

FIG. 6. Genome mutations of HCV secreted from H751JFH1/Zeo cells. (A) RT-PCR of HCV genome extracted from the culture supernatant of H751JFH1/Zeo cells. Viral RNA sequences were reverse transcribed [RT ( + )] or not [RT ( - )], followed by amplification with primer pairs encompassing the specified HCV genome regions. (B) Comparison of the genome mutations of HCV secreted from H751JFH1/Zeo cells cultured for 120 days (upper panel) and JFH-1 RNA-transfected cells cultured for 36 days (lower panel). The positions of original (wild-type) and mutated (day 120, day 36) nucleotides are indicated under the schematic diagram of the HCV genome. Amino acid residues and their positions are marked in parentheses. Del, deletion.

SGR-Luc and pCAG/C-NS2, with an 10-fold increase in activity observed at 2 to 5 days p.t. In contrast, luciferase signal in the Huh7.5.1 cells inoculated from supernatant of cells transfected with pHH/SGR-Luc with polymerase-deficient mutation (GND) showed background levels. There was a faint luciferase signal in the cells inoculated from supernatant of cells transfected with pHH/SGR-Luc in the absence of pCAG/C-NS2, suggesting carryover of a low level of cells with the supernatants. Transfer of supernatant from infected cells to naive Huh7.5.1 cells did not result in infection, as judged by undetectable luciferase activity (data not shown). To examine whether NS2 is important for HCV production as previously demonstrated (17–19, 52), we compared the expression of core-NS2 versus core-p7 in the packaged cells (Fig. 8C). The reporter activity in cells inoculated with virus trans-packaged by core-p7 was 100-fold lower than the virus trans-packaged by core-NS2, indicating that NS2 needs to be expressed with the structural proteins for efficient assembly and/or infectivity. CD81-dependent infection of HCV-LP was further confirmed by demonstrating reduced reporter activity in the presence of anti-CD81 antibody (Fig. 8D). Thus, we developed a simple trans-encapsulation system based on transient two-plasmid transfection which permits experimental separation of HCV genome replication and virion assembly.

DISCUSSION

Here, we exploited Pol I-derived vectors for expression of the HCV genome, a strategy that generates viral RNAs from the Pol I promoter and terminator. We demonstrated that the HCV JFH-1 RNA produced using this system is unspliced with precise sequences at both ends and that it is replicated in the cytoplasm of transfected cells to produce infectious particles. This approach was used to establish a replicon trans-packaging system based on transient two-plasmid transfection and enables the production of a stable cell line capable of constitutive HCV production. The cell line produced using this method can be used to screen a large number of potential antiviral agents by assessing their ability to interfere with HCV replication and/or virion formation. The Pol I-mediated transcription system was originally developed to perform reverse genetics on influenza A viruses (12, 29) which replicate in the nucleus. This system has also been shown useful in the development of reverse genetics for negative-strand RNA viruses having a cytoplasmic replication cycle (3, 10, 11, 31). The results of the present study suggest that the Pol I system can also be used to perform reverse genetics on a cytoplasmically replicating positive-strand RNA virus.

Although viral RNA transfection by electroporation is the most commonly used method to perform reverse genetics on

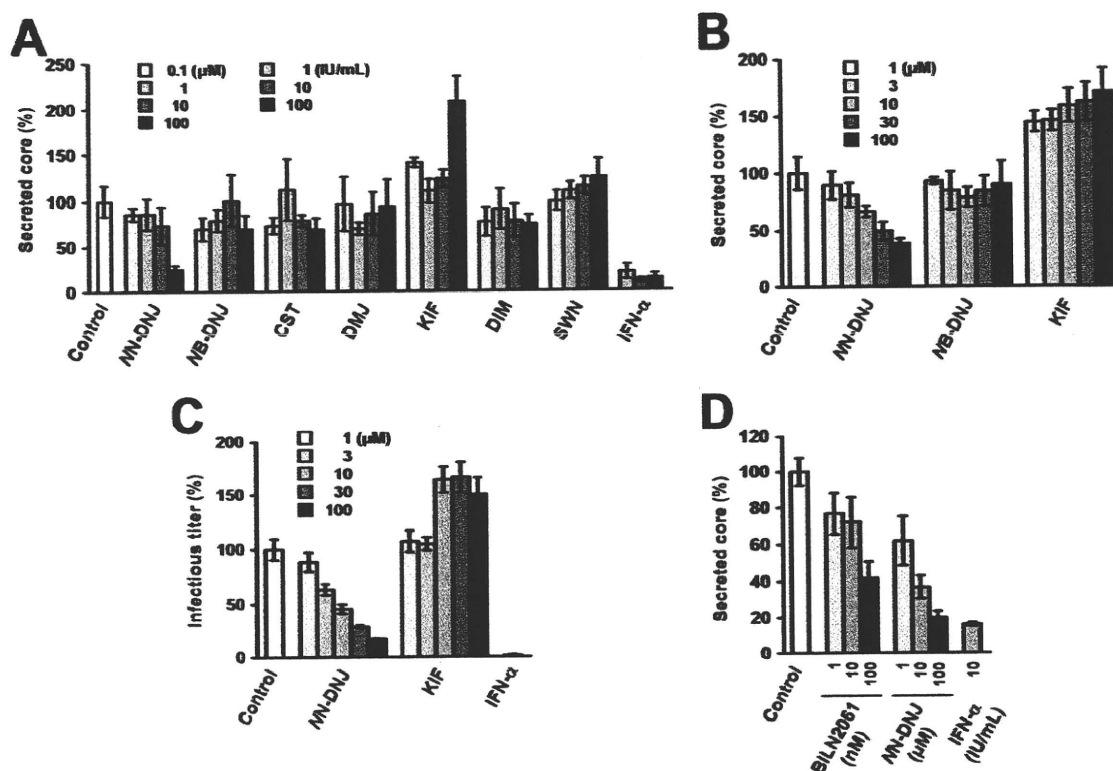


FIG. 7. Effects of glycosylation inhibitors on HCV production from H751JFH1/Zeo cells. (A and B) Effects of glycosylation inhibitors on the secretion of HCV core protein. H751JFH1/Zeo cells were seeded at a density of  $1 \times 10^4$  cells/well in a 96-well culture plate (A) or  $3 \times 10^4$  cells/well in a 12-well cell culture plate (B). One day later, each compound was added to the cell culture supernatant at the indicated concentrations. The culture supernatant was collected after a further 3-day culture and processed by core protein-specific ELISA. The control represents an untreated cell culture. The level of secreted core protein was normalized by setting the control value at 100%. The data for each experiment are averages of triplicate values with error bars showing standard deviations. (C) Effects of NN-DNJ and KIF on infectious HCV production. The culture supernatant obtained in panel B was used to infect naive Huh7.5.1 cells. At 72 h after infection, the inoculated cells were fixed and immunostained as described in Materials and Methods for titration of virus infectivity. The infectious titer was normalized by setting the control value at 100%. Cells were treated with IFN- $\alpha$  at 100 IU/ml as a positive control. The data for each experiment are averages of triplicate values with error bars showing standard deviations. The control represents an untreated cell culture. (D) After 1 year of culturing H751JFH1/Zeo cells, antiviral effects of NN-DNJ and BILN 2061 were evaluated. H751JFH1/Zeo cells were seeded at a density of  $3 \times 10^4$  cells/well in a 12-well cell culture plate. One day later, each compound was added to the cell culture supernatant at the indicated concentrations. The culture supernatant was collected after a further 3-day culture and processed by core protein-specific ELISA. The control represents an untreated cell culture. The level of secreted core protein was normalized by setting the control value at 100%. The data for each experiment are averages of triplicate values with error bars showing standard deviations.

HCV (23, 49, 53), it is comparatively difficult to manipulate. RNA electroporation requires high-quality in vitro-synthesized RNA and a large quantity of exponential-growth-phase cells, which may be hard to provide when a number of different RNA constructs are being examined in the same experiment. In addition to the Pol I system, other DNA expression systems have been examined with regard to HCV particle production (5, 15, 21). These systems require ribozyme sequences to be inserted at either end of the HCV genomic cDNA sequence in order to generate appropriately processed viral RNA. However, Heller et al. have reported that the HCV RNA generated by in vitro transcription of a HCV-ribozyme plasmid contains uncleaved or prematurely terminated forms of HCV RNA. These authors have also demonstrated that HCV RNA from the culture supernatant of HCV-ribozyme plasmid-transfected cells possesses nucleotide changes at the 5' and 3' ends (15), suggesting that the ribozyme is less reliable at generating cor-

rect transcripts compared to our Pol I system. In fact, there is evidence to suggest that a mouse Pol I terminator is significantly more effective than an HDV ribozyme in generating precise 3' ends of RNA, as demonstrated in a plasmid-based influenza virus rescue system (9). Recently, it has been demonstrated that Pol I-catalyzed rRNA transcription is activated in Huh-7 cells following infection with JFH-1 or transfection with a subgenomic HCV replicon (34). HCV NS5A has been shown to upregulate the transcription of Pol I, but not Pol II, through phosphorylation of an upstream binding factor, a Pol I DNA binding transcription factor. These observations indicate that a Pol I-mediated expression system is suitable for efficient production of infectious HCV by DNA transfection.

We established a stable cell line, H751JFH1/Zeo, that constitutively and efficiently produced infectious HCV particles by introducing a Pol I-driven plasmid containing a selection marker into Huh7.5.1 cells. Interestingly, the established cell

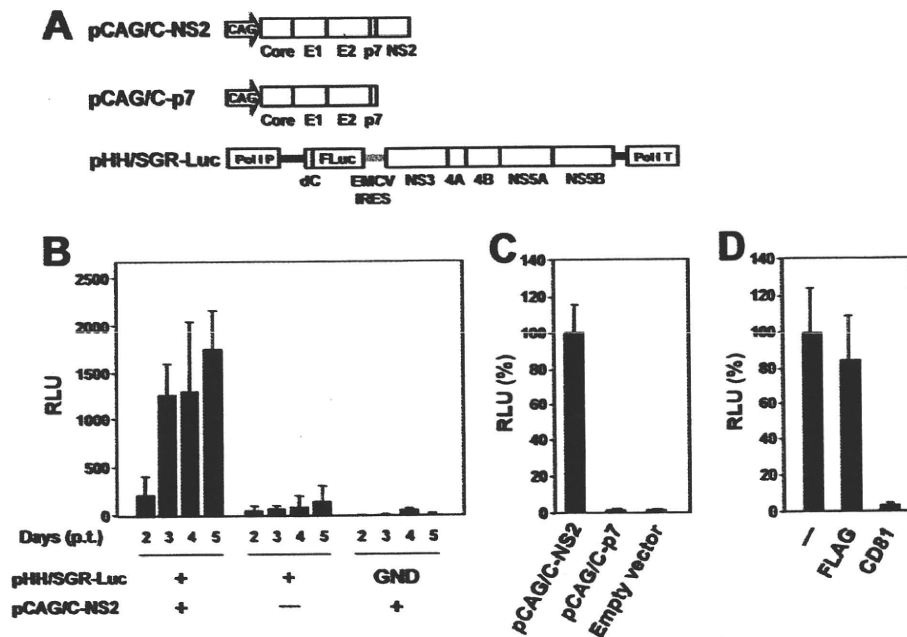


FIG. 8. Establishment of a trans-packaging system involving two-plasmid transfection. (A) Schematic representation of the plasmids used for the production of HCV-LP. HCV polyproteins are indicated by the open boxes. Bold lines indicate the HCV UTR. EMCV IRES is denoted by gray bars. The firefly luciferase gene (F Luc) is depicted as a gray box. CAG, CAG promoter; Pol I P, Pol I promoter; dC, 5' region of Core gene; Pol I T, Pol I terminator. (B) Luciferase activity in Huh7.5.1 cells inoculated with culture supernatant from cells transfected with the indicated plasmids. Luciferase activity is expressed in terms of relative luciferase units (RLU). The data for each experiment are averages of triplicate values with error bars showing standard deviations. (C) Culture supernatant from cells cotransfected with pHH/SGR-Luc and the indicated plasmids was collected 4 days p.i. The luciferase activity in Huh7.5.1 cells inoculated with culture supernatant was determined 3 days postinoculation and expressed as relative luciferase units (RLU). The RLU was normalized according to the luciferase activity observed in the pCAG/C-NS2-transfected sample (C-NS2), which was set at 100%. The data for each experiment are averages of triplicate values with error bars showing standard deviations. (D) Huh7.5.1 cells were inoculated with HCVLP in the absence ( / ) or presence of 5  $\mu$ g of anti-CD81 or anti-FLAG antibody/ml. The luciferase activity was determined 72 h postinoculation and is expressed as relative luciferase units (RLU). The RLU was normalized to the level of luciferase activity observed in the antibody-untreated sample ( / ), which was set at 100%. The data for each experiment are averages of triplicate values with error bars showing standard deviations.

clones exhibited little to no surface expression of CD81, one of the key features of HCV glycoprotein-mediated infection (Fig. 5). Defective expression of receptor molecules might be advantageous in generating stable cell lines for robust production of HCV. HCV-induced cytotoxicity has been reported (7, 45, 54). Persistent HCV infection was established after electroporation of JFH-1 genomic RNA, and a variable cytopathic effect was observed at the peak of acute HCV infection, as well as during the persistent phase of infection (54). A recent study has demonstrated that the cytopathic effect triggered by HCV RNA transfection and viral infection is characterized by massive apoptotic cell death with expression of several ER stress markers, such as GRP78 and phosphorylated eIF2- (39). Therefore, in the present study, it is likely that selective forces to evade cell death during high levels of HCV replication produced cell populations resistant to virus infection. As a consequence, H751JFH1/Zeo cells maintained robust production of infectious HCV particles over a long period of time without gross cytopathic effects or changes in cell morphology.

Substantial evidence demonstrates that the mutation rate of the HCV genome produced in H751JFH1/Zeo was low (Fig. 6) presumably because of consistent expression of wild-type HCV RNA from the chromosomally integrated gene. Nevertheless, a considerable proportion of the genome was mutated, with

two nonsynonymous mutations in the E1 (V293A) and E2 (I414T) regions identified in the culture supernatant of H751JFH1/Zeo cells after 4 months of passages (Fig. 6). A I414T mutation has also been reported after long-term propagation of HCV in culture after JFH-1-RNA transfection (54). This mutation is located between the hypervariable regions 1 and 2 within the N terminus of E2 (51). Adaptive mutations in this region have been shown to enhance virus expansion, presumably by enabling more efficient virus entry (6, 36, 54). A possible CD81-independent mechanism for cell-to-cell transmission of HCV has been proposed (48, 50). However, the mechanisms governing cell-to-cell spread of HCV are not well understood. Further investigation into the importance of envelope protein mutations in HCV transmission independent of CD81 provide a better understanding of the complex interactions required for HCV infection.

In the present study we assessed the effects of N-linked glycosylation inhibitors on HCV production using H751JFH1/Zeo (Fig. 7) and found that an  $\alpha$ -glucosidase inhibitor NN-DNJ inhibits the production of infectious HCV, which has also been observed in previous studies (43, 47). In contrast, HCV production is increased in the presence of an ER  $\alpha$ -mannosidase inhibitor KIF, but not in the presence of the Golgi  $\alpha$ -mannosidase inhibitors DMJ, DIM, and SWN. KIF inhibits  $\alpha$ -man-

nosidase I, which primarily functions to remove the middle mannose branch from  $\text{Man}_9\text{GlcNAc}_2$  to form  $\text{Man}_8\text{GlcNAc}_2$  after the removal of glucose residues by glucosidases I and II (8, 24). Experiments to elucidate the role of mannose trimming of N-glycans in the HCV life cycle are currently under way.

It has recently been demonstrated that subgenomic replicons or defective genomes of HCV that have the potential of translation and self-replication can be encapsidated into infectious viruslike particles by trans-complementation of the viral structural proteins (1, 17, 32, 41, 44). In these studies, the viral RNAs were generally generated by in vitro transcription from linearized corresponding plasmids, followed by electroporation into the cells. Structural proteins or Core to NS2 proteins were then provided by DNA or RNA transfection, viral-vector-based transduction, or stable packaging cell lines established. Here, we achieved the replicon trans-encapsidation via transient cotransfection with two DNA plasmids. This system, which is apparently easier to manipulate and allows production of trans-encapsidated materials more rapidly compared to the systems published, can be applied to the study for understanding phenomenon and biological significance of a variety of naturally occurring HCV subgenomic deletion variants that possibly circulate in hepatitis C patients.

