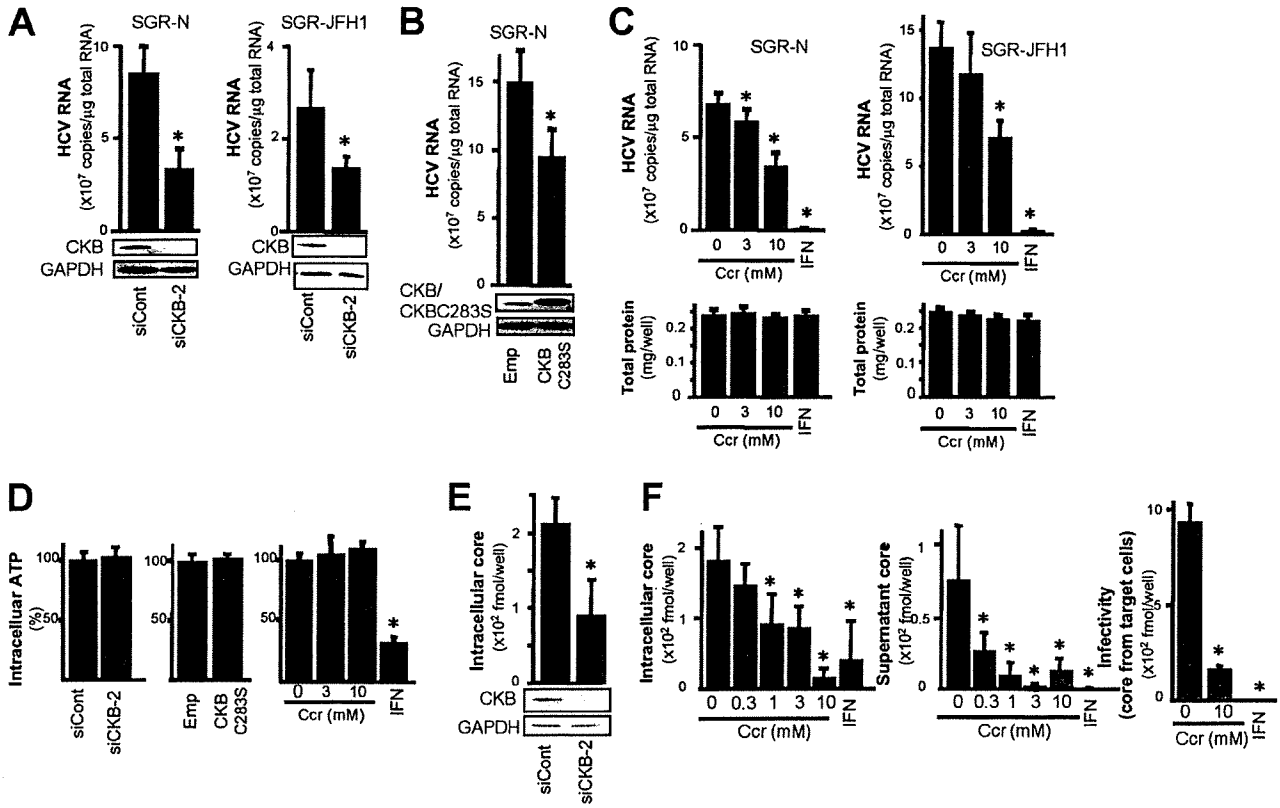


FIG. 2. Involvement of CKB in HCV replication. (A and E) Knockdown of endogenous CKB in SGR-N and SGR-JFH1 cells (A) or HCVcc-infected cells (E). Cells were transfected with siRNA against CKB (siCKB-2) or control siRNA (siCont) and were harvested at 72 h posttransfection. Real-time RT-PCR for HCV RNA levels and immunoblotting for CKB and GAPDH were performed. (B) SGR-N cells were transfected with pCAGCKB-C283S or empty vector, and HCV RNA levels and expression of CKB and CKB-C283S were determined 72 h posttransfection. SGR-N and SGR-JFH1 cells (C) or HCVcc-infected cells (F) were treated with Ccr at various concentrations for 72 h, followed by quantification of HCV RNAs and total cellular proteins. ATP levels (D) were determined after transfection with siCKB-2, pCAGCKB-C283S, or treatment with Ccr for 72 h in SGR-N cells. The ATP levels in the cells transfected with negative control siRNA (left), empty vector (middle), and no treatment (right) were set at 100%, respectively. (F) HCVcc-infected cells were treated with Ccr, and the viral core protein levels in cells (left) and supernatants (middle) were determined at 72 h postinfection. Collected culture supernatants were inoculated into naive Huh-7.5.1 cells after the removal of Ccr. After 72 h, the core proteins in cells were determined (right panel). All data are presented as averages and standard deviation values for at least triplicate samples. *, $P < 0.05$ against control such as transfection with siCont (A and E) or empty vector (B) or nontreatment (C, D, and F).

ported that interaction of CKB with some cellular proteins is required for local availability of CKB activity and local generation of ATP (22, 29). To examine the possible interaction of CKB with HCV NS proteins, HA-tagged CKB (HA-CKB) was coexpressed with FLAG-tagged NS proteins (NIHJ1 strain), followed by immunoprecipitation with an anti-FLAG antibody. CKB was shown to specifically interact with NS4A. No or little interaction was observed between CKB and either NS3, NS4B, NS5A, or NS5B (Fig. 3B). CKB-NS4A interaction was also found with the JFH-1 strain (Fig. 3C).

To identify the CKB region required for the interaction with NS4A, various deletion mutants of CKB were generated (Fig. 3A). An immunoprecipitation assay indicated that NS4A was coimmunoprecipitated with either a full-length CKB, a C-terminal deletion (aa 1 to 357), an N-terminal deletion (aa 297 to 381), or CKB-C283S, but not with aa 1 to 296, aa 1 to 247, or aa 1 to 184 (Fig. 3D, upper middle panel). Further, internal deletions of CKB (CKBdel297-357 and CKB-C283Sdel297-357) failed to interact with NS4A (Fig. 3D, lower panel), suggesting that aa 297 to 357 of CKB are important for its interaction with NS4A. It is noted that the expression of CKB aa 297 to 357 in cells was undetected, presumably due to its misfolding and/or instability. To verify a role for CKB-NS4A interaction in HCV RNA replication, we further determined the effect of expression of either CKB-C283S or its internal deletion lacking aa 297 to 357 (CKB-C283Sdel297-357) on viral replication in SGR-JFH1 cells. As expected, the HCV RNA level was significantly decreased by CKB-C283S, whereas this effect was not observed by CKB-C283Sdel297-357 (Fig. 3E).

NS4A is a 54-residue small protein composed of three domains: the N-terminal membrane anchor, the central domain responsible for interacting with NS3, and the C-terminal acidic domain. To define the portion in NS4A responsible for its interaction with CKB, we constructed three NS4A deletion mutants, each separately expressing one of the NS4A domains, with a FLAG tag (Fig. 3F). CKB proved to interact with the central domain, aa 21 to 39, of NS4A, which is involved in

