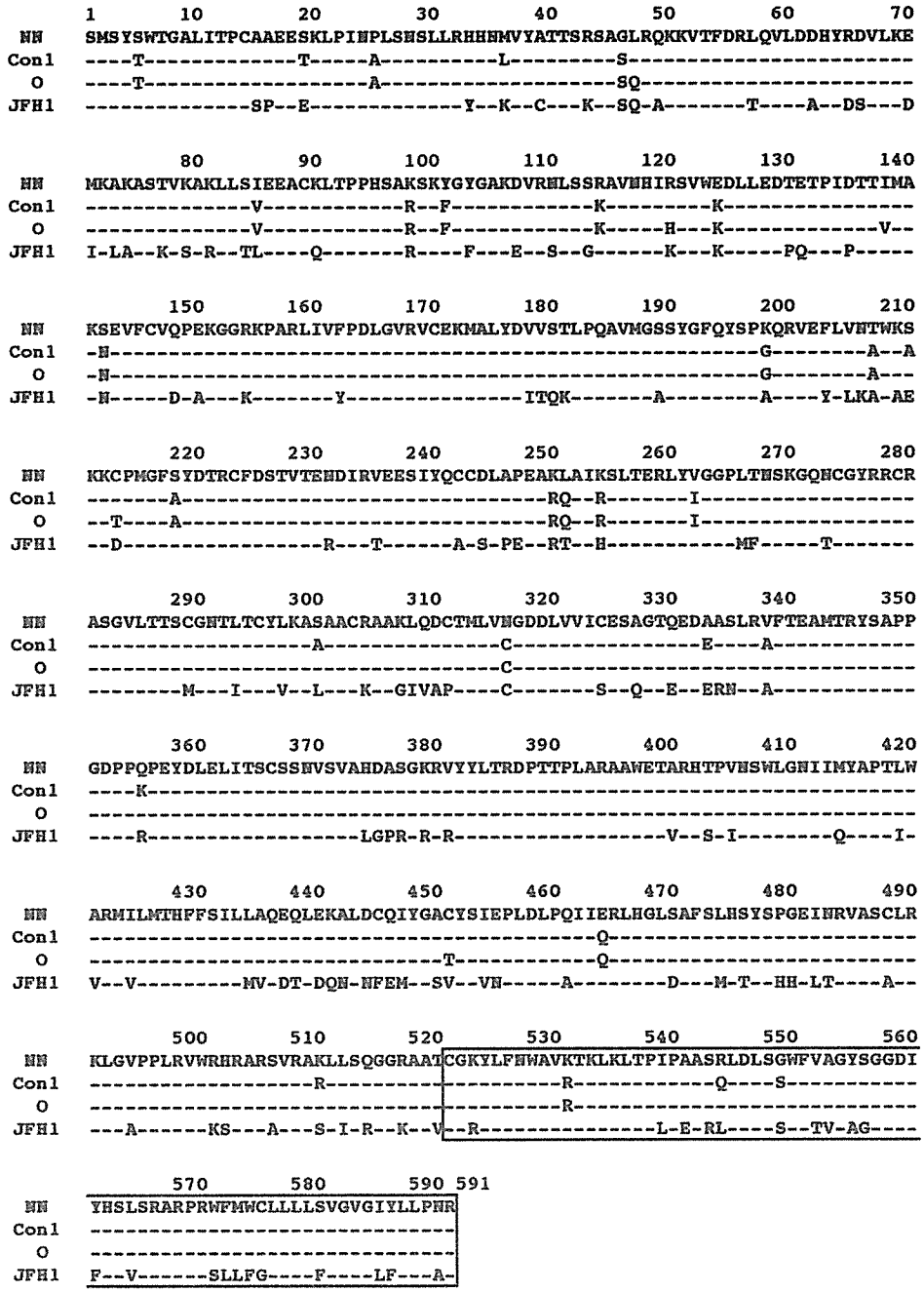

FIG. 8. Amino acid sequence alignment of NS5B encoded by HCV strains NN, Con1, O, and JFH1. The numbers above the sequence indicate the amino acid numbers. Conserved residues are shown by dashes. The region spanning 521 to 591 aa, which is involved in the interaction with CyPB, is boxed.

recombinant GST-CyPB. The addition of recombinant GST-CyPB increased the binding of genotype 1b NS5B to poly(U) RNA (Fig. 7B and C). However, this augmentation of RNA binding was not observed with NS5B from the JFH1 strain (Fig. 7B and C). From the above results, it is suggested that the RNA binding of JFH1 NS5B is free from regulation by CyPB.