In summary, we have established a Pol I-based reverse-genetics system for the efficient production of infectious HCV. This methodology can be applied to develop (i) a stable HCV-producing cell line with a low mutation frequency of the viral genome and (ii) a simple trans-encapsidation system with the flexibility of genome packaging and improved biosafety. This may be useful for antiviral screening and may assist in the development of a live-attenuated HCV vaccine.

#### ACKNOWLEDGMENTS

We are grateful to Francis V. Chisari (The Scripps Research Institute) for providing Huh7.5.1 cells and to Y. Kawaoka (School of Veterinary Medicine, University of Wisconsin-Madison) for providing the pH21 vector. We thank A. Murayama and T. Date for their help in sequence and Northern blot analyses and our coworkers for their helpful discussions. We also thank S. Yoshizaki, T. Shimoji, M. Kaga, and M. Sasaki for their technical assistance and T. Mizoguchi for secretarial work.

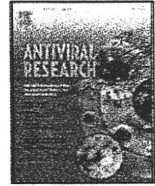
This study was supported by grants-in-aid from the Ministry of Health, Labor, and Welfare; by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, R&D Promotion, and Product Review of Japan (01-3); and by Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation, Japan.

#### REFERENCES

- Adair, R., A. H. Patel, L. Corless, S. Griffin, D. J. Rowlands, and C. J. McCormick. 2009. Expression of hepatitis C virus (HCV) structural proteins in trans facilitates encapsidation and transmission of HCV subgenomic RNA. *J. Gen. Virol.* 90:833–842.
- Akazawa, D., T. Date, K. Morikawa, A. Murayama, M. Miyamoto, M. Kaga, H. Barth, T. F. Baumert, J. Dubuisson, and T. Wakita. 2007. CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. *J. Virol.* 81:5036–5045.
- Billecocq, A., N. Gaudin, N. Le May, R. M. Elliott, R. Flick, and M. Bouloy. 2008. RNA polymerase I-mediated expression of viral RNA for the rescue of infectious virulent and avirulent Rift Valley fever viruses. *Virology* 378:377–384.
- Cabral, C. M., P. Choudhury, Y. Liu, and R. N. Sifers. 2000. Processing by endoplasmic reticulum mannosidases partitions a secretion-impaired glycoprotein into distinct disposal pathways. *J. Biol. Chem.* 275:25015–25022.
- Cai, Z., C. Zhang, K. S. Chang, J. Jiang, B. C. Ahn, T. Wakita, T. J. Liang, and G. Luo. 2005. Robust production of infectious hepatitis C virus (HCV) from stably HCV cDNA-transfected human hepatoma cells. *J. Virol.* 79:13963–13973.
- Delgrange, D., A. Pillez, S. Castelain, L. Cocquerel, Y. Rouille, J. Dubuisson, T. Wakita, G. Duverlie, and C. Wychowski. 2007. Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins. *J. Gen. Virol.* 88:2495–2503.
- Deng, L., T. Adachi, K. Kitayama, Y. Bungyoku, S. Kitazawa, S. Ishido, I. Shoji, and H. Hotta. 2008. Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. *J. Virol.* 82:10375–10385.
- Elgaard, L., M. Molinari, and A. Helenius. 1999. Setting the standards: quality control in the secretory pathway. *Science* 286:1882–1888.
- Feng, L., F. Li, X. Zheng, W. Pan, K. Zhou, Y. Liu, H. He, and L. Chen. 2009. The mouse Pol I terminator is more efficient than the hepatitis delta virus ribozyme in generating influenza-virus-like RNAs with precise 3' ends in a plasmid-only-based virus rescue system. *Arch. Virol.* 154:1151–1156.
- Flick, R., K. Flick, H. Feldmann, and F. Elgh. 2003. Reverse genetics for Crimean-Congo hemorrhagic fever virus. *J. Virol.* 77:5997–6006.
- Flick, R., and R. F. Pettersson. 2001. Reverse genetics system for *Unkiunemi* virus (Bunyaviridae): RNA polymerase I-catalyzed expression of chimeric viral RNAs. *J. Virol.* 75:1643–1655.
- Fodor, E., L. Devenish, O. G. Engelhardt, P. Palese, G. G. Brownlee, and A. Garcia-Sastre. 1999. Rescue of influenza A virus from recombinant DNA. *J. Virol.* 73:9679–9682.
- Groseth, A., H. Feldmann, S. Theriault, G. Mehmetoglu, and R. Flick. 2005. RNA polymerase I-driven minigenome system for Ebola viruses. *J. Virol.* 79:4425–4433.
- Hamamoto, I., Y. Nishimura, T. Okamoto, H. Aizaki, M. Liu, Y. Mori, T. Abe, T. Suzuki, M. M. Lai, T. Miyamura, K. Moriishi, and Y. Matsuura. 2005. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. *J. Virol.* 79:13473–13482.
- 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. U. S. A.* 102:2579–2583.
- Hoofnagle, J. H. 2002. Course and outcome of hepatitis C. *Hepatology* 36:S21–S29.
- Ishii, K., K. Murakami, S. S. Hmwe, B. Zhang, J. Li, M. Shirakura, K. Morikawa, R. Suzuki, T. Miyamura, T. Wakita, and T. Suzuki. 2008. Trans-encapsidation of hepatitis C virus subgenomic replicon RNA with viral structure proteins. *Biochem. Biophys. Res. Commun.* 371:446–450.
- Jirasko, V., R. Montserret, N. Appel, A. Janvier, L. Eustachi, C. Brohm, E. Steinmann, T. Pietschmann, F. Penin, and R. Bartenschlager. 2008. Structural and functional characterization of nonstructural protein 2 for its role in hepatitis C virus assembly. *J. Biol. Chem.* 283:28546–28562.
- Jones, C. T., C. L. Murray, D. K. Eastman, J. Tassello, and C. M. Rice. 2007. Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. *J. Virol.* 81:8374–8383.
- 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.
- Kato, T., T. Matsumura, T. Heller, S. Saito, R. K. Sapp, K. Murthy, T. Wakita, and T. J. Liang. 2007. Production of infectious hepatitis C virus of various genotypes in cell cultures. *J. Virol.* 81:4405–4411.
- Liang, T. J., B. Rehmann, L. B. Seef, and J. H. Hoofnagle. 2000. Pathogenesis, natural history, treatment, and prevention of hepatitis C. *Ann. Intern. Med.* 132:296–305.
- 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.
- Liu, Y., P. Choudhury, C. M. Cabral, and R. N. Sifers. 1999. Oligosaccharide modification in the early secretory pathway directs the selection of a misfolded glycoprotein for degradation by the proteasome. *J. Biol. Chem.* 274:5861–5867.
- Manns, M. P., H. Wedemeyer, and M. Cornberg. 2006. Treating viral hepatitis C: efficacy, side effects, and complications. *Gut* 55:1350–1359.
- Masaki, T., R. Suzuki, K. Murakami, H. Aizaki, K. Ishii, A. Murayama, T. Date, Y. Matsuura, T. Miyamura, T. Wakita, and T. Suzuki. 2008. Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles. *J. Virol.* 82:7964–7976.
- Meusser, B., C. Hirsch, E. Jarosch, and T. Sommer. 2005. ERAD: the long road to destruction. *Nat. Cell Biol.* 7:766–772.
- Neumann, G., and Y. Kawaoka. 2001. Reverse genetics of influenza virus. *Virology* 287:243–250.
- Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, G. Hobom, and Y. Kawaoka. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. U. S. A.* 96:9345–9350.
- Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193–199.
- Ogawa, Y., K. Sugiyama, K. Kato, Y. Tohya, and H. Akashi. 2007. Rescue of



- Akabane virus (family Bunyaviridae) entirely from cloned cDNAs by using RNA polymerase I. *J. Gen. Virol.* **88**:3385–3390.
32. Pacini, L., R. Graziani, L. Bartholomew, R. De Francesco, and G. Paonessa. 2009. Naturally occurring hepatitis C virus subgenomic deletion mutants replicate efficiently in Huh-7 cells and are trans-packaged in vitro to generate infectious defective particles. *J. Virol.* **83**:9079–9093.
  33. Poyndar, T., M. F. Yuen, V. Ratzl, and C. L. Lai. 2003. Viral hepatitis C. *Lancet* **362**:2095–2100.
  34. Raychaudhuri, S., V. Fontanes, B. Barat, and A. Dasgupta. 2009. Activation of rRNA transcription by hepatitis C virus involves upstream binding factor phosphorylation via induction of cyclin D1. *Cancer Res.* **69**:2057–2064.
  35. Ruddock, L. W., and M. Molinari. 2006. N-glycan processing in ER quality control. *J. Cell Sci.* **119**:4373–4380.
  36. Russell, R. S., J. C. Meunier, S. Takikawa, K. Faulk, R. E. Engle, J. Bukh, R. H. Purcell, and S. U. Emerson. 2008. Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus. *Proc. Natl. Acad. Sci. U. S. A.* **105**:4370–4375.
  37. Seeff, L. B., and J. H. Hoofnagle. 2003. Appendix: National Institutes of Health Consensus Development Conference Management of Hepatitis C 2002. *Clin. Liver Dis.* **7**:261–287.
  38. Seeff, L. B., and J. H. Hoofnagle. 2002. National Institutes of Health Consensus Development Conference: management of hepatitis C: 2002. *Hepatology* **36**:S1–S2.
  39. Sekine-Osajima, Y., N. Sakamoto, K. Mishima, M. Nakagawa, Y. Itsui, M. Tasaka, Y. Nishimura-Sakurai, C. H. Chen, T. Kanai, K. Tsuchiya, T. Wakita, N. Enomoto, and M. Watanabe. 2008. Development of plaque assays for hepatitis C virus-JFH1 strain and isolation of mutants with enhanced cytopathogenicity and replication capacity. *Virology* **371**:71–85.
  40. Shi, S. T., K. J. Lee, H. Aizaki, S. B. Hwang, and M. M. Lai. 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. *J. Virol.* **77**:4160–4168.
  41. Steinmann, E., C. Brohm, S. Kallis, R. Bartenschlager, and T. Pietschmann. 2008. Efficient trans-encapsidation of hepatitis C virus RNAs into infectious virus-like particles. *J. Virol.* **82**:7034–7046.
  42. Steinmann, E., F. Penin, S. Kallis, A. H. Patel, R. Bartenschlager, and T. Pietschmann. 2007. Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. *PLoS Pathog.* **3**:e103.
  43. Steinmann, E., T. Whitfield, S. Kallis, R. A. Dwek, N. Zitzmann, T. Pietschmann, and R. Bartenschlager. 2007. Antiviral effects of amantadine and iminosugar derivatives against hepatitis C virus. *Hepatology* **46**:330–338.
  44. Sugiyama, K., K. Suzuki, T. Nakazawa, K. Funami, T. Hishiki, K. Ogawa, S. Saito, K. W. Shimotohno, T. Suzuki, Y. Shimizu, R. Tobita, M. Hijikata, H. Takaku, and K. Shimotohno. 2009. Genetic analysis of hepatitis C virus with defective genome and its infectivity in vitro. *J. Virol.* **83**:6922–6928.
  45. Sung, V. M., S. Shimodaira, A. L. Doughty, G. R. Picchio, H. Can, T. S. Yen, K. L. Lindsay, A. M. Levine, and M. M. Lai. 2003. Establishment of B-cell lymphoma cell lines persistently infected with hepatitis C virus in vivo and in vitro: the apoptotic effects of virus infection. *J. Virol.* **77**:2134–2146.
  46. Suzuki, T., K. Ishii, H. Aizaki, and T. Wakita. 2007. Hepatitis C viral life cycle. *Adv. Drug Deliv. Rev.* **59**:1200–1212.
  47. Tani, H., Y. Komoda, E. Matsuo, K. Suzuki, I. Hamamoto, T. Yamashita, K. Moriishi, K. Fujiyama, T. Kanto, N. Hayashi, A. Owsianka, A. H. Patel, M. A. Whitt, and Y. Matsuura. 2007. Replication-competent recombinant vesicular stomatitis virus encoding hepatitis C virus envelope proteins. *J. Virol.* **81**:8601–8612.
  48. Timpe, J. M., Z. Stamataki, A. Jennings, K. Hu, M. J. Farquhar, H. J. Harris, A. Schwarz, I. Desombere, G. L. Roels, P. Balfe, and J. A. McKeating. 2008. Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. *Hepatology* **47**:17–24.
  49. 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.
  50. Witteveldt, J., M. J. Evans, J. Bitzegeio, G. Koutsoudakis, A. M. Owsianka, A. G. Angus, Z. Y. Keck, S. K. Fong, T. Pietschmann, C. M. Rice, and A. H. Patel. 2009. CD81 is dispensable for hepatitis C virus cell-to-cell transmission in hepatoma cells. *J. Gen. Virol.* **90**:48–58.
  51. Yagnik, A. T., A. Lahm, A. Meola, R. M. Roccasecca, B. B. Ercole, A. Nicosia, and A. Tramontano. 2000. A model for the hepatitis C virus envelope glycoprotein E2. *Proteins* **40**:355–366.
  52. Yi, M., Y. Ma, J. Yates, and S. M. Lemon. 2009. Trans-complementation of an NS2 defect in a late step in hepatitis C virus (HCV) particle assembly and maturation. *PLoS Pathog.* **5**:e1000403.
  53. 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. U. S. A.* **102**:9294–9299.
  54. Zhong, J., P. Gastaminza, J. Chung, Z. Stamataki, M. Isogawa, G. Cheng, J. A. McKeating, and F. V. Chisari. 2006. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. *J. Virol.* **80**:11082–11093.



## Identification of hepatitis C virus genotype 2a replicon variants with reduced susceptibility to ribavirin

Su Su Hmwe<sup>a,b</sup>, Hideki Aizaki<sup>a</sup>, Tomoko Date<sup>a</sup>, Kyoko Murakami<sup>a</sup>, Koji Ishii<sup>a</sup>, Tatsuo Miyamura<sup>a</sup>, Kazuhiko Koike<sup>b</sup>, Takaji Wakita<sup>a</sup>, Tetsuro Suzuki<sup>a</sup>

<sup>a</sup> Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

<sup>b</sup> Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

### article info

#### Article history:

Received 8 April 2009

Received in revised form 19 October 2009

Accepted 18 December 2009

#### Keywords:

Hepatitis C virus

Replication

Ribavirin

Drug resistance

### abstract

Ribavirin (RBV), a nucleoside analogue, is used in the treatment of hepatitis C virus (HCV) infection in combination with interferons. However, potential mechanisms of RBV resistance during HCV replication remain poorly understood. Serial passage of cells harboring HCV genotype 2a replicon in the presence of RBV resulted in the reduced susceptibility of the replicon to RBV. Transfection of fresh cells with RNA from RBV-resistant replicon cells demonstrated that the RBV resistance observed is largely replicon-derived. Four major amino acid substitutions: T1134S in NS3, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B region, were identified. Site-directed mutagenesis of these mutations into the replicon indicated that Y2471H plays a role in the reduced susceptibility to RBV and leads to decrease in replication fitness. The results, in addition to analysis of sequence database, suggest that HCV variants with reduced susceptibility to RBV identified are preferential to genotype 2a.

© 2010 Elsevier B.V. All rights reserved.

### 1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases, such as chronic hepatitis, cirrhosis and hepatocellular carcinoma, affecting approximately 170 million people worldwide (WHO, 2000). HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae*, and its genome is a single-stranded, positive-sense RNA of 9.6 kb. HCV displays marked genetic heterogeneity and is currently classified into 6 major genotypes and more than 50 subtypes. HCV genotypes have regional distribution and, of those, genotypes 1 and 2 are detected worldwide (Simmonds et al., 2000). Current standard therapy for chronic hepatitis C consists of the combination of pegylated interferon alpha (IFN- $\alpha$ ) in combination with ribavirin (RBV). However, approximately 50% of treated patients infected with genotype 1 do not respond or show only a partial or transient response and treatment is limited by the adverse effects of both agents (Manns et al., 2001; Fried et al., 2002).