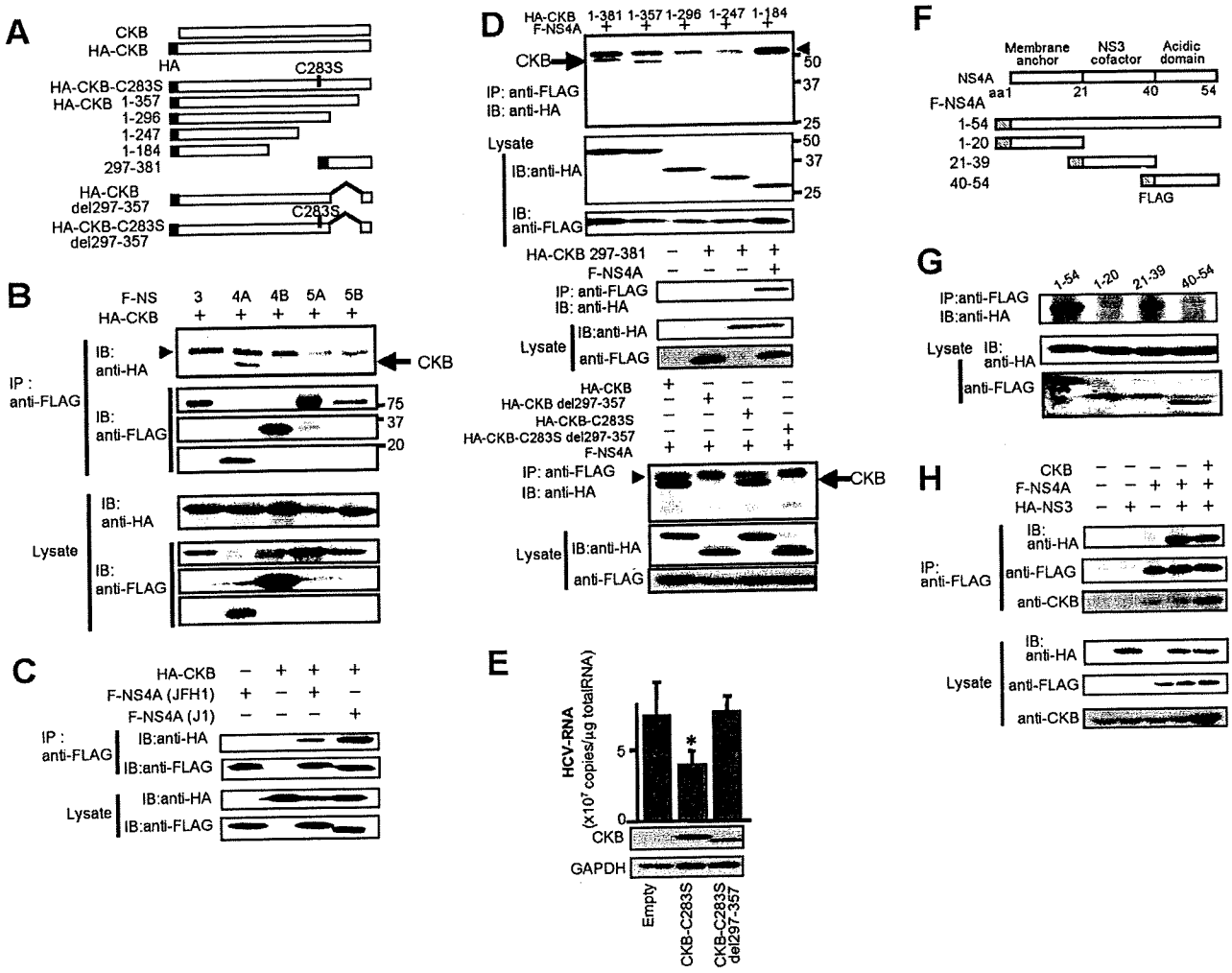


FIG. 3. CKB interacts with HCV NS4A. (A) Structures of CKB constructs used in the present study. A full-length wild-type CKB without an epitope tag (CKB) or with an N-terminal HA tag (HA-CKB), HA-CKB with deletions (aa 1 to 357, aa 1 to 296, aa 1 to 247, aa 1 to 184, and aa 297 to 381 and del297-357), CKB mutant at the catalytic site, Cys-283 (CKB-C283S) or CKB-C283S lacking aa 297 to 357 (CKB-C283Sdel297-357) are shown. HA-CKB was coexpressed with FLAG-tagged versions of each NS protein of strain NIHJ1 (B) or with NS4A of strain JFH-1 (C) in 293T cells and immunoprecipitated (IP) with an anti-FLAG antibody. Immunoprecipitates were subjected to immunoblotting (IB) with anti-HA or anti-FLAG antibody. (D) Each CKB deletion mutant was coexpressed with FLAG-NS4A in 293T cells. Immunoprecipitates were analyzed by immunoblotting. Arrow, CKB; arrowhead, immunoglobulin heavy chain. (E) SGR-JFH1 cells were transfected with the expression plasmid for CKB-C283S, CKB-C283Sdel297-357 or empty vector. At 72 h posttransfection, HCV RNA levels and the expression of CKB and CKB-C283S were determined by real-time RT-PCR and immunoblotting with anti-HA antibody, respectively. For HCV RNA quantitation, data are indicated as averages and standard deviations ($n = 3$). *, $P < 0.05$ against the empty vector control. (F) Structure of NS4A and NS4A constructs. FLAG-tagged NS4A (aa 1 to 54) or its truncated mutants (aa 1 to 20, aa 21 to 39, or aa 40 to 54) are shown. (G) Each NS4A deletion mutant was coexpressed with HA-CKB and analyzed as described above. (H) FLAG-NS4A was coexpressed with HA-NS3 or HA-NS3 and CKB, followed by immunoprecipitation with anti-FLAG antibody. Immunoprecipitates were analyzed by immunoblotting with anti-HA, anti-FLAG or anti-CKB antibody.

formation of the NS3-NS4A complex (Fig. 3G). We therefore investigate whether NS3-NS4A interaction is affected in the presence of CKB and found that exogenous expression of CKB has no influence on NS3-NS4A interaction, and a putative NS3-NS4A-CKB complex was detected in the coimmunoprecipitation analysis (Fig. 3H). Collectively, these results strongly suggest that CKB plays a key role in HCV RNA replication via interaction with NS4A.

Subcellular localization of CKB and NS4A in cells replicating HCV RNA. CKB is distributed throughout cells but is mainly localized in the perinuclear area (31), whereas NS4A is predominantly localized at the endoplasmic reticulum and mitochondrial membranes (37). We examined the possible subcellular colocalization of CKB and NS4A in SGR-N cells by immunofluorescence staining (Fig. 4A). CKB tended to gather in the perinuclear area of HCV replicating cells and was partially colocalized with NS4A in the area, sharing a dotlike structure. To further analyze the subcellular compartments in which CKB and NS4A coexist, we used double-labeling immunoelectron microscopy on SGR-N cells using antibodies against CKB and NS4A, with secondary antibodies coupled to 12- and 18-nm gold particles, respectively. One fraction of

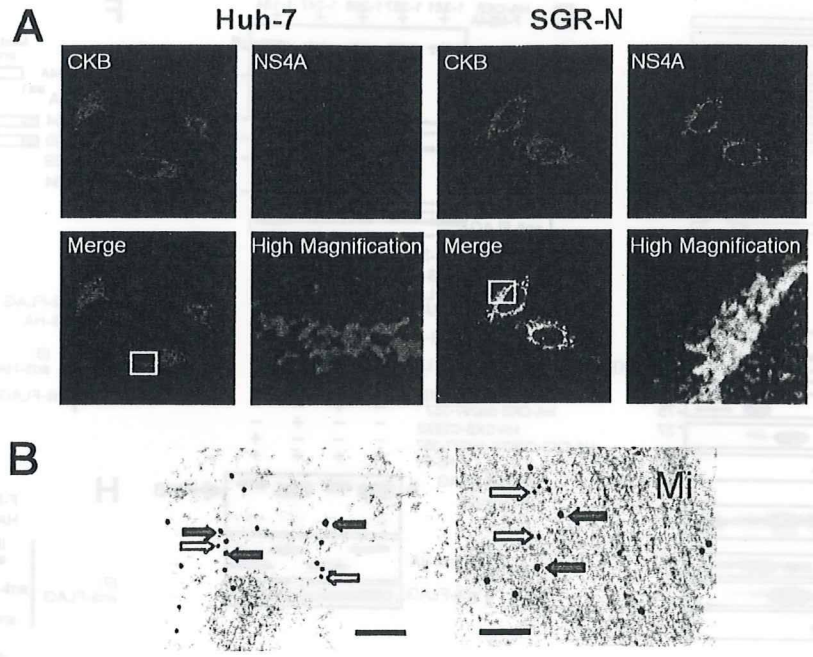


FIG. 4. Colocalization of CKB with HCV NS4A. (A) Indirect immunofluorescence analysis. The primary antibodies used were anti-CKB goat PAb (red) and anti-NS4A MAb (green). Merged images of red and green signals are shown. High-magnification panels are enlarged images of white squares in the merge panels. (B) Immunoelectron microscopic localization of CKB and NS4A. SGR-N cells were double-immunolabeled for CKB (12-nm gold particles; white arrows) and for NS4A (18-nm gold particle; gray arrows). Mi, mitochondria. Bars, 200 nm.

CKB colocalized with NS4A in the cytoplasmic electron-dense regions, presumably derived from altered or folded membrane structures (Fig. 4B, left panel) and mitochondria (Fig. 4B, right panel).