DISCUSSION

Until now, we and another group have utilized subgenomic replicons carrying genotype 1b NN and HCV-N strains to demonstrate that CsA suppresses HCV genome replication (22, 29). This study reveals that CsA is effective on full-genome replicons to almost the same extent. In addition, other available genotype 1b replicons carrying the Con1 and O strains also have a high sensitivity to CsA, consistent with our proposal that HCV genotype 1b is highly sensitive to CsA. However, a fulminant-type genotype 2a replicon, JFH1, was less responsive to CsA, although a high dose of CsA suppressed the replication of this strain.

CyPB interacts with genotype 1b NS5B to stimulate its RNA

binding activity. In contrast, CyPB binds JFH1 NS5B but does not regulate the function of JFH1 NS5B. This is consistent with a previous speculation that genotype 1b and JFH1 replicons utilize the same cellular factors in distinct manners (21). The NS5B sequence of NN strain has 95.0, 95.9, and 70.4% homology to that of Con1, O, and JFH1, respectively (Fig. 8). The region spanning amino acids (aa) 521 to 591 of NS5B, which is involved in the interaction with CyPB (31), is highly conserved among genotype 1b strains NN, Con1, and O while that of JFH1 has 21 substituted residues in this region. The proline at 540 aa, which is important for CyPB binding (31), is conserved but the adjacent residues such as isoleucine at 539 aa and alanine at 541 aa are replaced by leucine and glutamic acid, respectively, in JFH1. Through molecular interactions, CyPB seems to make the conformation of NS5B of genotype 1b strains but not JFH1 suitable for RNA binding (31). The diverse regulation system of NS5B by CyPB among strains may be due to differences in either the sequence or the entire conformation of NS5B. Further study is important for elucidating the regulation mechanism of RNA binding activity of NS5B by CyPB.

Thus, replication in JFH1 replicon is independent of CyPB. Interestingly, human immunodeficiency virus type 1 (HIV-1) strains also have a diversity of CyP dependence on viral proliferation (3, 33). CyPA plays an important role in the life cycle of HIV-1. The interaction of the HIV-1 capsid protein with CyPA that resides within the target cells of infection is critical for HIV-1 replication (7, 24). In peripheral blood mononuclear cells or Jurkat T cells, CsA suppresses the proliferation of HIV-1 group main (M) strain (3). However, certain strains of group outlier (O), such as MVP5180 and MVP9435, are resistant to CsA (3, 33), suggesting the different dependency of the replication on CyPA. Authors have suggested that MVP5180 and MVP9435 clones adapt to replicate independently of CyPA and that this adaptation provides a significant replication advantage for the virus in vivo (3). In vesicular stomatitis virus (VSV) strains, a role for CyPA in virus replication also has been reported (2). CyPA is required for the infection of the VSV-NJ strain but not the VSV-IND strain. These authors proposed that during evolutionary divergence from the ancestral lineages that initially were dependent on CyPA for replication, VSV-IND may have adapted to reduce its dependency on CyPA (2). In the case of HCV, a fulminant type genotype 2a replicon (JFH1) replicates independently of CyPB. It has previously been reported that JFH1 has a much higher competency of replication in the cells than other strains (13). The adaptation to independence from CyPB may contribute to the high capacity of replication of JFH1.

Although the JFH1 replicon is less sensitive to CsA, high concentrations of CsA still suppress replication of the JFH1 replicon. Moreover, the introduction of the siRNA designed to recognize several CyP subtypes [si-CyP(broad)] moderately diminishes HCV RNA in the JFH1 replicon. We suspect that a CyP family member other than CyPB is involved in HCV genome replication. Further analysis is needed on the role of other CyP subtypes.