HCV replication is associated with a high rate of mutation that gives rise to a mixed and changing population of mutants, known as quasispecies (Martell et al., 1992; Domingo, 1996). The characteristic of HCV may have important implications concerning viral persistence, pathogenicity and resistance to antiviral agents

(Domingo, 1996; Forns et al., 1999; Farci and Purcell, 2000). Most previous studies on the possible relationship between HCV quasispecies and response to chemotherapy have been carried out in HCV genotype 1 patients. In addition, several studies have successfully demonstrated that the HCV subgenomic replicon is derived from genotype 1, which typically contains HCV nonstructural genes placed downstream of the neomycin phosphotransferase gene, in selecting variants resistant to antiviral inhibitors. Two studies have demonstrated the identification of HCV genotype 1 mutants responsible for decreased sensitivity to RBV (Young et al., 2003; Pfeiffer and Kirkegaard, 2005). However, little is known about the generation of genotype 2 isolates resistant to antivirals including RBV, or the molecular mechanisms that confer resistance.

In this study, we report the generation and characterization of HCV genotype 2a replicon variants with reduced susceptibility to RBV. The impacts of major amino acid substitutions observed on RBV susceptibility and viral replication capacity were also examined.

### 2. Materials and methods

#### 2.1. Compounds

RBV and IFN- $\alpha$  were purchased from MP Biomedicals (Eschwege, Germany) and Daiinippon Sumitomo Pharma (Osaka, Japan), respectively.

Corresponding author. Tel.: +81 3 5285 1111; fax: +81 3 5285 1161.  
E-mail address: [tesuzuki@nih.go.jp](mailto:tesuzuki@nih.go.jp) (T. Suzuki).

Table 1  
Primers used for PCR and nucleotide sequencing.

Region	Primer name	Nucleotide sequence	Position <sup>a</sup>	Polarity
NS3–4A–4B region	PCR primers			
	JF1S	GAAAAACACGATGATACCATG	1756–1776	Sense
	JF1AS	AADCCAGTCCACACGTC	4650–4633	Antisense
	Sequencing primers			
	JF5S	CACTTTCAGTGACAACAGCA	2322–2341	Sense
	JF6S	CGCCACCGACGCGCTCATGA	3003–3022	Sense
NS5A–NS5B region	PCR primers			
	JF2S	TGCTCCGGATCCTGGCTC	4612–4629	Sense
	JF2AS	TACCTAGTGTGTGCGCTCTA	7786–7806	Antisense
	Sequencing primers			
	JF3S	TGAGGTCCATGCTAACAGA	5208–5228	Sense
	JF4S	TCGAGGGGGAGCGCTGGAGAT	5870–5889	Sense
	JF3AS	GAGTGTCTAACTGTTTCCACG	7220–7200	Antisense

<sup>a</sup> Reference strain: Gene Bank accession no. AB114136.

## 2.2. Cell culture

The human hepatoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with MEM non-essential amino acids (Invitrogen) 100 units/ml penicillin, 100 g/ml streptomycin, and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO<sub>2</sub> incubator. HCV replicon cells JFH-1/4-1 (Miyamoto et al., 2006), which are Huh-7-derived cells carrying a subgenomic replicon of JFH-1 (Kato et al., 2003) were maintained in the Huh-7 medium as above, supplemented with 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan).

## 2.3. Quantification of HCV RNA

Total RNA was isolated from harvested cells using Trizol (Invitrogen). Copy numbers of the viral RNA were determined by real-time RT-PCR involving single-tube reactions and performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, CA, USA), as described previously (Aizaki et al., 2003; Takeuchi et al., 1999).

## 2.4. Cell viability assay

Cells were seeded at density of 5 × 10<sup>4</sup> cells/well in 24-well plates and RBV at various concentrations was added on the next day. Cultures were further incubated for 3 days at 37 °C under a humidified 5% CO<sub>2</sub> atmosphere. Cytotoxicity assay was performed by Cell Titer-GLO<sup>TM</sup> Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activities were quantified with LUMAT LB 9501 (Berthold Technologies, Bad Wilbad, Germany).

## 2.5. Isolation and nucleotide sequencing of HCV nonstructural regions from replicon-containing cells

Total cellular RNA was isolated from replicon cells with or without RBV treatment as described above. cDNA synthesis was carried out by using Super Script<sup>TM</sup> III First-Strand Synthesis System for RT-PCR (Invitrogen) with primer JF1AS for NS3/4B region and JF2AS for NS5A region. Two cDNA fragments, corresponding to NS3–NS4B and NS5A–NS5B regions, were amplified by PCR using Takara EX Taq DNA polymerase (Takara BIO, Kyoto, Japan) and specific primers (Table 1; Date et al., 2004). PCR products were subcloned into pGEM-T vector (Promega) and inserts were sequenced using QIA prep<sup>R</sup> Spin Mini Prep kit (QIAGEN, Tokyo, Japan). Nucleotide sequences were analyzed with the 3100 Avant Genetic Analyzer (PE Applied Biosystems).

## 2.6. Plasmid constructions

pSGR-JFH1/luc, a subgenomic replicon construct with luciferase reporter derived from HCV genotype 2a JFH-1 isolate was reported previously (Miyamoto et al., 2006). Mutant replicons carrying T1134S, P1969S, V2405A, and Y2471H were created by PCR-based site-directed mutagenesis and cDNA fragments containing the above mutations were inserted into the corresponding sites of pSGR-JFH1/luc. All plasmids were confirmed by sequencing the entire PCR-generated inserts. Each mutant is referred to by the original amino acid (one letter code) followed by the residue positions within the complete open reading frame of full-length JFH-1 and the substituted amino acid (one letter code).

## 2.7. RNA synthesis and transient replication assay

The transient replication assay method was described previously (Kato et al., 2005). Briefly, purified plasmids of pSGR-JFH1/luc, -JFH1/luc-T1134S, -JFH1/luc-P1969S, -JFH1/luc-V2405A and -JFH1/luc-Y2471H were linearized with XbaI and were treated with proteinase K and SDS, followed by phenol–chloroform extraction. RNA was synthesized with Ampliscribe<sup>TM</sup> T7 Transcription Kits (Epicentre BIO Technologies, Madison, WI, USA). Each transcribed RNA (5 g) was electroporated into 2.5 × 10<sup>6</sup> of Huh7 cells pulsed at 290 mV, 975 μFD with Gene pulser II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Transfected cells were resuspended in growth medium without selection antibiotics and were plated in 24-well plates at 6 × 10<sup>4</sup> cells per well. Cells were harvested at different time points post-transfection and were lysed in Passive Lysis Buffer (Promega). Luciferase activity in cells was determined using the Luciferase Assay System (Promega).

## 3. Results

### 3.1. Selection of replicon variants derived from genotype 2a with reduced susceptibility to RBV

It has been reported that RBV inhibits HCV RNA replication in Huh-7 cells bearing the viral subgenomic replicon RNAs with the EC<sub>50</sub> (50% effective concentration) values of 15–225 μM (Zhou et al., 2003; Tanaka et al., 2004; Kato et al., 2005; aus dem Siepen et al., 2007). To select for RBV-associated replicon variants, cells bearing a genotype 2a HCV replicon were serially passed in the presence of 200 μM RBV as well as 1 mg/ml G418. After 20-week treatment, variant cells were then tested for RBV resistance. HCV RNA levels were determined after a 72-h incubation with various concentrations of RBV in the absence of G418, and about 5-fold-reduced susceptibility to RBV was observed in the variant replicon

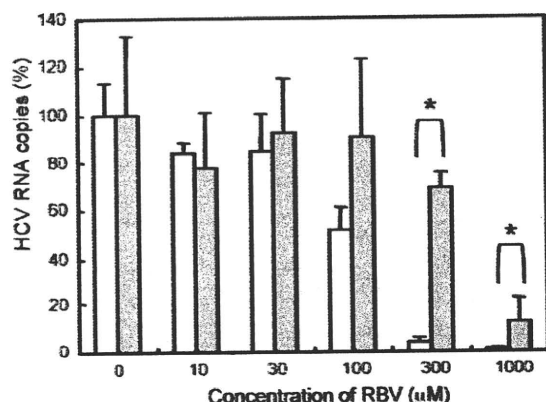


Fig. 1. Inhibitory effect of RBV on HCV RNA levels in genotype 2a replicon cells after long-term treatments with RBV. The replicon cells were serially passaged in 0 or 200  $\mu$ M RBV for 20 weeks. The cells were then split and incubated with fresh RBV at various concentrations in the absence of G418 for 3 days, followed by the determination of HCV RNA. Clear bars, passage in the absence of RBV; gray bars, passage in the presence of RBV. HCV RNA copies per microgram of total RNA were normalized as percentages of those of untreated (RBV 0  $\mu$ M). Each data point is presented as the mean of three independent determinations with standard deviation. \* $p < 0.05$ .

cells; the  $EC_{50}$  values for the variant and wild-type replicon cells were 470 and 102  $\mu$ M, respectively (Fig. 1). Comparable cytotoxic effects of RBV were observed against wild-type and variant replicon cells, with the  $CC_{50}$  (50% cytotoxicity concentration) values of 151 and 156  $\mu$ M, respectively (data not shown).

### 3.2. Mapping RBV resistance to cell line or replicon RNA

To test whether reduced susceptibility to RBV in the variant cells observed as above was due to the appearance of mutations within the viral RNA or was cell-derived, total RNAs from the variant and wild-type replicon cells were extracted and used for retransfection of naïve Huh7 cells. Retransfected cells resistant to G418 were established after 4 weeks of cultures in the presence of 1 mg/ml G418 and were assessed for HCV RNA replication sensitivity to RBV (Fig. 2A). HCV RNA levels in the cells obtained from the wild-type replicon were inhibited by 56, 89 and 97% with 100, 300 and 1000  $\mu$ M RBV, respectively. By contrast, the culture retransfected with RNA derived from the variant replicon cells exhibited inhibition levels of 13, 29 and 89% with the corresponding concentrations of RBV.  $EC_{50}$  values were calculated to be 93 and 449  $\mu$ M, respectively. We confirmed the presence of replicon mutations, as described below, in the cells retransfected with RNA derived from the variant replicon cells.

In order to explore the possibility for cell-derived resistance, both wild-type and variant replicon cells were cured of viral RNAs by IFN- $\alpha$  treatment; cells were passaged with media containing 100 IU/mL IFN- $\alpha$  in the absence of G418 for 2 months. To compare RBV sensitivity, cured cells were transiently transfected with the wild-type JFH-1 subgenomic replicon RNA and were treated with various concentrations of RBV for 72 h. Similar anti-HCV effects of RBV were observed in the cured cells derived from wild-type and variant replicons, with the  $EC_{50}$  values of 147 and 118  $\mu$ M, respectively (Fig. 2B). Thus, the results suggest that the RBV resistance observed may arise by mutations in the replicon rather than by changes in the cells.

### 3.3. HCV mutations in replicon variant with reduced susceptibility to RBV

It has been reported that mutations in RNA virus genomes responsible for RBV resistance are mostly present in the coding region for the viral RNA-dependent RNA polymerase (RdRp). On the other hand, it is known that RBV works as an RNA mutagen to generate rapidly mutating viral RNA and that NS5B RdRp and other nonstructural proteins in HCV are involved in the viral replication complex, playing key roles in genome replication. Therefore, we sequenced the coding regions for NS3 through NS5B proteins of the replicon molecules in order to determine whether mutations associated with RBV resistance were generated. As shown in Table 2, there were numerically more synonymous and non-synonymous mutations in the RBV-resistant variant replicon cells (RBV treatment) when compared with untreated replicative conditions (No-treatment) across most regions examined. Mutation frequencies of NS3, NS4B and NS5A regions of RBV treatment were significantly higher than those of No-treatment. The total number of synonymous mutations in the RBV-resistant variant replicon cells was 3 times higher than that under untreated replicative conditions, and the number of non-synonymous mutations in the RBV-resistant variant replicon cells was 1.5 times higher than that under untreated replicative conditions. The number of both synonymous and non-synonymous mutations (NS3, NS4B, NS5A and NS5B regions) in the RBV-resistant replicon cells was greater than that in the control cells. We also found a large number of transition

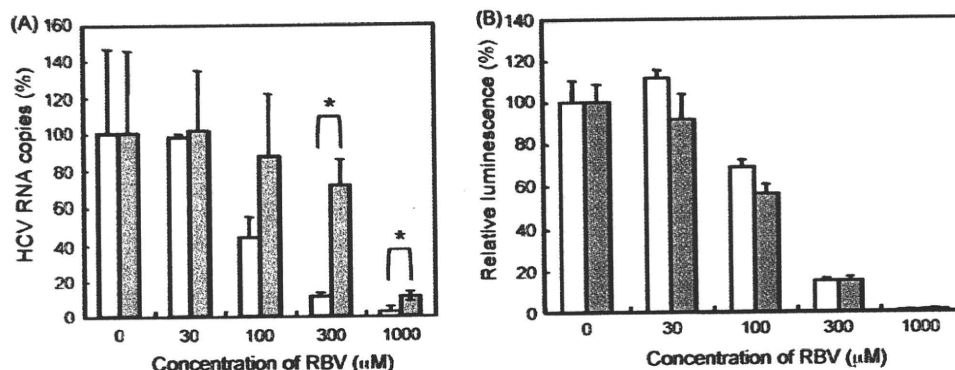


Fig. 2. Testing for replicon-derived resistance (A) or for cell-derived resistance (B). (A) Total RNA from RBV-resistant- or wild-type replicon cells was transfected into naïve Huh7 cells. After selection in 1 mg/ml G418 for 4 weeks, re-established replicon cells, wild-type derived (clear bars) and RBV resistance derived (gray bars), were treated with increasing concentrations of RBV in the absence of G418 for 3 days. HCV RNA copies per microgram total RNA were assessed and the levels from wild-type cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations. \* $p < 0.05$ . (B) RBV-resistant- or wild-type replicon cells were cured by passage in IFN- $\alpha$  in the absence of G418. Cured cells were transiently transfected with the replicon RNA derived from pSGR-JFH1/luc. Transient replication assay of transfectants derived from wild-type (clear bars) and RBV resistance (gray bars) was performed after treatment with various concentrations of RBV for 72 h. The values for wild-type-derived cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations.



**Table 2**  
Mutation frequencies in HCV NS regions after 20-weeks culture with or without RBV treatment.

Region	nt length	No-treatment			RBV treatment		
		No. of non-synonymous mutations <sup>a</sup>	No. of synonymous mutations <sup>a</sup>	Mutation frequency (10 <sup>-3</sup> )	No. of non-synonymous mutations <sup>a</sup>	No. of synonymous mutations <sup>a</sup>	Mutation frequency (10 <sup>-3</sup> )
NS3	1893	1.7 ± 2.1	2.3 ± 1.5	2.1	4.7 ± 2.4	6.5 ± 2.5	5.9 <sup>b</sup>
NS4A	165	1.0 ± 1.0	0.3 ± 0.6	8.1	0.3 ± 0.5	0.5 ± 0.9	4.4
NS4B	780	1.3 ± 1.2	0.3 ± 0.6	2.1	2.3 ± 1.5	2.5 ± 1.2	4.7 <sup>c</sup>
NS5A	1380	4.0 ± 1.2	2.0 ± 1.2	4.3	5.9 ± 1.2	6.2 ± 2.4	12.2 <sup>c</sup>
NS5B	1773	4.5 ± 1.5	2.3 ± 1.5	3.8	4.8 ± 1.8	4.2 ± 1.1	9.0
NS3–NS5B	5991	12.5 ± 2.7	7.3 ± 2.7	–	17.8 ± 4.5	20.1 ± 4.6	–

<sup>a</sup> Values are means ± standard deviations.

<sup>b</sup>  $p < 0.05$  relative to No-treatment by the unpaired t-test.

<sup>c</sup>  $p < 0.01$  relative to No-treatment by the unpaired t-test.

mutations in RBV-resistant cells, particularly G-to-A and C-to-U transitions, as expected from previous studies. Although mutations were distributed throughout nonstructural regions, four major amino acid substitutions; T1134S in the NS3 region, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B, not seen in wild-type cells were observed in most of the subclones among RBV-resistant replicon cells. T1134S, P1969S, V2405A, and Y2471H were present, respectively, in 7 of 11, 6 of 11, 8 of 13, and 7 of 13 PCR subclones sequenced.

#### 3.4. Effects of T1134S, P1969S, V2405A, and Y2471H on RBV susceptibility

To test the possibility that any of the four mutations as identified confer resistance to RBV, we introduced these mutations individually into the JFH-1 subgenomic replicon containing a luciferase reporter gene. Cells transfected with mutant- or wild-type replicon RNA grown in the presence of various concentrations of RBV for 2 or 3 days. As demonstrated in Fig. 3A, the replication levels of all four mutant replicons (SGR-JFH1/Luc-T1134S, -P1969S, -V2405A, and -Y2471H) in the presence of 125 or 500 M RBV were higher than those of the wild-type replicon. In particular, the Y2471H mutant significantly reduced susceptibility to RBV; replication levels of SGR-JFH1/Luc-Y2471H were 3–5-fold higher when compared to those of wild-type under the present assay conditions.