CKB enhances functional HCV replicase and NS3-4A helicase. NS4A is known to mediate membrane association of the NS3-4A complex and to function as a cofactor in NS3 enzyme activity. To understand the mechanism(s) underlying positive regulation of HCV RNA replication through CKB via its interaction with NS4A, we first investigated whether CKB modulates NS3-4A helicase activity. NS3-4A helicase is a member of the superfamily-2 DexH/D-box helicase, which unwinds RNA-RNA substrates in a 3′-to-5′ direction. During RNA replication, the NS3-4A helicase is believed to translocate along the nucleic acid substrate by changing its protein conformation, utilizing the energy of ATP hydrolysis (9). We then tested the effect of CKB on RNA- or DNA-unwinding activity using purified recombinant full-length NS3 and NS3-4A complex (12). As shown in Fig. 5A (left middle panel), both NS3 and NS3-4A helicase activity unwound dsRNA substrate most efficiently when CKB, ATP, and pCr were added to the reaction mixture. The enhancing effect of CKB was observed in the presence of pCr but not in the absence of it, suggesting that catalytic activity of CKB is important for its effect on the HCV helicase activity. Similar results were obtained from the DNA helicase assay using dsDNA substrate (Fig. 5B). To address the specificity of the stimulation by the CKB/pCr system, effects of PK and pPy, which are also involved in the ATP generation, were determined (Fig. 5A, right panels). Exogenous PK and pPy at the same concentrations as those of CKB and pCr used in the study exhibited no effect on the HCV helicase activity.

The effect of CKB on NS3-4A serine protease activity, which is considered to be ATP-independent, was also assessed in an in vitro protease assay using the purified viral proteins as mentioned above (Fig. 5C). As expected, NS3-4A complex exhibited significantly higher activity than NS3 alone; however, CKB did not affect the protease activities of NS3 or NS3-4A.

Finally, we investigated loss and gain of function of CKB in HCV replicase activity, which requires high-energy phosphate, in the context of semi-intact replicon cells. Miyanari et al. (33) reported that the function of the active HCV RC can be monitored in permeabilized replicon cells treated with digitonin. Thus, permeabilized replicon cells in the presence or absence of exogenous CKB were incubated with [α-^{32}P]UTP to detect newly synthesized RNA. As indicated in Fig. 5D, an ~8-kb band corresponding to HCV subgenomic RNA was most abundant in cells in the presence of exogenous CKB, ATP and pCr. The enhancing effect of CKB was observed in the presence but not in the absence of pCr, suggesting that catalytic activity of CKB is important for its effect on the replicase activity. As for the RNA helicase assay, exogenous PK and pPy did not enhance the replicase activity (data not shown). HCV replicase activity in permeabilized cells to which we had introduced siCKB-2 was diminished compared to that in siRNA control-treated cells. Interestingly, the replicase activity in the CKB-depleted cells was recovered by the addition of CKB. Thus, our findings suggest that CKB functions as a key regulator of HCV genome replication by controlling energy-dependent viral enzyme activities.

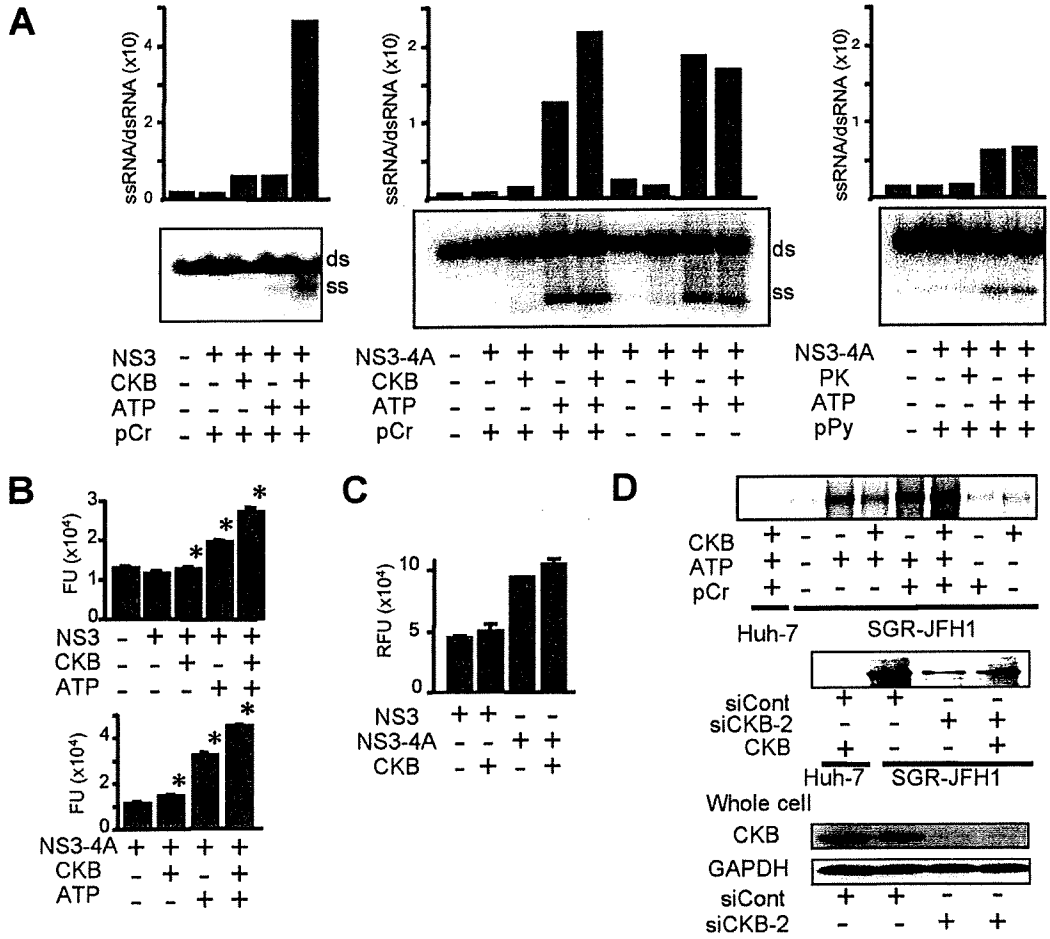


FIG. 5. CKB enhances NS3-4A helicase and HCV replicase activities. (A) In vitro RNA helicase activity of NS3-4A or NS3 was determined by detecting unwound single-strand RNA (ss) derived from the partially dsRNA substrate (ds). Band intensities corresponding to unwound products and those to dsRNA substrates were determined by ImageQuant 5.2 (Molecular Dynamics), and the ssRNA/dsRNA ratios were calculated. The results are representative of three similar experiments. (B) In vitro DNA helicase activity of NS3-4A or NS3 was analyzed by using a commercially available kit. The data represent averages and standard deviations ($n = 3$). *, $P < 0.05$ against the value without supplementation of CKB and ATP. (C) The in vitro HCV protease activity of NS3-4A or NS3 in the presence or absence of CKB was analyzed. Error bars represent standard deviations ($n = 3$). (D) Replicase activity in permeabilized replicon cells. The upper panel shows the activity for synthesis of HCV subgenomic RNA in the digitonin-permeabilized SGR-JFH1 cells with or without supplementation of CKB was measured. The middle panel shows results for SGR-JFH1 or Huh-7 cells that were transfected with siCKB-2 or siCont and permeabilized at 72 h posttransfection. The permeabilized cells with or without supplementation of CKB were subjected to the replicase assay. The lower panel shows the immunoblotting results for whole-cell lysates of siRNA-transfected cells.

DISCUSSION

Viral replication requires energy and macromolecule synthesis, and host cells provide the viruses with metabolic resources necessary for their efficient replication. Thus, it is highly likely that interaction of viruses with host cell metabolic pathways, including energy-generating systems, contributes to the virus growth cycle. In the regulation of HCV genome replication, the functions of the viral NS proteins that comprise the RC might be regulated by association in individual host cell factors. For example, hVAP-A and -B function as cofactors of modulating RC formation via interacting with NS5A and NS5B (13, 18). Cyclophilin B is involved in stimulating viral RNA binding activity via interacting with NS5B (49). FKBP8 (39) and hB-ind1 (45) play an important role in recruiting Hsp90 to RC via interacting with NS5A. However, the association of viral protein(s) with the cellular energy-generating system to directly regulate the activity of the RC has not been well understood.