As there a replicon system for a fulminant-type genotype 1b replicon or chronic-type genotype 2a replicon does not yet exist, we cannot conclude whether chronic-type genotype 2a replicons or fulminant-type replicons are less sensitive to CsA or not. However, there is a clinical report describing cotreatment of patients with chronic hepatitis C with IFN and CsA that resulted in a higher sustained virological rate than with treatment of IFN alone (11). In this report, increase in the sustained virological rate was prominent with patients carrying genotype 1 HCV (51.7% versus 21.9%), while it was relatively weak in patients carrying genotype 2 HCV (66.7% versus 58.3%) (11). Thus, genotype may affect the sensitivity of HCV replication to CsA. However, we cannot exclude the possibility that the diminished sensitivity to CsA is a characteristic only of the fulminant-type genotype 2a strain.

Our results suggest that sensitivity to CsA and replication dependency to CyPB is different among HCV strains. This finding is an important insight into the diversity of the mechanism of HCV genome replication and its sensitivity to antiviral agents.

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REFERENCES

1. **Bartenschlager, R., and V. Lohmann.** 2001. Novel cell culture systems for the hepatitis C virus. Antiviral Res. **52:**1–17.
2. **Bose, S., M. Mathur, P. Bates, N. Joshi, and A. K. Banerjee.** 2003. Requirement for cyclophilin A for the replication of vesicular stomatitis virus New Jersey serotype. J. Gen. Virol. **84:**1687–1699.
3. **Braaten, D., E. K. Franke, and J. Luban.** 1996. Cyclophilin A is required for the replication of group M human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus $SIV_{CPZ}GAB$ but not group O HIV-1 or other primate immunodeficiency viruses. J. Virol. **70:**4220–4227.
4. **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.
5. **Frese, M., V. Schwarzle, K. Barth, N. Krieger, V. Lohmann, S. Mihm, O. Haller, and R. Bartenschlager.** 2002. Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. Hepatology **35:**694–703.
6. **Grakoui, A., C. Wychowski, C. Lin, S. M. Feinstone, and C. M. Rice.** 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. J. Virol. **67:**1385–1395.
7. **Hatziioannou, T., D. Perez-Caballero, S. Cowan, and P. D. Bieniasz.** 2005. Cyclophilin interactions with incoming human immunodeficiency virus type 1 capsids with opposing effects on infectivity in human cells. J. Virol. **79:**176–183.
8. **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.
9. **Hosui, A., K. Ohkawa, H. Ishida, A. Sato, F. Nakanishi, K. Ueda, T. Takehara, A. Kasahara, Y. Sasaki, M. Hori, and N. Hayashi.** 2003. Hepatitis C virus core protein differently regulates the JAK-STAT signaling pathway under interleukin-6 and interferon-gamma stimuli. J. Biol. Chem. **278:**28562–28571.
10. **Ikeda, M., M. Yi, K. Li, and S. M. Lemon.** 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J. Virol. **76:**2997–3006.
11. **Inoue, K., K. Sekiyama, M. Yamada, T. Watanabe, H. Yasuda, and M.**