The relative replication activity of these mutant replicons was further determined in 3-day replication assay without drug treatment (Fig. 3B). All mutant replicons exhibited reduced efficiency

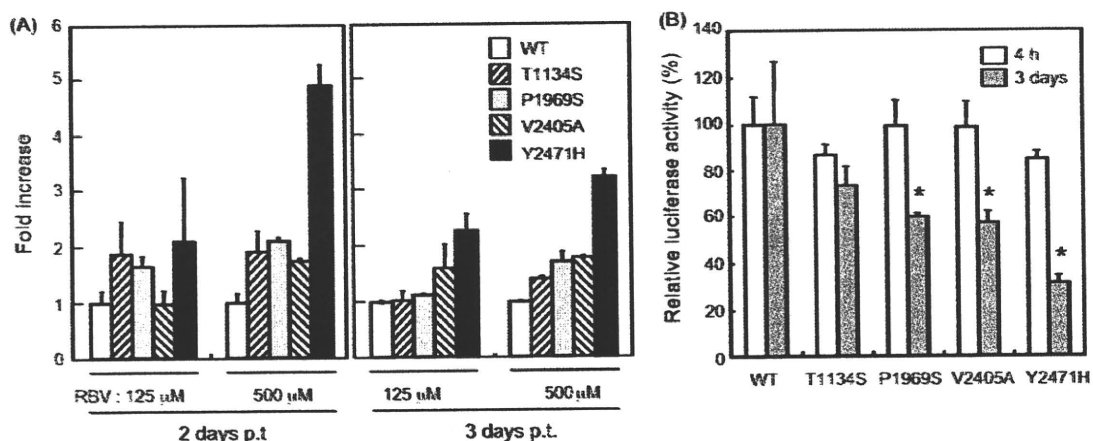
relative to the wild-type replicon. Levels of the Y2471H-mutated replicon were approximately 30% of those of the wild-type, thus suggesting that replicon mutants with reduced sensitivity to RBV are associated with decreased replication fitness.

#### 4. Discussion

It is generally accepted that, during chemotherapy against viral infection, high rates of viral replication and high frequencies of mutation lead to generation of drug-resistant mutants. Although several potential mechanisms for the inhibition of HCV replication by RBV have been proposed, the molecular mechanisms involved in the generation of RBV-resistant HCV remain poorly understood.

This study found that long-term treatment of HCV JFH-1-derived replicon cells with RBV leads to selection of preferential mutations in NS3 (T1134S), NS4B (P1969S), NS5A (V2405A) and NS5B (Y2471H) genes. Each mutation only required a single nucleotide change, and P1969S, V2405A and Y2471H are transition mutations, which are known to be commonly caused by incorporated RBV. Site-directed mutagenesis of these mutations into the replicon demonstrated that Y2471H plays a role in reduced susceptibility to RBV.

Crystal structure information revealed that HCV RdRp is organized into an arrangement with palm, fingers, and thumb subdomains (Lesburg et al., 1999). Residue 2471 (the 33rd position of NS5B) is present in the N-terminal loop region that bridges the fingers. Although this site is apparently distant from the active site of the polymerase in the palm region, it has been reported



**Fig. 3.** Impact of major mutations in NS3–NS5B regions on RBV susceptibility (A) and replication capacity (B). Mutated replicons carrying single residue substitutions (T1134S, P1969S, V2405A, and Y2471H) were constructed and used for transient replication assay. Cells were transfected with either wild-type (WT) or with mutant replicon RNA in the absence or presence (125, 500 M) of RBV. Luciferase activity was assessed at 4 h, 2 days and 3 days post-transfection (p.t.). (A) Luciferase activities of WT were set at 1, and the fold increases in the activities of mutants were plotted. (B) Luciferase activities in the absence of RBV at 4 h and 3 days post-transfection were shown. The activities of mutants were normalized as percentages of the WT activities. Data from triplicate samples were averaged and indicated with standard deviations. \* $p < 0.05$  against WT.

that small molecules, such as benzimidazole compounds, are able to specifically bind the fingers-thumb interface and inhibit polymerase activity (Herlihy et al., 2008), thus suggesting that amino acid substitutions in the loop region may affect RNA polymerization. The involvement of tyrosine residue at position 415 of HCV NS5B in RBV resistance has been previously described for patients with genotype 1a infection and for the genotype 1b replicon (Young et al., 2003). Although the mechanism for resistance remains elusive, it has been hypothesized that RBV interacts with RdRp around this residue, which is located in the thumb subdomain, thus affecting RNA polymerization (Young et al., 2003).

Based on analysis of available sequences from Genbank, tyrosine at the 33rd residue of NS5B is conserved in all isolates of genotype 2a, but not in other genotypes. In genotype 1a and 1b isolates, 96% contain histidine and only a small population contains tyrosine or asparagine at the site. All the isolates of genotypes 3, 4, 5 and 6 contain histidine, whereas phenylalanine is conserved for genotype 2b. It should be noted that V2405 and P1969 are also completely conserved for genotype 2a but not for other genotypes. Therefore, it is likely that the identified HCV variants with reduced susceptibility to RBV are genotype-specific. It will be of interest to determine whether HCV genotype 2a is intrinsically more sensitive to RBV when compared with other genotypes.

At present, at least 4 mechanisms of action of RBV are proposed (Lau et al., 2002). They include (1) direct inhibition of the HCV replication machinery, (2) as an RNA mutagen that drives a rapidly mutating RNA virus over the threshold to "error catastrophe", (3) inhibition of the host enzyme inosine monophosphate dehydrogenase (IMPDH), and (4) enhancement of host T-cell-mediated immunity against viral infection. In addition to the direct inhibition, it is also possible that other mechanisms such as error-prone and IMPDH-inhibition are involved in HCV escape from RBV treatment. Further investigation of the interaction of HCV variants with the viral and cellular factors involved in viral resistance may improve understanding of the mechanism(s) of RBV resistance.

In conclusion, RBV encountered resistance from the HCV genotype 2a replicon largely mediated by mutations in the N-terminal region of NS5B. Although whether these mutagenic effects are also demonstrable in IFN-RBV combination therapy will require further studies, the mutations identified in this study represent the first drug-resistant variants belonging to HCV genotype 2a. The drug resistance patterns found in this study may be of benefit in prediction *in vivo* resistance profiles and the development of next-generation nucleoside analogues as anti-HCV drugs.

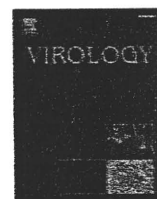
#### Acknowledgments

We thank M. Matsuda, S. Yoshizaki, M. Ikeda, T. Shimoji, M. Kaga and M. Sasaki for their technical assistance. This work was supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labour and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science and Technology, and by Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation, Japan and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of

Biomedical Innovation of Japan. S.S.H. is the recipient of a Research Resident Fellowship from Viral Hepatitis Research Foundation of Japan.

#### References

- Aizaki, H., Nagamori, S., Matsuda, M., Kawakami, H., Hashimoto, O., Ishiko, H., Kawada, M., Matsuura, T., Hasumura, S., Matsuura, Y., Suzuki, T., Miyamura, T., 2003. Production and release of infectious hepatitis C Virus for human liver cell cultures in the three-dimensional radial-flow bioreactor. *Virology* 314, 16–25.
- aus dem Siepen, M., Oniangue-Ndza, C., Wiese, M., Ross, S., Roggendorf, M., Viazov, S., 2007. Interferon-alpha and ribavirin resistance of Huh7 cells transfected with HCV subgenomic replicon. *Virus Res.* 125, 109–113.
- Date, T., Kato, T., Miyamoto, M., Zhao, Z., Yasui, K., Mizokami, M., Wakita, T., 2004. Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells. *J. Biol. Chem.* 279, 22371–22376.
- Domingo, E., 1996. Biological significance of viral quasispecies. *Viral Hep. Rev.* 2, 247–261.
- Farci, P., Purcell, R.H., 2000. Clinical significance of hepatitis C virus genotypes and quasispecies. *Semin. Liver Dis.* 20, 103–126.
- Forbes, X., Purcell, R.H., Bulk, J., 1999. Quasispecies in viral persistence and pathogenesis of hepatitis C virus. *Trends Microbiol.* 7, 402–410.
- Fried, T.R., Bradley, E.H., Towle, V.R., Altore, H., 2002. Understanding the treatment preferences of seriously ill patients. *N. Engl. J. Med.* 346, 1061–1066.
- Herlihy, K.J., Graham, J.P., Kumpf, R., Patrick, A.K., Duggal, R., Shi, S.T., 2008. Development of intragenotypic chimeric replicons to determine the broad-spectrum antiviral activities of hepatitis C virus polymerase inhibitors. *Antimicrob. Agents Chemother.* 52, 3523–3531.
- Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M., Wakita, T., 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* 125, 1808–1817.
- Kato, T., Date, T., Miyamoto, M., Sugiyama, M., Tanaka, Y., Orito, E., Ohno, T., Sugihara, K., Hasegawa, I., Fujiwara, K., Ito, K., Ozasa, A., Mizokami, M., Wakita, T., 2005. Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. *J. Clin. Microbiol.* 43, 5679–5684.
- Lau, J.Y., Tam, R.C., Liang, T.J., Hong, Z., 2002. Mechanism of action of ribavirin in the combination treatment of chronic HCV infection. *Hepatology* 35, 1002–1009.
- Lesburg, C.A., Cable, M.B., Ferrari, E., Hong, Z., Mannarino, A.F., Weber, P.C., 1999. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat. Struct. Biol.* 6, 937–943.
- Manns, M.P., McHutchison, J.G., Gordon, S.C., Rustgi, V.K., Shiffman, M., Reindollar, R., Goodman, Z.D., Koury, K., Ling, M., Albrecht, J.K., 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.
- Martell, M., Esteban, J.L., Quer, J., Genesca, J., Weiner, A., Esteban, R., Guardia, J., Gomez, I., 1992. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *J. Virol.* 66, 3225–3229.
- Miyamoto, M., Kato, T., Date, T., Mizokami, M., Wakita, T., 2006. Comparison between subgenomic replicons of hepatitis C virus genotypes 2a (JFH-1) and 1b (con1 NKS.1). *Intervirology* 49, 37–43.
- Pfeiffer, J.K., Kirkegaard, K., 2005. RBV resistance in hepatitis C virus replication containing cells conferred by changes in the cell line or mutations in the replicon RNA. *J. Virol.* 79, 2346–2355.
- Simmonds, P., Gallin, J.L., Farrei, A.S., 2000. Hepatitis C virus genotypes. *Biomed. Res. Rep.* 2, 53–70.
- Takeuchi, T., Katsume, A., Tanaka, T., Abe, A., Inoue, K., Tsukiyama Kohara, K., Kawaguchi, R., Tanaka, S., Kohara, M., 1999. Real-time detection system for quantification of Hepatitis C virus genome. *Gastroenterology* 116, 636–642.
- Tanaka, Y., Sakamoto, N., Enomoto, N., Kurosaki, M., Ueda, E., Maekawa, S., Yamashiro, T., Nakagawa, M., Chen, C.-H., Kanazawa, N., Kakinuma, S., 2004. Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. *J. Infect. Dis.* 189, 1129–1139.
- World Health Organization (WHO), 2000. Hepatitis C: global prevalence (update). *Weekly Epidemiological Record, WHO* 75, 18–19.
- Young, K.C., Lindsay, K.L., Lee, K.J., Liu, W.C., He, J.W., Milstein, S.L., Lai, M.M., 2003. Identification of a ribavirin-resistant NS5B mutation of hepatitis C virus during ribavirin monotherapy. *Hepatology* 38, 869–878.
- Zhou, S., Liu, R., Baroudy, B.M., Malcolm, B.A., Reyes, G.R., 2003. The effect of ribavirin and IMPDH inhibitors on hepatitis C virus subgenomic replicon RNA. *Virology* 310, 333–342.



## Chaperonin TRiC/CCT participates in replication of hepatitis C virus genome via interaction with the viral NS5B protein

Yasushi Inoue<sup>a,b,c</sup>, Hideki Aizaki<sup>a</sup>, Hiromichi Hara<sup>a</sup>, Mami Matsuda<sup>a</sup>, Tomomi Ando<sup>a</sup>, Tetsu Shimoji<sup>a</sup>, Kyoko Murakami<sup>a</sup>, Takahiro Masaki<sup>a</sup>, Ikuo Shoji<sup>d</sup>, Sakae Homma<sup>b</sup>, Yoshiharu Matsuura<sup>e</sup>, Tatsuo Miyamura<sup>a</sup>, Takaji Wakita<sup>a</sup>, Tetsuro Suzuki<sup>a,f,\*</sup>

<sup>a</sup> Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

<sup>b</sup> Department of Respiratory Medicine, Toho University School of Medicine, Tokyo 143-8541, Japan

<sup>c</sup> International University of Health and Welfare, Mita Hospital, Tokyo 108-8329, Japan

<sup>d</sup> Division of Microbiology, Kobe University Graduate School of Medicine, Hyogo 650-0017, Japan

<sup>e</sup> Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan

<sup>f</sup> Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan

### ARTICLE INFO

#### Article history:

Received 12 June 2010

Returned to author for revision 18 July 2010

Accepted 15 October 2010

Available online 18 November 2010

#### Keywords:

Hepatitis C virus

Replication

Non-structural protein

Chaperonin

### ABSTRACT

To identify the host factors implicated in the regulation of hepatitis C virus (HCV) genome replication, we performed comparative proteome analyses of HCV replication complex (RC)-rich membrane fractions prepared from cells harboring genome-length bicistronic HCV RNA at the exponential and stationary growth phases. We found that the eukaryotic chaperonin T-complex polypeptide 1 (TCP1)-ring complex/chaperonin-containing TCP1 (TRiC/CCT) plays a role in the replication possibly through an interaction between subunit CCT5 and the viral RNA polymerase NS5B. siRNA-mediated knockdown of CCT5 suppressed RNA replication and production of the infectious virus. Gain-of-function activity was shown following co-transfection with whole eight TRiC/CCT subunits. HCV RNA synthesis was inhibited by an anti-CCT5 antibody in a cell-free assay. These suggest that recruitment of the chaperonin by the viral nonstructural proteins to the RC, which potentially facilitate folding of the RC component(s) into the mature active form, may be important for efficient replication of the HCV genome.

© 2010 Elsevier Inc. All rights reserved.

### Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases, such as chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma (Hoofnagle, 2002; Manns et al., 2006; Saito et al., 1990; Seeff and Hoofnagle, 2003). HCV is an enveloped positive-strand RNA virus belonging to the *Hepacivirus* genus of the *Flaviviridae* family. Its genome of ~9.6 kb encodes a polyprotein precursor of ~3000 amino acids (aa) (Suzuki et al., 2007; Tagawa et al., 2008). The precursor polyprotein is post- or cotranslationally processed by both viral and host proteases into at least ten viral products. The nonstructural (NS) proteins NS3–NS5B are necessary and sufficient for autonomous HCV RNA replication. They form a membrane-associated replication complex (RC), in which NS5B is the RNA-dependent RNA polymerase (RdRp) that is responsible for copying the RNA genome of the virus during replication. The HCV RC has been detected in detergent-resistant membrane (DRM)

structures, possibly in a lipid-raft structure (Aizaki et al., 2004; Shi et al., 2003). Cell-free RC replication activity has also been demonstrated in crude membrane fractions of HCV subgenomic replicon cells (Aizaki et al., 2004; Ali et al., 2002; Hara et al., 2009; Hardy et al., 2003; Yang et al., 2004); these cell-free systems provide semi-intact RdRp assays for biochemical dissection of viral replication.

In general, any process that occurs during viral replication is dependent on the host cell machinery and requires close interaction between viral and cellular proteins. Although evidence that host cell factors interact with HCV NS proteins and are involved in viral replication is accumulating (Moriishi and Matsuura, 2007), the cellular components of HCV RC and their functional roles in viral replication are not fully understood.

Recently, using comparative proteome analysis, we identified 27 cellular proteins that were highly enriched in the DRM fraction of HCV replicon cells relative to parental cells. Subsequent analyses demonstrated that one of the identified proteins, creatine kinase B, a key ATP-generating enzyme, is important for efficient replication of the HCV genome and for production of the infectious virus (Hara et al., 2009).