In the present study, the accumulation of CKB, an ATP-generating enzyme, in the HCV RC-rich membrane fraction of viral replicating cells and its importance in replication of the HCV genome and production of infectious virions have been demonstrated. Enzymatic analyses with semi-intact replicon cells and purified NS3-4A protein revealed that CKB enhances the functional replicase and helicase of HCV. Its enhancing effect was observed in the presence of pCr but not in its absence, suggesting that the catalytic activity of CKB is important for enhancing the replicase and

helicase activities. Moreover, we clearly detected a CKB-NS4A complex using anti-tag antibodies in cotransfection experiments, but the endogenous complex could not be immunoprecipitated from cells expressing only endogenous levels of CKB, probably because of the inefficiency of the available antibodies. Further, a deletion of the NS4A-interacting region within an inactive mutant of CKB (CKB-C283S) resulted in the loss of its dominant-negative effect on HCV replication.

Creatine kinase, an evolutionarily conserved enzyme, is known to be critical for the maintenance and regulation of cellular energy stores in tissues with high and rapidly changing energy demands (48). In mammals, three cytosolic and two mitochondrial isoforms of CK, which share certain conserved regions, are expressed (35). The brain-type CK, CKB, plays a major role in cellular energy metabolism of nonmuscle cells, reversibly catalyzing the ATP-dependent phosphorylation of creatine and, hence, providing an ATP buffering system in subcellular compartments of high and fluctuating energy demand (21, 29). CKB is overexpressed in a wide range of tumor tissues and tumor cell lines, including hepatocellular carcinoma (32), and is used as a prognostic marker of cancer.

Although CK and creatine phosphate have been supplemented to in vitro replicase assays of some RNA viruses (15, 33), understanding of CKB function in the virus life cycle has been limited. One study indicated that the CK substrate analog, Ccr, exhibits antiviral activity against several herpesviruses but not influenza viruses or vesicular stomatitis virus (26). We have demonstrated here that HCV genome replication is downregulated by either treatment with Ccr, siRNA-mediated knockdown of CKB, or the exogenous expression of CKB-C283S. Coimmunoprecipitation experiments revealed that the essential domain within NS4A for the interaction with CKB is the NS4A central domain, aa 21 to 39, which is also responsible for NS3-4A complex formation. However, the NS3-4A interaction was not impaired by overexpression of CKB, and CKB was found to be able to form a complex with NS3-4A (Fig. 3H). Since CKB does not directly interact with NS3 (Fig. 3A), it is likely that NS3-4A-CKB association occurs through two interactions of NS3-4A and NS4A-CKB. We examined whether the formation of the ternary complex affects HCV enzymatic activities, possibly through conformational changes in the viral proteins, and found that CKB has no influence on NS3-4A protease activity (Fig. 5C). With regard to helicase activity, the effect of CKB on RNA unwinding activity by NS3-4A was similar to the effect of NS3 alone in the presence of ATP (Fig. 5A). It is conceivable that interaction with CKB causes no or little global change in the NS3-4A conformation and does not affect the viral helicase and protease activities.

In general, translation initiation in eukaryotes includes an ATP-dependent process such as unwinding the secondary structure in the 5′-untranslated region to permit assembly of 48S ribosomal complexes. It was reported, however, that 48S complex formation on the HCV internal ribosome entry site (IRES) has no requirement for ATP hydrolysis (25). In fact, we found that Huh-7 cells with or without gene silencing of CKB exhibited the same level of HCV IRES activity by transfection with IRES-reporter constructs (data not shown).

Collectively, we conclude that CKB is targeted to the HCV RC through its interaction with NS4A and functions as a positive regulator for the viral replicase by providing ATP. It is likely that the catalytic activity of CKB that associates with the viral RC is important for enhancing the RNA replication. The role of CKB-NS4A interaction in the enhancing effect seems to be limited. Although either knocking down CKB, expression of the dominant-negative mutant of CKB, or Ccr treatment resulted in the reduction of HCV replication (Fig. 2A to C), the total cellular ATP levels were not changed under these conditions (Fig. 2D). This suggests that CKB contributes to enhancing HCV replication through controlling the ATP level in the particular RC compartment. A tight coupling of a fast ATP regeneration and delivery system to the viral RC is advantageous for achieving efficient replication of the viral genome. To our knowledge, the findings presented here provide the first experimental evidence of the involvement of viral protein in recruiting an ATP generating/buffering system to the subcellular compartment for viral genome replication, a site with high-energy turnover. Given that the levels of HCV RNA were not dramatically diminished by the knocking down, dominant-negative mutant or Ccr, CKB may not be absolutely critical for the viral replication. One would argue that energy required for HCV genome replication can be partly complemented from the intracellular ATP pool.

Although there are several isoforms of CK as described above, the most abundant CK species expressed in Huh-7 cells in the present study was CKB, and no other isoenzymes, including mitochondrial CK, were detected by an isoform analysis based on the overlay gel technique (32; data not shown). Thus, the CKB isoenzyme appears to be a key molecule in the energy metabolism of HCV replicating cells. To identify potential HCV RC components, we used a comparative proteome analysis of the DRM fraction in cells harboring HCV subgenomic replicon and the DRM fractions in parental cells and then identified proteins that were more abundant in the fraction of HCV replicating cells. In agreement with similar previously reported approaches using the DRM or lipid raft fraction (30, 53), the functional categories of identified proteins included protein folding or assembly, cell metabolism and biosynthesis, cellular processes, and cytoskeleton organization (Table 1). Interestingly, Mannova et al. found that CKB was upregulated in the fraction of Huh-7 cells carrying the genotype 1b Con1 isolate-derived HCV replicon, as determined using stable isotope labeling by amino acids combined with one-dimensional electrophoresis (30). However, the effect of CKB on regulation of the HCV life cycle was not examined in that study.

In conclusion, CKB interacts with HCV NS4A and is important for efficient replication of the viral genome. Recruitment of CKB to the HCV replication machinery through its interaction with NS4A may have important implications for the maintenance or enhancement of the functional replicase activity in the RC compartment, where high-energy phosphoryl groups are required. A strategy for specific interception of energy supply at the subcellular site of HCV genome replication by disruption of the NS4A-CKB interface may lead to development of a new type of antiviral agent.

ACKNOWLEDGMENTS

We thank Francis V. Chisari (The Scripps Research Institute) for providing Huh-7.5.1 cells; Raffaele De Francesco (Istituto di Ricerche

di Biologia Molecolare, P. Angeletti) for providing purified recombinant NS3 and NS3-4A proteins; Oriental Yeast Co., Ltd., for providing human CKB cDNA; Minoru Fukuda (Laboratory for Electron Microscopy, Kyorin University School of Medicine) for electron microscopy; S. Yoshizaki, T. Shimoji, M. Kaga, M. Sasaki, and T. Date for technical assistance; and T. Mizoguchi for secretarial work.

This study was supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labor, and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science, and Technology 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.