Yoshiba. 2003. Combined interferon α2b and cyclosporin A in the treatment of chronic hepatitis C: controlled trial. J. Gastroenterol. **38:**567–572.
12. **Kato, N., K. Sugiyama, K. Namba, H. Dansako, T. Nakamura, M. Takami, K. Naka, A. Nozaki, and K. Shimotohno.** 2003. Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. Biochem. Biophys. Res. Commun. **306:**756–766.
13. **Kato, T., T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, and T. Wakita.** 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology **125:**1808–1817.
14. **Kato, T., T. Date, M. Miyamoto, M. Sugiyama, Y. Tanaka, E. Orito, T. Ohno, K. Sugihara, I. Hasegawa, K. Fujiwara, K. Ito, A. Ozasa, M. Mizokami, and T. Wakita.** 2005. Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. J. Clin. Microbiol. **43:**5679–5684.
15. **Kishine, H., K. Sugiyama, M. Hijikata, N. Kato, H. Takahashi, T. Noshi, Y. Nio, M. Hosaka, Y. Miyanari, and K. Shimotohno.** 2002. Subgenomic replicon derived from a cell line infected with the hepatitis C virus. Biochem. Biophys. Res. Commun. **293:**993–999.
16. **Liang, T. J., and T. Heller.** 2004. Pathogenesis of hepatitis C-associated hepatocellular carcinoma. Gastroenterology **127:**S62–S71.
17. **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.
18. **Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285:**110–113.
19. **Manns, M. P., J. G. McHutchison, S. C. Gordon, V. K. Rustgi, M. Shiffman, R. Reindollar, Z. D. Goodman, K. Koury, M. Ling, and J. K. Albrecht.** 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet **358:**958–965.
20. **McHutchison, J. G., S. C. Gordon, E. R. Schiff, M. L. Shiffman, W. M. Lee, V. K. Rustgi, Z. D. Goodman, M. H. Ling, S. Cort, J. K. Albrecht, et al.** 1998. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N. Engl. J. Med. **339:**1485–1492.
21. **Miyamoto, M., T. Kato, T. Date, M. Mizokami, and T. Wakita.** 2006. Comparison between subgenomic replicons of hepatitis C virus genotypes 2a (JFH-1) and 1b (Con1 NK5.1). Intervirology **49:**37–43.
22. **Nakagawa, M., N. Sakamoto, N. Enomoto, Y. Tanabe, N. Kanazawa, T. Koyama, M. Kurosaki, S. Maekawa, T. Yamashiro, C. H. Chen, Y. Itsui, S. Kakinuma, and M. Watanabe.** 2004. Specific inhibition of hepatitis C virus replication by cyclosporin A. Biochem. Biophys. Res. Commun. **313:**42–47.
23. **Ohno, O., M. Mizokami, R. R. Wu, M. G. Saleh, K. Ohba, E. Orito, M. Mukaide, R. Williams, and J. Y. Lau.** 1997. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. J. Clin. Microbiol. **35:**201–207.
24. **Sokolskaja, E., D. M. Sayah, and J. Luban.** 2004. Target cell cyclophilin A modulates human immunodeficiency virus type 1 infectivity. J. Virol. **78:**12800–12808.
25. **Taylor, D. R., S. T. Shi, P. R. Romano, G. N. Barber, and M. M. Lai.** 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science **285:**107–110.
26. **Tellinghuisen, T. L., and C. M. Rice.** 2002. Interaction between hepatitis C virus proteins and host cell factors. Curr. Opin. Microbiol. **5:**419–427.
27. **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.
28. **Waldmeier, P. C., K. Zimmermann, T. Qian, M. Tintelnot-Blomley, and J. J. Lemasters.** 2003. Cyclophilin D as a drug target. Curr. Med. Chem. **10:**1485–1506.
29. **Watashi, K., M. Hijikata, M. Hosaka, M. Yamaji, and K. Shimotohno.** 2003. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. Hepatology **38:**1282–1288.
30. **Watashi, K., M. Hijikata, A. Tagawa, T. Doi, H. Marusawa, and K. Shimotohno.** 2003. Modulation of retinoid signaling by a cytoplasmic viral protein via sequestration of Sp110b, a potent transcriptional corepressor of retinoic acid receptor, from the nucleus. Mol. Cell. Biol. **23:**7498–7509.
31. **Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno.** 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol. Cell **19:**111–122.
32. **Watashi, K., and K. Shimotohno.** 2003. The roles of hepatitis C virus proteins in modulation of cellular functions: a novel action mechanism of the HCV core protein on gene regulation by nuclear hormone receptors. Cancer Sci. **94:**937–943.
33. **Wiegers, K., and H. G. Krausslich.** 2002. Differential dependence of the infectivity of HIV-1 group O isolates on the cellular protein cyclophilin A. Virology **294:**289–295.
34. **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.