In this study, to extend our investigation and to increase our understanding of the precise components of HCV RC and the

\* Corresponding author. Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan. Fax: +81 53 435 2337.

E-mail address: [tesuzuki@hama-med.ac.jp](mailto:tesuzuki@hama-med.ac.jp) (T. Suzuki).

mechanisms of viral genome replication, we designed another comparative proteomic approach in which cells harboring genome-length bicistronic HCV RNA at the exponential growth phase (showing rapid replication of viral RNA) were compared with cells at the confluent-growth phase (showing poor replication of viral RNA). This strategy revealed that the chaperonin T-complex polypeptide (TCP1)-ring complex/chaperonin-containing TCP1 (TRiC/CCT) participates in HCV RNA replication and virion production possibly through an interaction between CCT5 (chaperonin-containing TCP1, subunit 5) and NS5B.

## Results

### *CCT5 and Hsc70 are enriched in the DRM fraction containing the HCV RC*

Recently, we analyzed the protein content of DRM fractions prepared from HCV subgenomic replicons and parental Huh-7 cells and identified 27 cellular proteins that were enriched in the DRM fraction prepared from the replicon cells (Hara et al., 2009). These were identified as factors that may be involved in the HCV RC and in viral replication. In fact, subsequent silencing of several genes coding for these proteins resulted in the inhibition of HCV RNA replication (Hara et al., 2009). However, it is likely that proteins unrelated to HCV replication are also included in the identified groups because long-term culture of the replicon cells under the selective pressure of G418 selects for a subpopulation of the parental cells and may induce changes in their protein expression profiles. Thus, to minimize interline differences in culture background, we further designed a comparative proteome analysis using a single cell line as follows.

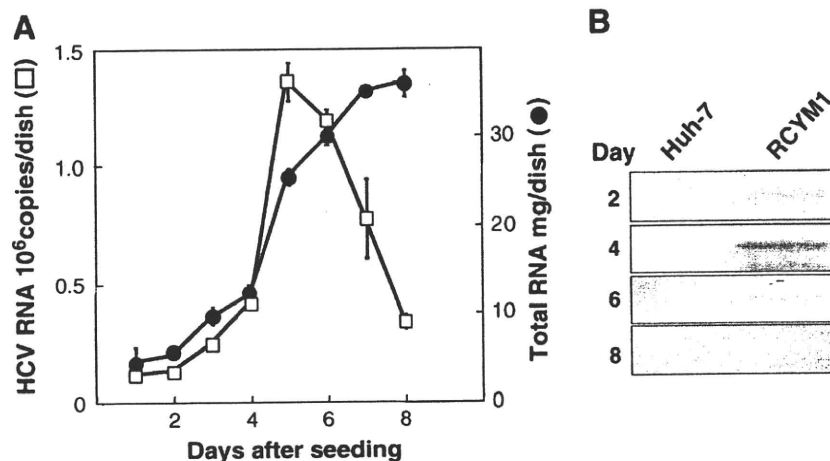
HCV replication efficiency is dependent on the conditions of host cell growth. High cell density of the replicon culture has a reversible inhibitory effect on viral replication (Nelson and Tang, 2006; Pietschmann et al., 2001). Fig. 1A demonstrates that a high level of HCV RNA was detected in cells harboring the genome-length bicistronic HCV RNA, Con1 strain of genotype 1b (RCYM1) in the growth phase, whereas the RNA level declined sharply when the cells reached the stationary phase. We further compared the synthesis of HCV RNA in cell-free reaction mixtures containing the viral RC isolated from the RCYM1 cells at various cell densities (Fig. 1B). Replication activity was highest at the mid-log phase of cell growth (day 4 after seeding). By contrast, little or no RNA synthesis was observed under the confluent-growth cell culture (day 8), confirming the critical role of host cell growth conditions in the replication of the HCV genome.

Thus, to identify the host cell proteins required for HCV replication, we designed a two-dimensional fluorescence difference gel electro-

phoresis (2D-DIGE)-based comparative proteomics analysis of RC-rich DRM fractions prepared from RCYM1 cells at the mid-log and confluent-growth phases. Protein spots that reproducibly showed a greater than 1.5-fold difference in the mid-log growth- and the confluent phases were excised and digested by trypsin or lysylendo-peptidase. Matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry (MS), which allows identification of the corresponding proteins in 9 cases (Table 1). Two increased spots that showed an increase in levels (their stereoscopic images are shown in Fig. 2A) were identified as CCT5 and Hsc70. CCT5, an epsilon subunit of chaperonin TRiC/CCT, is a 900-kDa toroid-shaped complex consisting of eight different subunits (Valpuesta et al., 2002; Yaffe et al., 1992). Hsc70, a member of the HSP70 family, is a 71-kDa heat shock cognate protein (Dworkiczak and Mirault, 1987). Independent of the proteome analyses, DRM fractions and whole cell lysates were prepared from RCYM1 cells at two different growth phases (as above) and were analyzed by immunoblotting (Fig. 2B). Steady-state levels of CCT5 and Hsc70 were obviously higher in the DRM fraction prepared from the cells that were at the mid-log growth phase compared with those at the confluent phase. However, in the whole cell analyses, they were shown to be present at comparable levels during the two different growth phases. These results suggest that expression of CCT5 and Hsc70 is not enhanced in proliferating cells and that the enrichment of these proteins in the DRM fraction is possibly due to their post-translational modification. It should be noted that in the previous proteome analysis, CCT5 and other TRiC/CCT subunits, such as CCT1 and CCT2, were identified as proteins that were enriched in the DRM fraction prepared from subgenomic replicon-containing cells compared with that prepared from parental cells (Hara et al., 2009). We showed that CCT5 and CCT1 were enriched in the DRM fractions of cells transfected with the HCV genomic RNA derived from JFH-1 isolate as well as of subgenomic replicon cells (Fig. 2C).

### *TRiC/CCT participates in replication of the HCV genome*

We investigated gain- and loss-of-functions of TRiC/CCT and Hsc70 with respect to the replication of HCV RNA. Seventy-two hours after RCYM1 cells were transfected with eight plasmids corresponding to each of the TRiC/CCT subunits, the level of HCV RNA in the cells (determined by quantitative RT-PCR) significantly increased to 2-fold that observed in the control cells. However, exogenous expression of Hsc70 in the RCYM1 cells showed no effect on the viral RNA (Fig. 3A). siRNAs targeted to CCT5 or Hsc70 and consisting of pools of three target-specific siRNAs or control nonspecific siRNAs were transfected



**Fig. 1.** Effect of cell growth on HCV RNA replication. (A) Measurement of HCV RNA (open squares) and total cellular RNA (closed circles) in RCYM1 cells at the time of harvest (days after seeding). (B) DRM fractions obtained from RCYM1 and parental Huh-7 cells harvested as indicated (day) were analyzed by cell-free RNA replication assay. RNA extracted from each sample was analyzed by agarose gel electrophoresis and autoradiograph.



**Table 1**  
Selected cellular proteins that reproducibly increased and decreased in membrane fraction of RCYM1 cells at exponential growth phase.

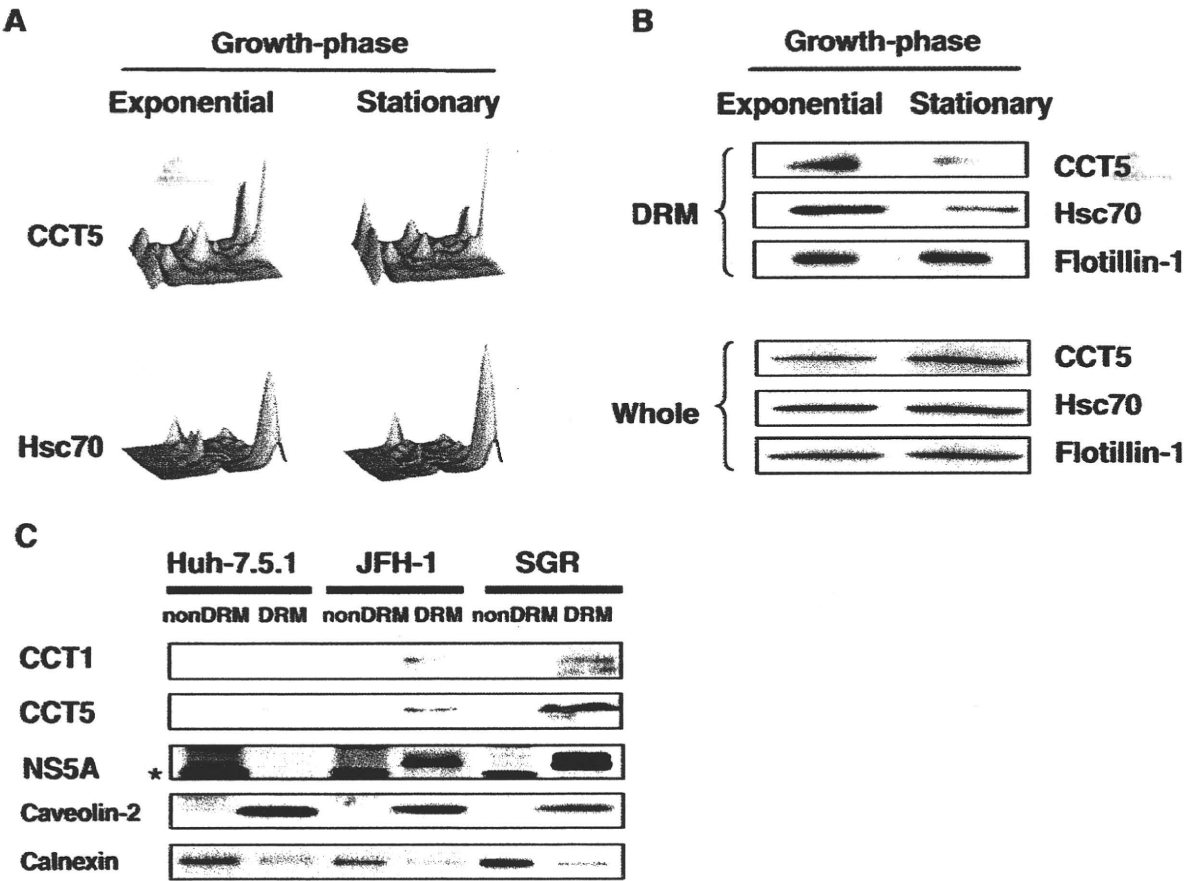
Av. ratio	T-test	Coverage (%)	Protein name	Molecular function	GI
<i>Increased proteins</i>					
1.58	0.017	31	CCT5	Protein folding	33879913
1.54	0.005	35	HSPA8 (Hsc70)	Protein folding	24657660
<i>Decreased proteins</i>					
−1.95	0.028	44	Creatine kinase isozyme CK-B gene, exon 8	Energy pathway/metabolism	180568
−1.53	0.011	16	Chain C, Human Sirt2 Histone deacetylase	Cell cycle control	15826438
−2.14	0.001	33	Proteasome regulatory particle subunit p44S10	Metabolism	15341748
−1.71	0.004	21	Aldehyde dehydrogenase	Metabolism	178388
−1.85	0.004	40	Aminoacylase 1	Metabolism	12804328
−2.77	0.003	15	Eukaryotic translation initiation factor 3, subunit 3 gamma	Metabolism (translation regulator activity)	6685512
−2.43	0.014	20	Intraflagellar transport protein 74 homolog (Coiled-coil domain-containing protein 2)	Cell growth and/or maintenance	10439078

Three paired samples of RC-rich membrane fractions at the exponential- and confluent-growth phases of RCYM1 cultures were analyzed. The proteins representing a more than 1.5-fold increase or decrease (−) reproducibly and significantly are indicated.  
Coverage (%): the ratio of the portion of protein sequence covered by matched peptides to the whole sequence.  
GI: GenInfo Identifier number.

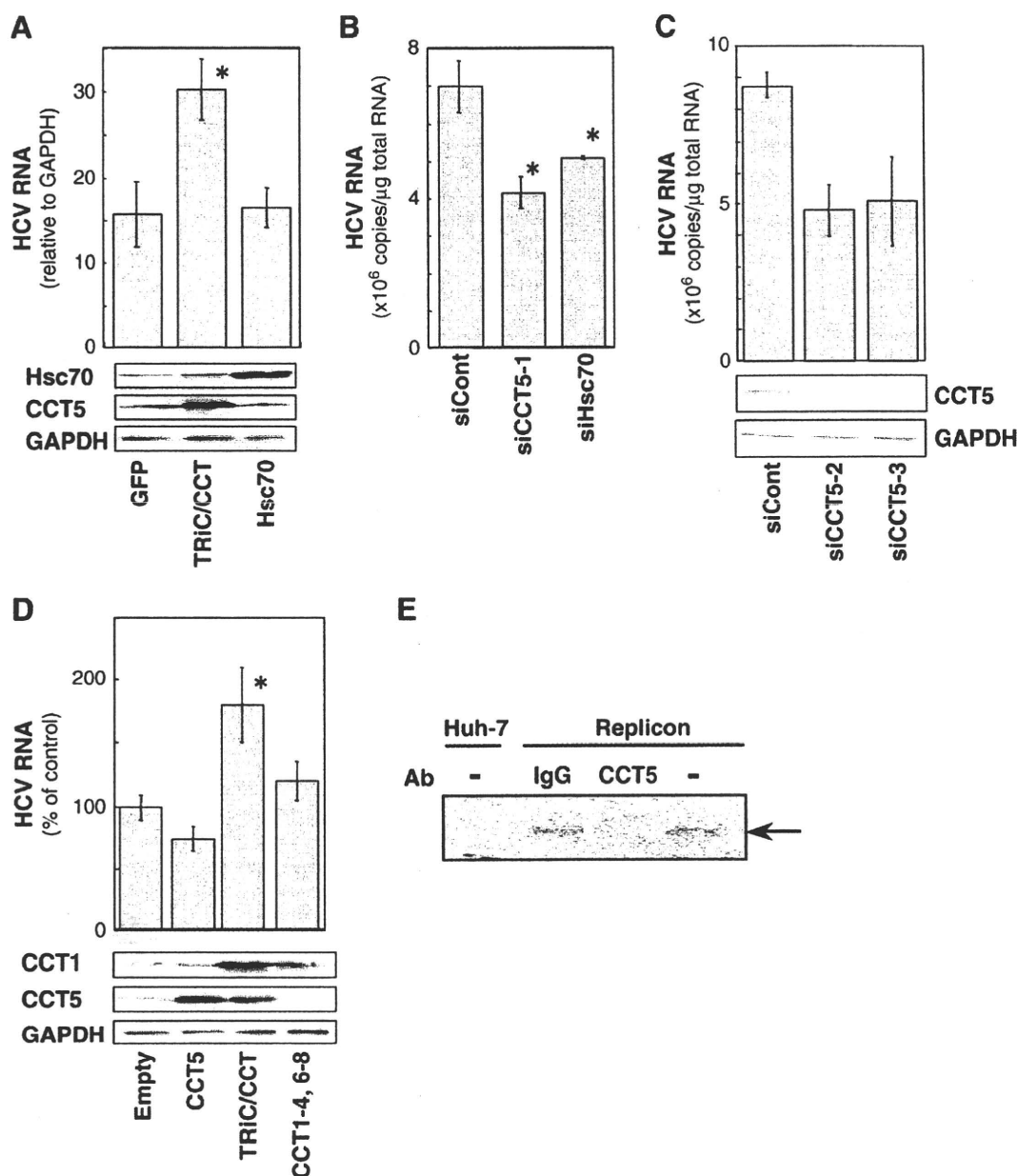
into RCYM1 cells. After 72 h, the HCV RNA level was reduced by 42% and 27% in the cells transfected with siRNAs against CCT5 and Hsc70, respectively, compared with controls (Fig. 3B). TRiC/CCT possibly interacts with Hsc70, and its complex formation contributes to increasing the efficiency of protein folding (Cuéllar et al., 2008). Our results suggest the involvement of TRiC/CCT and Hsc70 in the HCV

life cycle. In particular, TRiC/CCT may play an important role in the replication of the viral genome.