REFERENCES

1. **Aizaki, H., Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsuura, and T. Miyamura.** 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. Hepatology **27:** 621–627.
2. **Aizaki, H., K. J. Lee, V. M. Sung, H. Ishiko, and M. M. Lai.** 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology **324:**450–461.
3. **Aizaki, H., K. Morikawa, M. Fukasawa, H. Hara, Y. Inoue, H. Tani, K. Saito, M. Nishijima, K. Hanada, Y. Matsuura, M. M. Lai, T. Miyamura, T. Wakita, and T. Suzuki.** 2008. Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J. Virol. **82:**5715–5724.
4. **Alter, H. J., and L. B. Seeff.** 2000. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. Semin. Liver Dis. **20:**17–35.
5. **Aoyagi, K., C. Ohue, K. Iida, T. Kimura, E. Tanaka, K. Kiyosawa, and S. Yagi.** 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J. Clin. Microbiol. **37:**1802–1808.
6. **Appel, N., T. Schaller, F. Penin, and R. Bartenschlager.** 2006. From structure to function: new insights into hepatitis C virus RNA replication. J. Biol. Chem. **281:**9833–9836.
7. **Bartenschlager, R., and V. Lohmann.** 2001. Novel cell culture systems for the hepatitis C virus. Antivir. Res. **52:**1–17.
8. **Brass, V., J. M. Berke, R. Montserret, H. E. Blum, F. Penin, and D. Moradpour.** 2008. Structural determinants for membrane association and dynamic organization of the hepatitis C virus NS3-4A complex. Proc. Natl. Acad. Sci. USA.
9. **Dumont, S., W. Cheng, V. Serebrov, R. K. Beran, I. Tinoco, Jr., A. M. Pyle, and C. Bustamante.** 2006. RNA translocation and unwinding mechanism of HCV NS3 helicase and its coordination by ATP. Nature **439:**105–108.
10. **Failla, C., L. Tomei, and R. De Francesco.** 1994. Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. J. Virol. **68:**3753–3760.
11. **Gallinari, P., D. Brennan, C. Nardi, M. Brunetti, L. Tomei, C. Steinkuhler, and R. De Francesco.** 1998. Multiple enzymatic activities associated with recombinant NS3 protein of hepatitis C virus. J. Virol. **72:**6758–6769.
12. **Gallinari, P., C. Paolini, D. Brennan, C. Nardi, C. Steinkuhler, and R. De Francesco.** 1999. Modulation of hepatitis C virus NS3 protease and helicase activities through the interaction with NS4A. Biochemistry **38:**5620–5632.
13. **Gao, L., H. Aizaki, J. W. He, and M. M. Lai.** 2004. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. J. Virol. **78:**3480–3488.
14. **Gosert, R., D. Egger, V. Lohmann, R. Bartenschlager, H. E. Blum, K. Bienz, and D. Moradpour.** 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J. Virol. **77:**5487–5492.
15. **Green, K. Y., A. Mory, M. H. Fogg, A. Weisberg, G. Belliot, M. Wagner, T. Mitra, E. Ehrenfeld, C. E. Cameron, and S. V. Sosnovtsev.** 2002. Isolation of enzymatically active replication complexes from feline calicivirus-infected cells. J. Virol. **76:**8582–8595.
16. **Guidotti, L. G., and F. V. Chisari.** 2006. Immunobiology and pathogenesis of viral hepatitis. Annu. Rev. Pathol. **1:**23–61.
17. **Guo, J. T., V. V. Bichko, and C. Seeger.** 2001. Effect of alpha interferon on the hepatitis C virus replicon. J. Virol. **75:**8516–8523.
18. **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.
19. **Hoofnagle, J. H.** 2002. Course and outcome of hepatitis C. Hepatology **36:**S21–S29.
20. **Ichimura, T., H. Yamamura, K. Sasamoto, Y. Tominaga, M. Taoka, K. Kakiuchi, T. Shinkawa, N. Takahashi, S. Shimada, and T. Isobe.** 2005. 14-3-3 proteins modulate the expression of epithelial Na+ channels by phosphorylation-dependent interaction with Nedd4-2 ubiquitin ligase. J. Biol. Chem. **280:**13187–13194.
21. **Inoue, K., S. Ueno, and A. Fukuda.** 2004. Interaction of neuron-specific K^+-Cl^- cotransporter, KCC2, with brain-type creatine kinase. FEBS Lett. **564:**131–135.
22. **Inoue, K., J. Yamada, S. Ueno, and A. Fukuda.** 2006. Brain-type creatine kinase activates neuron-specific K^+-Cl^- cotransporter KCC2. J. Neurochem. **96:**598–608.
23. **Kato, T., T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, and T. Wakita.** 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology **125:**1808–1817.
24. **Kato, T., A. Furusaka, M. Miyamoto, T. Date, K. Yasui, J. Hiramoto, K. Nagayama, T. Tanaka, and T. Wakita.** 2001. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J. Med. Virol. **64:**334–339.
25. **Lancaster, A. M., E. Jan, and P. Sarnow.** 2006. Initiation factor-independent translation mediated by the hepatitis C virus internal ribosome entry site. RNA **12:**894–902.
26. **Lillie, J. W., D. F. Smee, J. H. Huffman, L. J. Hansen, R. W. Sidwell, and R. Kaddurah-Daouk.** 1994. Cyclocreatine (1-carboxymethyl-2-iminoimidazolidine) inhibits the replication of human herpesviruses. Antivir. Res. **23:**203–218.
27. **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.
28. **Lindenbach, B. D., B. M. Pragai, R. Montserret, R. K. Beran, A. M. Pyle, F. Penin, and C. M. Rice.** 2007. The C terminus of hepatitis C virus NS4A encodes an electrostatic switch that regulates NS5A hyperphosphorylation and viral replication. J. Virol. **81:**8905–8918.
29. **Mahajan, V. B., K. S. Pai, A. Lau, and D. D. Cunningham.** 2000. Creatine kinase, an ATP-generating enzyme, is required for thrombin receptor signaling to the cytoskeleton. Proc. Natl. Acad. Sci. USA **97:**12062–12067.
30. **Mannova, P., R. Fang, H. Wang, B. Deng, M. W. McIntosh, S. M. Hanash, and L. Beretta.** 2006. Modification of host lipid raft proteome upon hepatitis C virus replication. Mol. Cell Proteomics **5:**2319–2325.
31. **Manos, P., and J. Edmond.** 1992. Immunofluorescent analysis of creatine kinase in cultured astrocytes by conventional and confocal microscopy: a nuclear localization. J. Comp. Neurol. **326:**273–282.
32. **Meffert, G., F. N. Gellerich, R. Margreiter, and M. Wyss.** 2005. Elevated creatine kinase activity in primary hepatocellular carcinoma. BMC Gastroenterol. **5:**9.
33. **Miyanari, Y., M. Hijikata, M. Yamaji, M. Hosaka, H. Takahashi, and K. Shimotohno.** 2003. Hepatitis C virus nonstructural proteins in the probable membranous compartment function in viral genome replication. J. Biol. Chem. **278:**50301–50308.
34. **Moradpour, D., F. Penin, and C. M. Rice.** 2007. Replication of hepatitis C virus. Nat. Rev. Microbiol. **5:**453–463.
35. **Muhlebach, S. M., M. Gross, T. Wirz, T. Wallimann, J. C. Perriard, and M. Wyss.** 1994. Sequence homology and structure predictions of the creatine kinase isoenzymes. Mol. Cell Biochem. **133–134:**245–262.
36. **Murakami, K., K. Ishii, Y. Ishihara, S. Yoshizaki, K. Tanaka, Y. Gotoh, H. Aizaki, M. Kohara, H. Yoshioka, Y. Mori, N. Manabe, I. Shoji, T. Sata, R. Bartenschlager, Y. Matsuura, T. Miyamura, and T. Suzuki.** 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:**381–392.
37. **Nomura-Takigawa, Y., M. Nagano-Fujii, L. Deng, S. Kitazawa, S. Ishido, K. Sada, and H. Hotta.** 2006. Non-structural protein 4A of Hepatitis C virus accumulates on mitochondria and renders the cells prone to undergoing mitochondria-mediated apoptosis. J. Gen. Virol. **87:**1935–1945.
38. **Ohara-Imaizumi, M., T. Fujiwara, Y. Nakamichi, T. Okamura, Y. Akimoto, J. Kawai, S. Matsushima, H. Kawakami, T. Watanabe, K. Akagawa, and S. Nagamatsu.** 2007. Imaging analysis reveals mechanistic differences between first- and second-phase insulin exocytosis. J. Cell Biol. **177:**695–705.
39. **Okamoto, T., Y. Nishimura, T. Ichimura, K. Suzuki, T. Miyamura, T. Suzuki, K. Moriishi, and Y. Matsuura.** 2006. Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. EMBO J. **25:**5015–5025.
40. **Oliver, I. T.** 1955. A spectrophotometric method for the determination of creatine phosphokinase and myokinase. Biochem. J. **61:**116–122.
41. **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.
42. **Shirakura, M., K. Murakami, T. Ichimura, R. Suzuki, T. Shimoji, K. Fukuda, K. Abe, S. Sato, M. Fukasawa, Y. Yamakawa, M. Nishijima, K. Moriishi, Y. Matsuura, T. Wakita, T. Suzuki, P. M. Howley, T. Miyamura, and I. Shoji.** 2007. E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. J. Virol. **81:**1174–1185.
43. **Sunahara, Y., K. Uchida, T. Tanaka, H. Matsukawa, M. Inagaki, and Y. Matuo.** 2001. Production of recombinant human creatine kinase (r-hCK) isozymes by tandem repeat expression of M and B genes and characterization of r-hCK-MB. Clin. Chem. **47:**471–476.