To verify the specificity of the knockdown of CCT5 siRNA, we further synthesized two siRNAs targeted to different regions used in the above CCT5 siRNA and assessed their knockdown effect on HCV genome replication (Fig. 3C, upper panel). As expected, transfection of



**Fig. 2.** Comparison of protein levels in DRM fractions prepared from RCYM1 cells at the exponential and stationary growth phases. (A) Three-dimensional images of CCT5 and Hsc70 analyzed by Ettan DIGE (GE Healthcare). Spots corresponding to CCT5/Hsc70 at exponential and stationary growth phases of the cells, respectively, are shown in green and red. (B) Equal amounts of protein in the DRM fractions prepared from RCYM1 cells at the exponential and stationary growth phases or corresponding whole cell lysates were analyzed by immunoblotting with Abs against CCT5, Hsc70 or flotillin-1. (C) Enrichment of CCT1 and CCT5 in the DRM fractions of HCV RNA replicating cells. Equal amounts of DRM or non-DRM fractions from full-length JFH-1 RNA transfected cells (JFH-1), subgenomic replicon cells (SGR) and parental Huh-7.5.1 cells were analyzed by immunoblotting with antibodies against CCT1, CCT5, NS5A, caveolin-2 or calnexin. \*Non-specific bands.



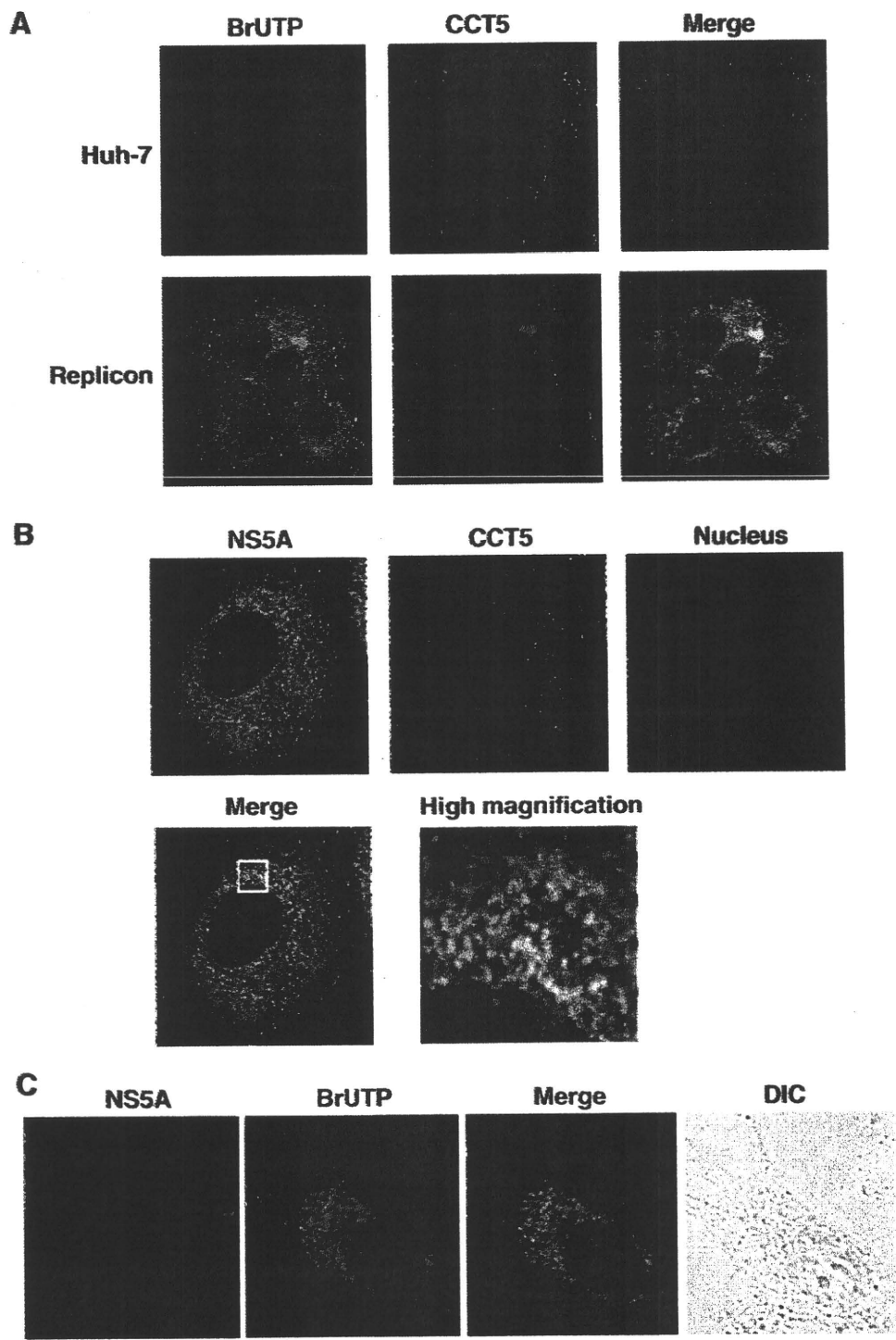
**Fig. 3.** Involvement of TRiC/CCT in HCV replication (A and D). Overexpression of all eight subunits of TRiC/CCT (TRiC/CCT); seven subunits, CCT1, 2, 3, 4, 6, 7, and 8 (CCT1–4, 6–8); subunit CCT5 only (CCT5); Hsc70; or control GFP in RCYM1 cells. HCV RNA levels were determined 48 h post-transfection (B and C). Knockdown of endogenous CCT5 or Hsc70 in RCYM1 cells, which were transfected with three types of siRNAs against CCT5 (siCCT5-1, -2, and -3), siRNA against Hsc70 (siHsc70), or control siRNA (siCont), and were harvested at 72 h post-transfection. siCCT5-1 and siHsc70 consisted of pools of three target-specific siRNAs. Immunoblotting for CCT1, CCT5, Hsc70 and GAPDH was performed (A, C and D; lower). (E) Cell-free de novo viral RNA synthesis assays were performed in the presence of anti-CCT5 Ab or control mouse IgG. Cytoplasmic fractions from SGR-N (replicon) and parental Huh-7 cells were used. An arrow indicates the synthesized HCV RNA. Error bars denote standard deviations with asterisks indicating statistical significance (\* $P < 0.01$ ).

RCYM1 cells with each CCT5 siRNA resulted in a reduction in viral RNA to a level of about 50% of that observed in cells treated with control siRNAs. Immunoblotting confirmed the efficient reduction in expression of endogenous CCT5 and the lack of cytotoxic effect exerted by the CCT5 siRNAs (Fig. 3C, middle and lower panels).

Having confirmed the upregulation of HCV RNA by ectopic expression of all the TRiC/CCT subunits, we further addressed the possibility that CCT5, independent of the complete TRiC/CCT complex, might have a role in promoting replication of HCV RNA. Transfection with either a CCT5 expression plasmid alone or with seven plasmids expressing all the TRiC/CCT subunits except CCT5 resulted in no or only a slight increase in the level of HCV RNA, indicating that all CCT subunits are required for HCV replication (Fig. 3D).

TRiC/CCT is generally known as a cytosolic chaperone (Valpuesta et al., 2002). However, it is enriched in the DRM fraction of HCV-

replicating cells during the exponential growth phase (Fig. 2B). We used immunofluorescence staining to investigate whether TRiC/CCT is localized in the intracellular membrane compartments where replication of the viral genome occurs (Fig. 4). The de novo-synthesized RdRp was labeled by bromouridine triphosphate (BrUTP) incorporation in the presence of actinomycin D, and brominated nucleotides were detected with a specific antibody (Ab). Fluorescence staining in distinct speckles of various sizes was found in the cytoplasm of the HCV subgenomic replicon cells, whereas no signal was detected in the control cells, indicating that the observed BrUTP-incorporating RNA is mostly viral, newly synthesized viral RNA (Fig. 4A). Double immunofluorescence staining showed that a certain section of CCT5 co-distributed with the BrUTP-labeled RNA (Fig. 4A), which is known to co-exist with HCV NS proteins in viral replicating cells (Shi et al., 2003). We further observed that CCT5 was at least partially colocalized



**Fig. 4.** Immunofluorescence analysis of CCT5 in SGR-N and Huh-7 cells (A) and HCVcc-infected cells (B). The primary Abs used were anti-CCT5 goat polyclonal Ab (red), anti-BrUTP monoclonal Ab (green), and anti-NS5A monoclonal Ab (green). Merged images of red and green signals (A) or of red, green and blue (nucleus) signals (B) are shown. The high magnification panel is an enlarged image of a white square of the merge panel. (C) Colocalization of NS5A protein with the viral RNA. The replicon cells were permeabilized with lyssolecithin and labeled with BrUTP, followed by staining with anti-NS5A rabbit polyclonal Ab (red) and the anti-BrUTP monoclonal Ab (green). DIC, differential interference contrast.

with the viral NS protein in certain compartments sharing a dot-like structure in Huh-7 cells infected with HCV JFH-1 infectious HCV (HCVcc) derived from HCV genotype 2a (Fig. 4B) as well as in the replicon cells (data not shown). Fig. 4C indicated co-localization of BrUTP-labeled RNA with NS5A.

To further address the role of TRiC/CCT in HCV genome replication, we performed immunodepletion and in vitro replication analyses, which have been used for studying the genome replication of several

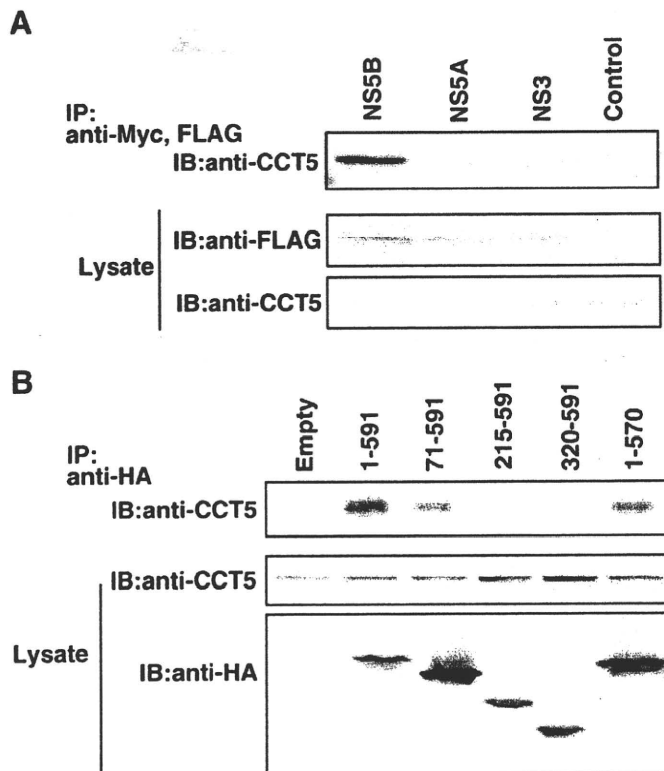
viruses (Daikoku et al., 2006; Garcin et al., 1993; Liu et al., 2009). Cell extracts prepared from the HCV-replicating cells were reacted with either a mouse monoclonal Ab against CCT5 or mouse IgG derived from preimmune serum, followed by cell-free synthesis of HCV RNA. Fig. 3E shows that treatment with anti-CCT5 Ab inhibited viral RNA synthesis, whereas the control IgG did not affect the process, suggesting that TRiC/CCT participates directly in HCV RNA replication.

### CCT5 interacts with HCV NS5B

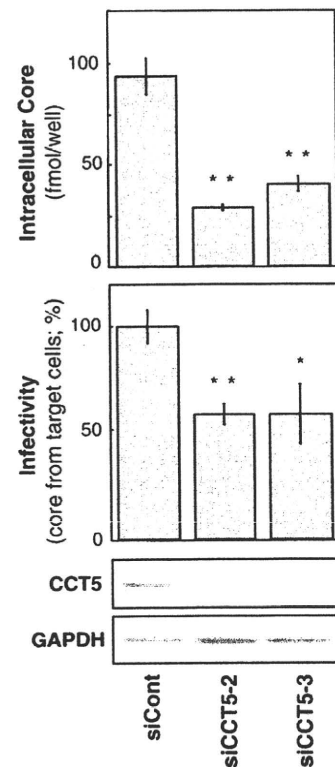
The genome replication machinery of HCV is a membrane-associated complex composed of multiple factors including viral NS proteins. Given the involvement of TRiC/CCT in HCV RNA synthesis, we next examined its possible interaction with HCV NS proteins. A first attempt to immunoprecipitate the viral proteins with antibodies against TRiC/CCT subunits in the replicon cells was unsuccessful (data not shown), suggesting that endogenous levels of TRiC/CCT is not sufficient to pull out NS5B. Next, dual (myc/FLAG)-tagged NS3, NS5A, or NS5B proteins derived from the genotype 1b NIHJ1 strain were co-expressed with CCT5 in Huh-7 cells and then subjected to two-step immunoprecipitation with anti-myc and anti-FLAG Abs (Ichimura et al., 2005; Shirakura et al., 2007). An empty plasmid was used as a negative control in the analyses. As shown in Fig. 5A, CCT5 specifically interacted with NS5B. Little or no interaction was found between CCT5 and NS3 or NS5A. To determine the NS5B region required for the interaction with CCT5, various deletion mutants of HA-NS5B were constructed and their interactions with CCT5 were analyzed as described above. CCT5 was shown to be coimmunoprecipitated with either a full-length NS5B (aa 1–591), an N-terminal deletion (aa 71–591) or a C-terminal deletion (aa 1–570), but not with deletions aa 215–591 or aa 320–591 (Fig. 5B), suggesting that aa 71–214 of NS5B are important for its interaction with CCT5.

### Knockdown of CCT5 results in the reduction of propagation of infectious HCV

We further examined whether the knockdown of CCT5 would abrogate the production of infectious HCV (HCVcc), derived from JFH-1 (Fig. 6). At 72 h post-transfection with each CCT5 siRNA, HCV RNA



**Fig. 5.** CCT5 interacts with HCV NS5B. (A) CCT5 was co-expressed with MEF-tagged-NS5B, -NS5A, or -NS3 protein of strain NIHJ1 in cells, followed by two-step immunoprecipitation (IP) with anti-FLAG and anti-myc Abs. Immunoprecipitates were subjected to immunoblotting with anti-CCT5 Ab (IB). (B) Full-length NS5B (1–591) or its deletions (71–591, 215–591, 320–591, 1–570) along with a HA tag were co-expressed with CCT5. IP and IB were performed as described above.



**Fig. 6.** Knockdown of endogenous CCT5 in HCVcc-infected cells. The cells were transfected with siRNAs against CCT5 (siCCT5-2, -3) or with control siRNAs (siCont). At 72 h post-transfection, the viral core protein levels in cells were determined (upper panel). Collected culture supernatants were inoculated into naïve Huh7.5.1 cells and intracellular core proteins were determined at 72 h post-infection (middle panel). Cells transfected with siRNAs were analyzed by immunoblotting with anti-CCT5 or anti-GAPDH Ab (lower panel). Error bars denote standard deviations with asterisks indicating statistical significance (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

levels in Huh-7 cells infected with HCVcc were reduced by 25–35% compared with controls. Accordingly, virion production from CCT5 siRNA-transfected cultures was significantly decreased, as determined by intracellular HCV core protein levels at 72 h after the infection of naïve cells with culture supernatants taken from transfected cells. These results demonstrate that reduction of the HCV RNA replication by siRNA-mediated knockdown of CCT5 results in reduction of the propagation of the infectious virus.

### Discussion

The chaperone-assisted protein-folding pathway is a process in living cells that results from coordinated interactions between multiple proteins that often form multi-component complexes. Several steps in the viral life cycle, such as protein processing, genome replication, and viral assembly, are regulated by cellular chaperones. Hsp90, one of the most abundant proteins in unstressed cells, has been implicated in HCV RNA replication (Nakagawa et al., 2007; Okamoto et al., 2006, 2008; Tagawa et al., 2008, 2009; Ujino et al., 2009). FKBP8, a member of the FKBP506-binding protein family, and hB-ind1, human butyrate-induced transcript 1, play key roles through their interaction with HCV NS5A and Hsp90 (Okamoto et al., 2006, 2008; Tagawa et al., 2008, 2009). Hsp90 has also been implicated in viral enzymatic activities including those of the influenza virus (Momose et al., 2002; Naito et al., 2007), herpes simplex virus (Burch and Weller, 2005), Flock house virus (Kampmueller and Miller, 2005), and hepatitis B virus (Hu et al., 2004).

In our former study, comparative proteome analyses of the viral RC-rich DRM fractions prepared from subgenomic replicon cells and Huh-7 cells were carried out to identify host factors involved



in HCV replication (Hara et al., 2009). We extended the proteomics by modifying our protocol of the analysis to reduce the interline differences in culture background and analyzed the DRM samples derived from the mid-log and confluent-growth phases of single cell line. Here, we identified two proteins, CCT5 and Hsc70, showing an increase in levels at the mid-log growth phase. Although CCT5 was also identified in the former study as expected, Hsc70 was not included in the list of proteins identified in the study (Hara et al., 2009). This difference may be due to the use of cells carrying the full-length replicon RNA in this study.

In this study, we demonstrated that TRiC/CCT participates in HCV RNA replication and virion production possibly through its interaction with NS5B. TRiC/CCT is a group II chaperonin that assists in protein folding in eukaryotic cells and forms a double-ring-like hexadecamer complex. Although relatively little is known about its function compared with that of the group I chaperonins such as bacterial GroEL, several mammalian proteins whose folding is mediated by TRiC/CCT have been identified, such as actin, tubulin, and von Hippel–Lindau tumor suppressor protein (Farr et al., 1997; Feldman et al., 2003; Frydman and Hartl, 1996; Meyer et al., 2003; Tian et al., 1995). With regard to viral proteins, the Epstein–Barr virus nuclear antigen, HBV capsid protein, and p4 of M-PMV have been identified as TRiC/CCT-interacting proteins (Yam et al., 2008). However, the functional significance of their interactions in the viral life cycles has yet to be determined. Here we demonstrated that the reduction in CCT5 expression in HCV replicon cells and in virus-infected cells inhibits HCV RNA replication (Figs. 3B and C) and virus production (Fig. 6) respectively. Gain-of-function was also shown by co-transfection of the replicon cells with eight constructs corresponding to all the TRiC/CCT subunits (Figs. 3A and D).

A recent study of the three-dimensional structure of the TRiC/CCT and Hsc70 complex has demonstrated that the apical domain of the CCT2 (CCT- $\beta$ ) subunit is involved in the interaction with Hsc70 (Cuéllar et al., 2008). The complex formation created by the TRiC/CCT and Hsc70 interaction may promote higher efficiency in the folding of certain proteins (Cuéllar et al., 2008). In our comparative proteome analyses, both CCT subunits and Hsc70 were enriched in the HCV RC-rich membrane fraction of the replicon cells that showed high viral replication activity (Fig. 2B). Transfection of Hsc70 siRNA into the replicon cells moderately inhibited viral RNA replication (Fig. 3B). However, upregulation of HCV replication was not observed by ectopic expression of Hsc70 (Fig. 3A), and little or no interaction was observed between Hsc70 and HCV NS proteins in the co-immunoprecipitation analysis (data not shown). Thus, it is likely that TRiC/CCT acts as a regulator of HCV replication through participating in the de novo folding of NS5B RdRp, and Hsc70 might serve to assist in folding through its interaction with TRiC/CCT. It was recently reported that Hsc70 is associated with HCV particles and modulates the viral infectivity (Parent et al., 2009). Here we showed an additional role of Hsc70 in the HCV life cycle.

HCV genomic single-stranded RNA serves as a template for the synthesis of the full-length minus strand that is used for the overproduction of the virus-specific genomic RNA. NS5B RdRp is a single subunit catalytic component of the viral replication machinery responsible for both of these processes. It is known that the in vitro RdRp activity of recombinant NS5B expressed in and purified from insect cells and *Escherichia coli* is low in many cases. This could be due to the lack of a suitable cellular environment for favorable RdRp activity, although the particular conformational features dependent on the viral isolates may also be involved (Lohmann et al., 1997; Weng et al., 2009). In fact, besides interacting with HCV NS proteins, NS5B has been reported to interact with several host cell proteins. For example, human vesicle-associated membrane protein-associated protein subtype A (VAP-A) and subtype B (VAP-B), which are involved in the regulation of membrane trafficking, lipid transport and metabolism, and the unfolded protein response, interact with NS5B and NS5A and

participate in HCV replication (Hamamoto et al., 2005). Recently, VAP-C, a splicing variant of VAP-B, was found to act as a negative regulator of viral replication through its interaction with NS5B but not with VAP-A (Kukihara et al., 2009). Cyclophilin A and B, peptidyl-prolyl isomerases that facilitate protein folding by catalyzing the *cis-trans* interconversion of peptide bonds at proline residues, play a role in stimulating HCV RNA synthesis through interaction with NS5B (Liu et al., 2009; Watashi et al., 2005). SNARE-like protein (Tu et al., 1999), eIF4AII (Kyono et al., 2002), protein kinase C-related kinase 2 (Kim et al., 2004), nucleolin (Kim et al., 2004; Hirano et al., 2003; Shimakami et al., 2006), and p68 (Goh et al., 2004) are also known to associate with NS5B and are possibly involved in HCV RNA replication.

We found that the aa 71–214 region in NS5B is important for interaction with TRiC/CCT. The catalytic domain of HCV RdRp has a “right-hand” configuration similar to other viral polymerases, such as HIV-1 reverse transcriptase (Huang et al., 1998) and poliovirus RdRp (Hansen et al., 1997), and is divided into the fingers, palm, and thumb functional subdomains (Lohmann et al., 2000). The region required for the interaction with TRiC/CCT has been mapped in a part of the fingers and palm domains of NS5B RdRp. To address how TRiC/CCT assists in the correct folding or disaggregation of NS5B through their interaction, leading to the formation of a functional RdRp, work based on an in vitro reconstitution system using purified proteins is under way. As all the TRiC/CCT subunits possess essentially identical ATPase domains, their protein-recognition regions are apparently divergent, allowing for substrate-binding specificity. It has recently been reported that TRiC/CCT interacts with the PB2 subunit of the influenza virus RNA polymerase complex and TRiC/CCT binding site is located in the central region of PB2, suggesting involvement of TRiC/CCT in the influenza virus life cycle (Fislová et al., 2010). Eukaryotic RNA polymerase subunit has also been identified as a binding partner of TRiC/CCT from interactome analysis (Yam et al., 2008). It would be interesting to examine how conserved the mechanisms of TRiC/CCT action that result in enhanced replication are among RNA polymerases.

The recruitment of a chaperonin by viral NS proteins may be important for understanding regulation of the viral genome replication. In this study, we demonstrated the involvement of TRiC/CCT in HCV RNA replication possibly through its interaction between TRiC/CCT and HCV NS5B. Although possible interaction of subunit CCT5 with NS5B was shown, considering involvement of whole TRiC/CCT complex in its chaperonin function, whether CCT5 directly interacts with NS5B is unclear. Further detailed studies are needed to make clear the manner of TRiC/CCT–NS5B interaction. NS5B RdRp is one of the main targets for HCV drug discovery. The search for NS5B inhibitors has resulted in the identification of several binding sites on NS5B, such as the domain adjacent to the active site and the allosteric GTP site (De Francesco and Migliaccio, 2005; Laporte et al., 2008). The findings obtained here suggest that disturbing the interaction between NS5B and TRiC/CCT may be a novel approach for an antiviral chemotherapeutic strategy.

## Materials and methods

### Cell culture, transfection, and infection

Human hepatoma Huh-7 and Huh-7.5.1 cells (kindly provided by Francis V. Chisari from The Scripps Research Institute) and human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Huh-7-derived SGR-N (Shi et al., 2003) and RCYM1 (Murakami et al., 2006) cells, which possess subgenomic replicon RNA from the HCV-N strain (Guo et al., 2001; Ikeda et al., 2002) and genome-length HCV RNA from the Con 1 strain (Pietschmann et al., 2002), were cultured in the above medium in the presence of 1 mg/ml G418. Cells were transfected with plasmid DNAs using FuGENE transfection reagents

(Roche Diagnostics, Tokyo, Japan). Culture media from Huh-7 cells transfected with in vitro-transcribed RNA corresponding to the full-length HCV RNA derived from the JFH-1 strain (Wakita et al., 2005) were collected, concentrated, and used for the infection assay (Aizaki et al., 2008).

## Ab

Primary Abs used in this study were mouse monoclonal Abs against FLAG (Sigma-Aldrich, St. Louis, MO), c-myc (Sigma-Aldrich), CCT5 (Abnova Corporation, Taipei City, Taiwan), flotillin-1 (BD Biosciences, San Jose, CA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon, Temecula, CA), BrdU (Caltag, CA) and HCV NS5A (Austral Biologicals, San Ramon, CA), a rabbit polyclonal Ab against hemagglutinin (HA; Sigma-Aldrich), a sheep polyclonal Ab against bromodeoxyuridine (Bioscience International, Saco, ME), and goat polyclonal Abs against the individual subunits of CCT (Santa Cruz Biotechnology, Santa Cruz, CA) and Hsc70 (Santa Cruz Biotechnology). Anti Hsc70 and CCT5 monoclonal rat Abs were obtained from Abcam (Tokyo, Japan) and AbD serotec (Oxford, UK). Rabbit polyclonal antibody to NS5A was described previously (Hamamoto et al., 2005). Anti NS5B monoclonal Ab was kindly provided by D. Moradpour (Centre Hospitalier Universitaire Vaudois, University of Lausanne; Moradpour et al., 2002).

## Plasmids

To generate expression plasmids for the NS proteins with dual epitope tags, DNA fragments encoding the NS3, NS5A, or NS5B proteins were amplified from HCV strain NIHJ1 (Aizaki et al., 1998) by PCR and cloned into the EcoRI–EcoRV sites of pCDNA3-MEF, which includes the MEF tag cassette containing the myc tag, TEV protease cleavage site, and FLAG tag sequences (Ichimura et al., 2005; Shirakura et al., 2007). To create a series of NS5B truncation mutants, each fragment was amplified by PCR and cloned into the EcoRI–XhoI site of pCMV-HA (Clontech, Mountain View, CA). To generate expression plasmids for the individual CCT subunits, cDNA fragments encoding human CCT1 through CCT8 were amplified from the total cellular RNA by RT-PCR and then cloned into the SmaI site of pCAGGS (Niwa et al., 1991). All PCR products were confirmed by nucleotide sequencing.

## Proteome analysis

RC-rich membrane fractions from the cells were isolated as described previously (Aizaki et al., 2004). Briefly, cells were lysed in hypotonic buffer. After removing the nuclei, the supernatants were mixed with 70% sucrose, overlaid with 55% and 10% sucrose, and centrifuged at 38,000 rpm for 14 h. Proteins from the membrane fractions were then analyzed by 2D-DIGE as described previously (Hara et al., 2009). Briefly, protein samples were resolved in protein solubilization buffer (Bio-Rad Laboratories, Tokyo, Japan) and washed with pH adjustment buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris–HCl [pH 10.0]), before being labeled with fluorescent dyes; the dyes used were Cy3 for RCYM1 cells samples taken at the exponential growth phase, Cy5 for cells samples taken at the confluent phase, and Cy2 for a protein standard containing equal amounts of both cell samples. Aliquots of the labeled samples were pooled and applied to Immobilized DryStrip (GE Healthcare, Tokyo, Japan) for first-dimension separation and to 12.5% polyacrylamide gels for second-dimension separation. Images of the 2-D gels were captured on a Typhoon scanner (GE Healthcare), and analyzed quantitatively using DeCyder v5.0 software (GE Healthcare). Samples were analyzed in triplicate as independent cultures and the Student's *t*-test was applied using the DeCyder biological variation analysis

module to validate the significance of the differences in spot intensity detected between the samples.

## In vitro RNA replication assay

In vitro replication of HCV RNA was performed as described previously (Hamamoto et al., 2005). Briefly, cytoplasmic fractions of subgenomic replicon cells were treated with 1% NP-40 at 4 °C for 1 h, followed by being incubated with 1 mM of ATP, GTP, and UTP; 10  $\mu$ M CTP; [<sup>32</sup>P]CTP (1 MBq; 15 TBq/mmol); 10  $\mu$ g/ml actinomycin D; and 800 U/ml RNase inhibitor (Promega, Madison, WI) for 4 h at 30 °C. RNA was extracted from the total mixture by using TRI Reagent (Molecular Research Center, Cincinnati, OH). The RNA was precipitated, eluted in 10  $\mu$ l of RNase-free water, and analyzed by 1% formaldehyde-agarose gel electrophoresis. For the immunodepletion assay, the cytoplasmic fractions were incubated with anti-CCT5 Ab in the presence of NP-40 for 4 h before NTP incorporation.

## MALDI-TOF MS analysis

Target spots were cut and collected from gels under UV luminescence and rechecked with Typhoon scanner. The spot gels of the target proteins were subjected to in-gel trypsin digestion and analyzed by MALDI-TOF MS meter (Voyager-DE STR, Applied Biosystems, Tokyo, Japan) as described previously (Yanagida et al., 2000). All proteins were identified by peptide mass fingerprinting.

## Immunoblot analysis and immunoprecipitation

Immunoblot analysis was performed essentially as described previously (Aizaki et al., 2004). The membrane was visualized with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). For immunoprecipitation, cells transfected with plasmids expressing epitope-tagged HCV protein or CCT5 were lysed and then subjected to two-step precipitations with anti-myc and anti-FLAG Abs according to the procedures described previously (Ichimura et al., 2005). In some experiments, HA-tagged full-length NS5B (aa 1–591) or its deletion mutants (aa 71–591, 215–591, 320–591, 1–570) were co-expressed with CCT5 in cells, followed by single-step immunoprecipitation and immunoblotting.

## Immunofluorescence staining

Cell permeabilization with lyssolecithin and detection of de novo-synthesized viral RNA was performed as described previously (Shi et al., 2003). Briefly, Huh-7 cells were plated on 8-well chamber slides at a density of  $5 \times 10^4$  cells per well. Cells were incubated with actinomycin D (5  $\mu$ g/ $\mu$ l) for 1 h and were washed twice with serum-free medium, before being incubated for 10 min on ice. The cells were then incubated in a transcription buffer containing 0.5 mM BrUTP for 30 min. The cells were fixed in 4% formaldehyde for 20 min and then incubated for 15 min in 0.1% Triton X-100 in phosphate-buffered saline (PBS). Primary Abs were diluted in 5% bovine serum albumin in PBS and were incubated with the cells for 1 h. After washing with PBS, fluorescein-conjugated secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA) were added to the cells at a 1:200 dilution for 1 h. The slides were then washed with PBS and mounted in ProLong Antifade (Molecular Probes, Eugene, OR). Confocal microscopy was performed on a Zeiss Confocal Laser Scanning Microscope LSM 510 (Carl Zeiss MicroImaging, Thornwood, NY).

## RNA interference

Small interfering RNAs (siRNAs) targeted to CCT5 or Hsc70 and scrambled negative control siRNAs were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Cells were plated on a 24-well plate with

antibiotic-free DMEM overnight, and each plate was transfected with 10 nM siRNAs by X-tremeGENE (Roche Diagnostics) according to the manufacturer's protocol. Forty-eight hours post-transfection, the total RNA and protein extracts were prepared and subjected to real-time RT-PCR and immunoblot analyses, respectively.

#### Quantitation of HCV RNA and core protein

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time RT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, CA) as described previously (Aizaki et al., 2004; Murakami et al., 2006). HCV core protein levels in the cells and in the supernatant were quantified using an HCV core enzyme-linked immunosorbent assay (Ortho-Clinical Diagnostics, Tokyo, Japan).

#### Acknowledgments

We thank Drs. F. V. Chisari (The Scripps Research Institute) and D. Moradpour (Centre Hospitalier Universitaire Vaudois, University of Lausanne) for providing the Huh-7.5.1 cells and anti-NS5B monoclonal antibody, respectively; S. Yoshizaki, M. Kaga, M. Sasaki, and T. Date for their technical assistance, and T. Mizoguchi for secretarial work. This work was supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labour and Welfare of Japan, and from the Ministry of Education, Culture, Sports, Science and Technology, and by Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation, and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan.