44. **Suzuki, T., K. Ishii, H. Aizaki, and T. Wakita.** 2007. Hepatitis C viral life cycle. Adv. Drug Deliv. Rev. **59**:1200–1212.
45. **Taguwa, S., T. Okamoto, T. Abe, Y. Mori, T. Suzuki, K. Moriishi, and Y. Matsuura.** 2008. Human butyrate-induced transcript 1 interacts with hepatitis C virus NS5A and regulates viral replication. J. Virol. **82**:2631–2641.
46. **Takeuchi, T., A. Katsume, T. Tanaka, A. Abe, K. Inoue, K. Tsukiyama-Kohara, R. Kawaguchi, S. Tanaka, and M. Kohara.** 1999. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology **116**:636–642.
47. **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.
48. **Wallimann, T., M. Wyss, D. Brdiczka, K. Nicolay, and H. M. Eppenberger.** 1992. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. Biochem. J. **281**(Pt. 1):21–40.
49. **Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno.** 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol. Cell **19**:111–122.
50. **Wolk, B., D. Sansonno, H. G. Krausslich, F. Dammacco, C. M. Rice, H. E. Blum, and D. Moradpour.** 2000. Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. J. Virol. **74**:2293–2304.
51. **Wyss, M., and R. Kaddurah-Daouk.** 2000. Creatine and creatinine metabolism. Physiol. Rev. **80**:1107–1213.
52. **Yi, M., R. A. Villanueva, D. L. Thomas, T. Wakita, and S. M. Lemon.** 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. USA **103**:2310–2315.
53. **Yi, Z., C. Fang, T. Pan, J. Wang, P. Yang, and Z. Yuan.** 2006. Subproteomic study of hepatitis C virus replicon reveals Ras-GTPase-activating protein binding protein 1 as potential HCV RC component. Biochem. Biophys. Res. Commun. **350**:174–178.
54. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA **102**:9294–9299.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 14, pp. 9237–9246, April 3, 2009

Hepatitis C Virus Nonstructural 4B Protein Modulates Sterol Regulatory Element-binding Protein Signaling via the AKT Pathway*

Received for publication, November 19, 2008, and in revised form, February 6, 2009 Published, JBC Papers in Press, February 9, 2009, DOI 10.1074/jbc.M808773200

Chul-Yong Park[‡], Hyun-Jeong Jun[‡], Takaji Wakita[§], Jae Hun Cheong[¶], and Soon B. Hwang[‡1]

From the [‡]National Research Laboratory of Hepatitis C Virus and Ilsong Institute of Life Science, Hallym University, Anyang 431-060, Korea, the [§]Department of Virology II, National Institute of Infectious Disease, Tokyo 162-8640, Japan, and the [¶]Department of Molecular Biology, Pusan National University, Pusan 609-735, Korea

Hepatitis C virus (HCV) infection is often associated with hepatic steatosis and yet the molecular mechanisms of HCV-associated steatosis are poorly understood. Because sterol regulatory element-binding proteins (SREBPs) are the major transcriptional factors in lipogenic gene expression including fatty acid synthase (FAS), we examined the effects of HCV nonstructural proteins on the signaling pathways of SREBP. In this study, we demonstrated that HCV nonstructural 4B (NS4B) protein increased the transcriptional activities of SREBPs. We also showed that HCV NS4B enhanced the protein expression levels of SREBPs and FAS. This was further confirmed in the context of viral RNA replication and HCV infection. The up-regulation of both SREBP and FAS by NS4B protein required phosphatidylinositol 3-kinase activity. We also demonstrated that NS4B protein induced a lipid accumulation in hepatoma cells. In addition, NS4B protein synergistically elevated the transcriptional activity of HCV core-mediated SREBP-1. These results strongly suggest that NS4B may play an important role in HCV-associated liver pathogenesis by modulating the SREBP signaling pathway.

Hepatitis C virus (HCV)[2] infection has a major impact on public health, affecting more than 170 million people worldwide (1). HCV infection often leads to chronic hepatitis, liver cirrhosis, and ultimately hepatocellular carcinoma (2). Although HCV infection is strongly associated with hepatic steatosis (3), the molecular events that lead to hepatic steatosis during HCV infection are poorly understood. HCV is an enveloped, positive-strand RNA virus classified in the *Hepacivirus* genus within the *Flaviviridae* family. HCV is highly heterogeneous and has been classified into six major genotypes and numerous subtypes (4). HCV genome encodes a polyprotein of more than 3,010 amino acids that is cleaved at the endoplasmic reticulum (ER) by host and viral proteases, yielding 3 structural (core, E1, and E2) and 7 nonstructural (p7, NS2 to NS5B) proteins (5). NS4B protein is released from the polyprotein by the NS3/4A serine protease (6). NS4B is a hydrophobic 27-kDa protein located in the ER membrane (7), and has four transmembrane domains with the N and C termini located in the cytoplasm. The N-terminal tail of NS4B has been suggested to be posttranslationally translocated to the ER lumen (8). NS4B protein is known to induce intracellular membrane changes that called a "membranous web" (9). Elazar *et al.* (10) suggest that a putative amphipathic helix within the N-terminal 26 residues of NS4B mediates membrane association, and these residues are critical for HCV replication in cell culture. Furthermore, NS4B can transform NIH-3T3 cells either in cooperation with Ras (11) or independent of Ras (12).

Hepatic steatosis is defined as an increased fat content in the liver, essentially accounted for by triglycerides (13). The prevalence of steatosis ranges from 40 to 86% in chronic hepatitis C patients (14). Interestingly, steatosis is more frequent in patients infected with HCV genotype 3 than in patients infected with other HCV genotypes, even though not all patients infected with genotype 3 have steatosis (15, 16). It has previously been reported that hepatic steatosis was induced by the HCV core protein through inhibition of the microsomal triglyceride transfer protein activity and very low density lipoprotein secretion (17), impairment of the expression and transcriptional activity of peroxisome proliferators-activated receptor (PPAR) α (18), and activation of the SREBP1 and PPARγ (19). Sterol regulatory element-binding proteins (SREBPs) are ER membrane-bound transcription factors, which activate genes encoding the enzymes that regulate the synthesis of cholesterol and fatty acids, and cellular uptake of lipoproteins (20).

In mammals, there are three SREBP isoforms, designated SREBP-1a, SREBP-1c, and SREBP-2 (20). SREBPs are synthesized as precursors bound to the ER membrane (21). Upon activation by SREBP cleavage-activating protein (SCAP), SREBPs are released from the membrane into the nucleus as mature proteins by sequential cleavage processes (21). The mature SREBP-1a activates target genes involved in both fatty acid and cholesterol biosynthesis, whereas SREBP-1c and SREBP-2 activate target genes involved in fatty acid and cholesterol biosynthesis, respectively (21).

* This work was supported by the National R&D Program for Cancer Control, Ministry of Health and Welfare Grant 0620100, Korea, the Research Program for New Drug Target Discovery (M10601000044-06N0100-04410) and Biotechnology Development (2008-04100), and National Research Laboratory Grant ROA-2007-000-20051-0 from the Ministry of Education, Science and Technology, Korea.

[1] To whom correspondence should be addressed: 1605-4 Gwanyang-dong, Dongan-gu, Anyang, 431-060, Korea. Fax: 82-31-384-5395; E-mail: sbhwang@hallym.ac.kr.

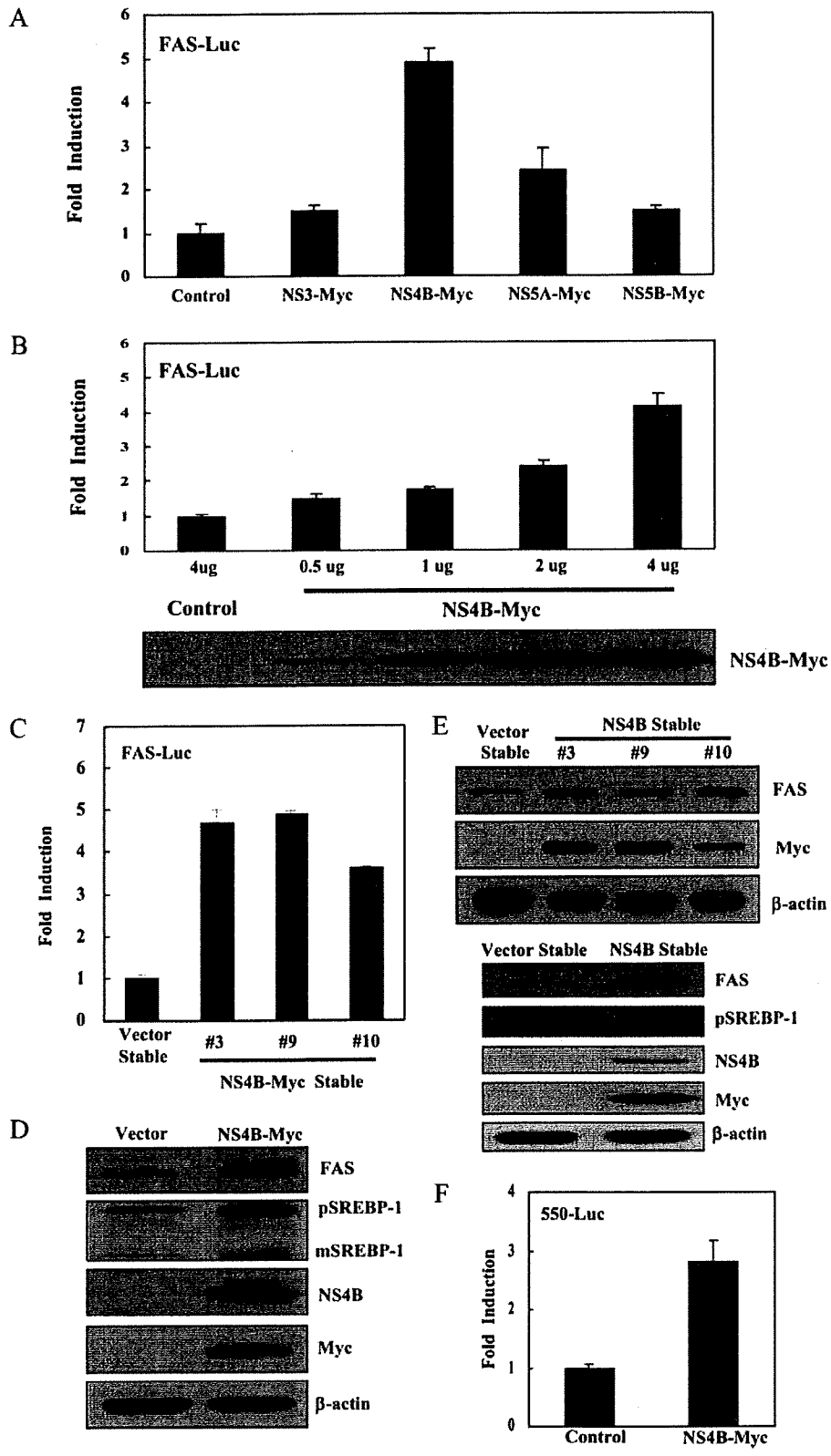
[2] The abbreviations used are: HCV, hepatitis C virus; NS4B, nonstructural 4B; HA, hemagglutinin; IFN, interferon; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; AKT, protein kinase B; FAS, fatty acid synthase; SREBP, sterol regulatory element-binding protein; ER, endoplasmic reticulum; PPAR, peroxisome proliferators-activated receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SCD, stearoyl-CoA desaturase; ACC, acetyl-CoA carboxylase.

Because both SREBP-1 and SREBP-2 are important transcriptional factors involved in lipid biosynthesis, we investigated the possible involvement of nonstructural proteins of HCV in hepatic lipid accumulation. In this study, we demonstrated that HCV NS4B protein promoted the transcriptional and translational activities of SREBPs and fatty acid synthase (FAS), and this was mediated through the AKT pathway. To our knowledge, this is the first report that NS4B mediates lipogenesis and hence this may provide a novel mechanism of hepatic steatosis associated with HCV infection.

EXPERIMENTAL PROCEDURES

Plasmids—NS4B-Myc of HCV (genotype 1b) was amplified by PCR using the Korean isolate of HCV (22) as a template and subcloned into the pEF6B/His-Myc (Invitrogen) vector. NS3-Myc, NS4B-Myc (genotype 2a, JFH-1), and NS5A-Myc expression plasmids were subcloned into the pEF6B/His-Myc vector. NS5B-Myc expression plasmid was described previously (23). FLAG-core expression plasmid was subcloned into the pFlag-CMV-2 vector. pcDNA3.1-Flag-SREBP-1a (human, amino acids 1–490), pGL2B-FAS-luc (fatty acid synthase promoter), and pSynSRE-luc (hamster HMG-CoA synthase promoter) vectors were kindly provided by Dr. T. R. Osborne (24–26). pcDNA3-HA-SREBP-1c (human, amino acids 1–447) was described previously (27). *pBP1c550-Luc*, the luciferase gene containing 550-bp fragments of the mouse SREBP-1c promoter, was a gift from Dr. H. Shimano (28).

Cell Culture, Transfection, and HCV Infection—Huh7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. For transfection, ~5 × 10^5 cells plated on 60-mm dishes were transfected with plasmid DNA using either Lipofectamine (Invitrogen) or polyethyleneimine reagent (Sigma) according to instructions from the manufac-



turer. Huh7 cells containing HCV subgenomic replicons were kindly provided by Dr. C. Seeger (Fox Chase Cancer Center, Philadelphia, PA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, 0.1 mM nonessential amino acids (Invitrogen), and 500 μg/ml G418 (Qbiogene, Inc., Irvine, CA). To establish interferon (IFN)-cured cells, HCV replicon cells were treated with 100 units/ml IFN-α (Sigma) for 2 weeks. Elimination of HCV replicon RNA was confirmed by reverse transcription-PCR, Western blotting, and loss of resistance to G418. The infectious HCVs were generated as described previously (29).

Establishment of Stable Cells Expressing NS4B-Myc Protein—To make the cell lines stably expressing NS4B-Myc, Huh7 cells were transfected with the pEF6B-NS4B (genotype 1b) expression plasmid and cultured for 4 weeks in the presence of 10 μg/ml blasticidin. Single positive clones were selected by Western blot analysis using anti-Myc monoclonal antibody. Huh7 cells transfected with empty vector (pEF6B only) were selected as described above and used as a control.

Reporter Assays—Huh7 cells were seeded in a 12-well culture plate and transfected with 0.2 μg of reporter plasmid (FAS-Luc, SRE-Luc, 550-Luc, individually) and 0.1 μg of pCH110 reference plasmid (Amersham Biosciences) containing the *Escherichia coli lacZ* gene under control of the simian virus 40 promoter. The total DNA amount in each transfection was kept constant by adjustment with empty vector. At 36 h after transfection, cells were harvested and luciferase activities were determined by measuring luminescence activity. Data were normalized by measuring β-galactosidase activity. Luciferase and β-galactosidase assays were performed as described previously (30).

Immunoblot Analysis—Cells were lysed in cell lysis buffer containing 50 mmol/liter Tris-HCl (pH 7.5), 150 mmol/liter NaCl, 1 mmol/liter EDTA, 1% Nonidet P-40, 10% glycerol, and protease inhibitor mixture (Roche) for 20 min on ice. The protein concentration was determined by the Bradford assay (Bio-Rad). Equal amounts of proteins were subjected to 10% SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was blocked in phosphate-buffered saline (PBS) containing 5% nonfat dry milk for 1 h and then incubated 2 h at room temperature with one of following antibodies: anti-FAS antibody (BD Transduction Laboratories), anti-SREBP-1 antibody (BD Transduction Laboratories, Santa Cruz Biotechnology, Inc.), anti-SREBP-2 antibody (BD Transduction Laboratories), anti-AKT and *p*-AKT antibody (Cell signaling Technology, Beverly, MA), anti-β-actin and anti-FLAG antibody (Sigma), anti-Myc and anti-HA antibody (Santa Cruz Biotechnology, Inc.), anti-NS4B antibody (Virostat), and rabbit anti-NS5A polyclonal antibody in Tris-buffered saline/Tween (20 mmol/liter Tris-HCl (pH 7.5), 500 mmol/liter NaCl, and 0.05% Tween 20). Following two washes in Tris-buffered saline/Tween, the membrane was incubated with either horseradish peroxidase-conjugated goat anti-rabbit antibody or goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in Tris-buffered saline/Tween for 1 h at room temperature. Proteins were detected using an ECL kit (Amersham Biosciences).