#### References

- Aizaki, H., Aoki, Y., Harada, T., Ishii, K., Suzuki, T., Nagamori, S., Toda, G., Matsuura, Y., Miyamura, T., 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. *Hepatology* 27 (2), 621–627.
- Aizaki, H., Lee, K.J., Sung, V.M., Ishiko, H., Lai, M.M., 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* 324 (2), 450–461.
- Aizaki, H., Morikawa, K., Fukasawa, M., Hara, H., Inoue, Y., Tani, H., Saito, K., Nishijima, M., Hanada, K., Matsuura, Y., Lai, M.M., Miyamura, T., Wakita, T., Suzuki, T., 2008. Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. *J. Virol.* 82 (12), 5715–5724.
- Ali, N., Tardif, K.D., Siddiqui, A., 2002. Cell-free replication of the hepatitis C virus subgenomic replicon. *J. Virol.* 76 (23), 12001–12007.
- Burch, A.D., Weller, S.K., 2005. Herpes simplex virus type 1 DNA polymerase requires the mammalian chaperone hsp90 for proper localization to the nucleus. *J. Virol.* 79 (16), 10740–10749.
- Cuéllar, J., Martín-Benito, J., Scheres, S.H., Sousa, R., Moro, F., López-Viñas, E., Gómez-Puertas, P., Muga, A., Carrascosa, J.L., Valpuesta, J.M., 2008. The structure of CCT-Hsc70 NBD suggests a mechanism for Hsp70 delivery of substrates to the chaperonin. *Nat. Struct. Mol. Biol.* 15 (8), 858–864.
- Daikoku, T., Kudoh, A., Sugaya, Y., Iwahori, S., Shirata, N., Isomura, H., Tsurumi, T., 2006. Postreplicative mismatch repair factors are recruited to Epstein-Barr virus replication compartments. *J. Biol. Chem.* 281 (16), 11422–11430.
- De Francesco, R., Migliaccio, G., 2005. Challenges and successes in developing new therapies for hepatitis C. *Nature* 436 (7053), 953–960.
- Dworkiczak, B., Mirault, M.E., 1987. Structure and expression of a human gene coding for a 71 kd heat shock 'cognate' protein. *Nucleic Acids Res.* 15 (13), 5181–5197.
- Farr, G.W., Scharl, E.C., Schumacher, R.J., Sondel, S., Horwich, A.L., 1997. Chaperonin-mediated folding in the eukaryotic cytosol proceeds through rounds of release of native and nonnative forms. *Cell* 89 (6), 927–937.
- Feldman, D.E., Spiess, C., Howard, D.E., Frydman, J., 2003. Tumorigenic mutations in VHL disrupt folding in vivo by interfering with chaperonin binding. *Mol. Cell* 12 (5), 1213–1224.
- Fislová, T., Thomas, B., Graef, K.M., Fodor, E., 2010. Association of the influenza virus RNA polymerase subunit PB2 with the host chaperonin CCT. *J. Virol.* 84 (17), 8691–8699.
- Frydman, J., Hartl, F.J., 1996. Principles of chaperone-assisted protein folding: differences between in vitro and in vivo mechanisms. *Science* 272 (5267), 1497–1502.
- Garcin, D., Rochat, S., Kolakofsky, D., 1993. The Tacaribe arenavirus small zinc finger protein is required for both mRNA synthesis and genome replication. *J. Virol.* 67 (2), 807–812.
- Goh, P.Y., Tan, Y.J., Lim, S.P., Tan, Y.H., Lim, S.G., Fuller-Pace, F., Hong, W., 2004. Cellular RNA helicase p68 relocalization and interaction with the hepatitis C virus (HCV) NS5B protein and the potential role of p68 in HCV RNA replication. *J. Virol.* 78 (10), 5288–5298.
- Guo, J.T., Bichko, V.V., Seeger, C., 2001. Effect of alpha interferon on the hepatitis C virus replicon. *J. Virol.* 75 (18), 8516–8523.
- Hamamoto, I., Nishimura, Y., Okamoto, T., Aizaki, H., Liu, M., Mori, Y., Abe, T., Suzuki, T., Lai, M.M., Miyamura, T., Moriishi, K., Matsuura, Y., 2005. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. *J. Virol.* 79 (21), 13473–13482.
- Hansen, J.L., Long, A.M., Schultz, S.C., 1997. Structure of the RNA-dependent RNA polymerase of poliovirus. *Structure* 5 (8), 1109–1122.
- Hara, H., Aizaki, H., Matsuda, M., Shinkai-Ouchi, F., Inoue, Y., Murakami, K., Shoji, I., Kawakami, H., Matsuura, Y., Lai, M.M., Miyamura, T., Wakita, T., Suzuki, T., 2009. Involvement of creatine kinase B in hepatitis C virus genome replication through interaction with the viral NS4A protein. *J. Virol.* 83 (10), 5137–5147.
- Hardy, R.W., Marcotrigiano, J., Blight, K.J., Majors, J.E., Rice, C.M., 2003. Hepatitis C virus RNA synthesis in a cell-free system isolated from replicon-containing hepatoma cells. *J. Virol.* 77 (3), 2029–2037.
- Hirano, M., Kaneko, S., Yamashita, T., Luo, H., Qin, W., Shiota, Y., Nomura, T., Kobayashi, K., Murakami, S., 2003. Direct interaction between nucleolin and hepatitis C virus NS5B. *J. Biol. Chem.* 278 (7), 5109–5115.
- Hoofnagle, J.H., 2002. Course and outcome of hepatitis C. *Hepatology* 36 (5 Suppl 1), S21–S29.
- Hu, J., Flores, D., Toft, D., Wang, X., Nguyen, D., 2004. Requirement of heat shock protein 90 for human hepatitis B virus reverse transcriptase function. *J. Virol.* 78 (23), 13122–13131.
- Huang, H., Chopra, R., Verdine, G.L., Harrison, S.C., 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* 282 (5394), 1669–1675.
- Ichimura, T., Yamamura, H., Sasamoto, K., Tominaga, Y., Taoka, M., Kakiuchi, K., Shinkawa, T., Takahashi, N., Shimada, S., Isobe, T., 2005. 14-3-3 proteins modulate the expression of epithelial Na<sup>+</sup> channels by phosphorylation-dependent interaction with Nedd4-2 ubiquitin ligase. *J. Biol. Chem.* 280 (13), 13187–13194.
- Ikeda, M., Yi, M., Li, K., Lemon, S.M., 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. *J. Virol.* 76 (6), 2997–3006.
- Kampmüller, K.M., Miller, D.J., 2005. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in *Drosophila* cells. *J. Virol.* 79 (11), 6827–6837.
- Kim, S.J., Kim, J.H., Kim, Y.G., Lim, H.S., Oh, J.W., 2004. Protein kinase C-related kinase 2 regulates hepatitis C virus RNA polymerase function by phosphorylation. *J. Biol. Chem.* 279 (48), 50031–50041.
- Kukihara, H., Moriishi, K., Tagawa, S., Tani, H., Abe, T., Mori, Y., Suzuki, T., Fukushima, T., Taketomi, A., Maehara, Y., Matsuura, Y., 2009. Human VAP-C negatively regulates hepatitis C virus propagation. *J. Virol.* 83 (16), 7959–7969.
- Kyono, K., Miyashiro, M., Taguchi, I., 2002. Human eukaryotic initiation factor 4AII associates with hepatitis C virus NS5B protein in vitro. *Biochem. Biophys. Res. Commun.* 292 (3), 659–666.
- Laporte, M.G., Jackson, R.W., Draper, T.L., Gaboury, J.A., Galie, K., Herbertz, T., Hussey, A.R., Rippin, S.R., Benetatos, C.A., Chunduru, S.K., Christensen, J.S., Coburn, G.A., Rizzo, C.J., Rhodes, G., O'Connell, J., Howe, A.Y., Mansour, T.S., Collett, M.S., Pevsner, D.C., Young, D.C., Gao, T., Tyrrell, D.L., Kneteman, N.M., Burns, C.J., Condon, S.M., 2008. The discovery of pyranoside [3, 4-b] indole-based allosteric inhibitors of HCV NS5B polymerase with in vivo activity. *ChemMedChem* 3 (10), 1508–1515.
- Liu, H.M., Aizaki, H., Choi, K.S., Machida, K., Ou, J.J., Lai, M.M., 2009. SYNCRIP (synaptotagmin-binding, cytoplasmic RNA-interacting protein) is a host factor involved in hepatitis C virus RNA replication. *Virology* 386 (2), 249–256.
- Lohmann, V., Körner, F., Herian, U., Bartenschlager, R., 1997. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J. Virol.* 71 (11), 8416–8428.
- Lohmann, V., Roos, A., Körner, F., Koch, J.O., Bartenschlager, R., 2000. Biochemical and structural analysis of the NS5B RNA-dependent RNA polymerase of the hepatitis C virus. *J. Viral Hepat.* 7 (3), 167–174.
- Manns, M.P., Wedemeyer, H., Cornberg, M., 2006. Treating viral hepatitis C: efficacy, side effects, and complications. *Gut* 55 (9), 1350–1359.
- Meyer, A.S., Gillespie, J.R., Walther, D., Millet, L.S., Doniach, S., Frydman, J., 2003. Closing the folding chamber of the eukaryotic chaperonin requires the transition state of ATP hydrolysis. *Cell* 113 (3), 369–381.
- Momose, F., Naito, T., Yano, K., Sugimoto, S., Morikawa, Y., Nagata, K., 2002. Identification of Hsp90 as a stimulatory host factor involved in influenza virus RNA synthesis. *J. Biol. Chem.* 277 (47), 45306–45314.
- Moradpour, D., Bieck, E., Hügle, T., Wels, W., Wu, J.Z., Hong, Z., Blum, H.E., Bartenschlager, R., 2002. Functional properties of a monoclonal antibody inhibiting the hepatitis C virus RNA-dependent RNA polymerase. *J. Biol. Chem.* 277 (1), 593–601.
- Moriishi, K., Matsuura, Y., 2007. Host factors involved in the replication of hepatitis C virus. *Rev. Med. Virol.* 17 (5), 343–354.
- Murakami, K., Ishii, K., Ishihara, Y., Yoshizaki, S., Tanaka, K., Gotoh, Y., Aizaki, H., Kohara, M., Yoshioka, H., Mori, Y., Manabe, N., Shoji, I., Sata, T., Bartenschlager, R., Matsuura, Y., Miyamura, T., Suzuki, T., 2006. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. *Virology* 351 (2), 381–392.
- Naito, T., Momose, F., Kawaguchi, A., Nagata, K., 2007. Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. *J. Virol.* 81 (3), 1339–1349.



- Nakagawa, S., Umehara, T., Matsuda, C., Kuge, S., Sudoh, M., Kohara, M., 2007. Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. *Biochem. Biophys. Res. Commun.* 353 (4), 882–888.
- Nelson, H.B., Tang, H., 2006. Effect of cell growth on hepatitis C virus (HCV) replication and a mechanism of cell confluence-based inhibition of HCV RNA and protein expression. *J. Virol.* 80 (3), 1181–1190.
- Niwa, H., Yamamura, K., Miyazaki, J., 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108 (2), 193–199.
- Okamoto, T., Nishimura, Y., Ichimura, T., Suzuki, K., Miyamura, T., Suzuki, T., Moriishi, K., Matsuura, Y., 2006. Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *EMBO J.* 25 (20), 5015–5025.
- Okamoto, T., Omori, H., Kaname, Y., Abe, T., Nishimura, Y., Suzuki, T., Miyamura, T., Yoshimori, T., Moriishi, K., Matsuura, Y., 2008. A single-amino-acid mutation in hepatitis C virus NSSA disrupting FKBP8 interaction impairs viral replication. *J. Virol.* 82 (7), 3480–3489.
- Parent, R., Qu, X., Petit, M.A., Beretta, L., 2009. The heat shock cognate protein 70 is associated with hepatitis C virus particles and modulates virus infectivity. *Hepatology* 49 (6), 1798–1809.
- Pietschmann, T., Lohmann, V., Rutter, G., Kurpanek, K., Bartenschlager, R., 2001. Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. *J. Virol.* 75 (3), 1252–1264.
- Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., Strand, D., Bartenschlager, R., 2002. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J. Virol.* 76 (8), 4008–4021.
- Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q.L., Houghton, M., Kuo, G., 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl Acad. Sci. USA* 87 (17), 6547–6549.
- Seeff, L.B., Hoofnagle, J.H., 2003. Appendix: the National Institutes of Health Consensus Development Conference: management of hepatitis C 2002. *Clin. Liver Dis.* 7 (1), 261–287.
- Shi, S.T., Lee, K.J., Aizaki, H., Hwang, S.B., Lai, M.M., 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. *J. Virol.* 77 (7), 4160–4168.
- Shimakami, T., Honda, M., Kusakawa, T., Murata, T., Shimotohno, K., Kaneko, S., Murakami, S., 2006. Effect of hepatitis C virus (HCV) NS5B-nucleolin interaction on HCV replication with HCV subgenomic replicon. *J. Virol.* 80 (7), 3332–3340.
- Shirakura, M., Murakami, K., Ichimura, T., Suzuki, R., Shimoji, T., Fukuda, K., Abe, K., Sato, S., Fukasawa, M., Yamakawa, Y., Nishijima, M., Moriishi, K., Matsuura, Y., Wakita, T., Suzuki, T., Howley, P.M., Miyamura, T., Shoji, I., 2007. E6AP ubiquitin ligase mediates ubiquitination and degradation of hepatitis C virus core protein. *J. Virol.* 81 (3), 1174–1185.
- Suzuki, T., Ishii, K., Aizaki, H., Wakita, T., 2007. Hepatitis C viral life cycle. *Adv. Drug Deliv. Rev.* 59 (12), 1200–1212.
- Tagawa, S., Okamoto, T., Abe, T., Mori, Y., Suzuki, T., Moriishi, K., Matsuura, Y., 2008. Human butyrate-induced transcript 1 interacts with hepatitis C virus NSSA and regulates viral replication. *J. Virol.* 82 (6), 2631–2641.
- Tagawa, S., Kambara, H., Omori, H., Tani, H., Abe, T., Mori, Y., Suzuki, T., Yoshimori, T., Moriishi, K., Matsuura, Y., 2009. Cochaperone activity of human butyrate-induced transcript 1 facilitates hepatitis C virus replication through an Hsp90-dependent pathway. *J. Virol.* 83 (20), 10427–10436.
- Tian, G., Vainberg, I.E., Tap, W.D., Lewis, S.A., Cowan, N.J., 1995. Specificity in chaperonin-mediated protein folding. *Nature* 375 (6528), 250–253.
- Tu, H., Gao, L., Shi, S.T., Taylor, D.R., Yang, T., Mircheff, A.K., Wen, Y., Gorbalenya, A.E., Hwang, S.B., Lai, M.M., 1999. Hepatitis C virus RNA polymerase and NSSA complex with a SNARE-like protein. *Virology* 263 (1), 30–41.
- Ujino, S., Yamaguchi, S., Shimotohno, K., Takaku, H., 2009. Heatshock protein 90 is essential for stabilization of the hepatitis C virus nonstructural protein NS3. *J. Biol. Chem.* 284 (11), 6841–6846.
- Valpuesta, J.M., Martín-Benito, J., Gómez-Puertas, P., Carrascosa, J.L., Willison, K.R., 2002. Structure and function of a protein folding machine: the eukaryotic cytosolic chaperonin CCT. *FEBS Lett.* 529 (1), 11–16.
- Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11 (7), 791–796.
- Watashi, K., Ishii, N., Hijikata, M., Inoue, D., Murata, T., Miyazawa, Y., Shimotohno, K., 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol. Cell* 19 (1), 111–122.
- Weng, L., Du, J., Zhou, J., Ding, J., Wakita, T., Kohara, M., Toyoda, T., 2009. Modification of hepatitis C virus 1b RNA polymerase to make a highly active JFH1-type polymerase by mutation of the thumb domain. *Arch. Virol.* 154 (5), 765–773.
- Yaffe, M.B., Farr, G.W., Miklos, D., Horwich, A.L., Sternlicht, M.L., Sternlicht, H., 1992. TCP1 complex is a molecular chaperone in tubulin biogenesis. *Nature* 358 (6383), 245–248.
- Yam, A.Y., Xia, Y., Lin, H.T., Burlingame, A., Gerstein, M., Frydman, J., 2008. Defining the TriC/CCT interactome links chaperonin function to stabilization of newly made proteins with complex topologies. *Nat. Struct. Mol. Biol.* 15 (12), 1255–1262.
- Yanagida, M., Miura, Y., Yagasaki, K., Taoka, M., Isobe, T., Takahashi, N., 2000. Matrix assisted laser desorption/ionization-time of flight-mass spectrometry analysis of proteins detected by anti-phosphotyrosine antibody on two-dimensional gels of fibroblast cell lysates after tumor necrosis factor- $\alpha$  stimulation. *Electrophoresis* 21 (9), 1890–1898.
- Yang, G., Pevear, D.C., Collett, M.S., Chunduru, S., Young, D.C., Benetatos, C., Jordan, R., 2004. Newly synthesized hepatitis C virus replicon RNA is protected from nuclease activity by a protease-sensitive factor(s). *J. Virol.* 78 (18), 10202–10205.