Confocal Microscopy—Huh7 cells grown on coverglass (Superior, 18 × 18 mm) were transfected with the NS4B-myc expression plasmid. At 36 h after transfection, cells were washed in PBS and fixed in 4% paraformaldehyde and 0.1% Triton X-100 for 20 min at 37 °C. Cells were incubated in 5% bovine serum albumin for 20 min at 37 °C and then incubated with anti-Myc (Santa Cruz) monoclonal antibody for 2 h at 37 °C. After being washed three times in PBS, cells were further incubated with TRITC-conjugated goat anti-mouse IgG (American Qualex, San Clemente, CA) and BODIPY 493/503 (1 μM, Invitrogen) for 1 h at 37 °C. After two washes with 0.1% Triton X-100 in PBS and three washes in PBS, cells were analyzed using the LSM 510 laser confocal microscopy system and BODIPY intensity was measured by imaging analysis (Carl Zeiss, Inc., Thornwood, NY).

RNA Isolation and Reverse Transcription-PCR—Total RNAs were isolated from vector, NS4B-myc stable, IFN-cured, and HCV subgenomic-replicon cells using TRIzol reagent (Invitrogen). The cDNAs were synthesized by avian myeloblastosis virus (AMV) reverse transcriptase (Promega) from 1 μg of total RNAs using oligo(dT) primer or poly(dA) primers (for NS4B amplification in replicon cells). To estimate transcriptional levels of SREBP-1 and its target genes, a one-tenth aliquot of cDNA was subjected to PCR amplification using SREBP-1, FAS, SCD, ACC, NS4B, and GAPDH primers: SREBP-1-f, 5-ACGGCAGCCCCTGTAACGACCACTGTGA-3 and SREBP-1-r, 5-TGCCAAGATGGTTCCGCCACTCACCAGG-3; FAS-f, 5-GAAACTGCAGGAGCTGTC-3 and FAS-r, 5-CACGGAGTTGAGGCGGAT-3; SCD-f, 5-CCTCTACTTGGAAGACGACATTCGC-3 and SCD-r, 5-GCAGCCGAGCTTTGTAAGAGCGGT-3; ACC-f, 5-GCTGCTCGGATCACTAGTGAA-3 and ACC-r, 5-TTCTGCTATCAGTCTGTCCAG-3; NS4B-f, 5-*cgcggatccatg*GCCTCACAACTTCCT-3 and NS4B-r, 5-*ggcgaattcca*TCCGCTGATGAAATT-3; GAPDH-f, 5-GCTCTCCAGAACATCATCCCTGCC-3 and GAPDH-r, 5-CGTTGTCATACCAGGAAATGAGCTT-3. Italic letters represent

FIGURE 1. **HCV NS4B protein increases the transcriptional activity of SREBP-1.** *A*, Huh7 cells were cotransfected with FAS-Luc reporter plasmid together with the indicated expression plasmids. At 36 h after transfection, cells were harvested and then luciferase activities were determined. The amount of DNA in each transfection was kept constant by adding an appropriate amount of pEF6-Myc empty vector. Data represent the mean of two independent experiments. *B*, HCV NS4B increases the transcriptional activity of SREBP-1 in a dose-dependent manner. Huh7 cells were cotransfected with FAS-Luc reporter plasmid together with increasing amounts of Myc-tagged NS4B expression plasmid. At 36 h after transfection, cells were harvested, and luciferase activities were determined (*upper panel*). Equal amounts of cell lysates were subjected to immunoblotting with anti-Myc monoclonal antibody (*lower panel*). *C*, both vector and NS4B-Myc stable cells were transfected with FAS-Luc reporter plasmid. At 36 h after transfection, cells were harvested and then luciferase activities were determined. *D*, Huh7 cells were transfected with either vector control or NS4B-Myc expression plasmid. At 36 h after transfection, total cell lysates were immunoblotted with the indicated antibodies. *E*, both vector stable and NS4B-Myc stable cell (#3) lysates harvested from each cell line were immunoblotted with the indicated antibodies. Protein expression of β-actin was used as a loading control for the same amount of cell lysates. *F*, Huh7 cells were cotransfected with 550-Luc reporter plasmid together with NS4B-Myc expression plasmid. At 36 h after transfection, cells were harvested and then luciferase activities were determined. Data represent the mean of two independent experiments.

irrelevant sequences and restriction sites (BamHI and EcoRI) for cloning. Amplified DNA was analyzed by agarose gel electrophoresis.

Statistical Analysis—The data are presented as mean ± S.D. The Student's *t* test was used for statistical analysis. $p < 0.05$ was considered statistically significant.

RESULTS

HCV NS4B Protein Increases the Transcriptional Activity of SREBP-1—Because SREBPs are the major transcription factors for lipogenic gene expression, we investigated the effects of HCV nonstructural proteins on transcriptional activity of SREBP-1. Huh7 cells were cotransfected with FAS-Luc reporter plasmid (reporter containing FAS promoter) and NS3-Myc, NS4B-Myc, NS5A-Myc, and NS5B-Myc expression plasmid, individually. We show that the NS4B protein strongly increases the promoter activity of the FAS gene (Fig. 1*A*). However, both NS3 and NS5B proteins do not increase the promoter activity of the FAS gene although the NS5A protein increases ~2-fold the promoter activity of the FAS gene as compared with the vector control. Because FAS is one of the downstream targets of SREBP-1, this result indicates that NS4B protein increases the transcriptional activity of SREBP-1. We found that the NS4B protein increased the promoter activity of the FAS gene in a dose-dependent manner (Fig. 1*B*). To further demonstrate the effect of NS4B on transcriptional activity of SREBP-1, we established NS4B stable cell lines by transfecting Huh7 cells with the NS4B-Myc expression plasmid. We identified 3 clones (3, 9, and 10) by selection with blasticidin. As demonstrated in Fig. 1*C*, the promoter activity of the FAS gene was also increased in all NS4B stable cells as compared with the vector stable cells. Next, we analyzed the effect of NS4B on gene expression of SREBP-1 using an immunoblot. For this purpose, Huh7 cells were transfected with either vector or NS4B-Myc expression plasmid and total cell lysates were immunoblotted with SREBP-1 antibody. As demonstrated in Fig. 1*D*, the protein expression level of FAS was increased in Huh7 cells transiently expressing the NS4B protein as compared with the vector control. It is noteworthy that protein expression levels of both precursor and mature forms of SREBP-1 (pSREBP-1 and mSREBP-1) were significantly increased in cells expressing NS4B protein. We further confirmed that protein expression levels of FAS were increased in all NS4B stable cells as compared with the vector stable cells (Fig. 1*E*, *upper panel*). In addition, the protein expression level of pSREBP-1 was also increased in the number 3 NS4B stable cell line (Fig. 1*E*, *lower panel*) as well as numbers 9 and 10 stable cells (data not shown). We then investigated whether promoter activity of SREBP-1 was increased by the NS4B protein. For this purpose, Huh7 cells were cotransfected with *pBP1c550-Luc* (28), the luciferase gene containing 550-bp fragments of the mouse SRBBP-1c promoter (550-Luc), and NS4B expression plasmid. As expected, luciferase activity was increased ~3-fold in cells expressing NS4B as compared with the control vector (Fig. 1*F*).

HCV NS4B Protein Increases the Transcriptional Activity and Gene Expression of SREBP-2—Because SREBP-2 is also a transcription factor for lipogenic gene expression, we investigated the effects of HCV nonstructural proteins on transcriptional activity of SREBP-2. Huh7 cells were cotransfected with pSynSRE-Luc (hamster HMG-CoA synthase promoter construct, SRE-Luc) and NS3-Myc, NS4B-Myc, NS5A-Myc, and NS5B-Myc expression plasmids, individually. We demonstrated that NS4B increased ~3-fold the promoter activity of the HMG-CoA synthase gene as compared with the vector control (Fig. 2*A*). However, other nonstructural proteins did not increase the promoter activity of the HMG-CoA synthase gene. Because HMG-CoA synthase is one of the downstream targets of SREBP-2, this indicates that the NS4B protein also increases the transcriptional activity of SREBP-2. We further showed that the NS4B protein increased promoter activity of the HMG-CoA synthase gene in a dose-dependent manner (Fig. 2*B*). We then investigated whether transcriptional activity of the SREBP-2 was increased in NS4B stable cells. For this purpose, we transfected either vector stable or NS4B-Myc stable cells with pSynSRE-Luc (SRE-Luc). As shown in Fig. 2*C*, the promoter activity of the HMG-CoA synthase gene was also increased in all NS4B stable cell lines as compared with the vector stable cells. We then analyzed the effect of NS4B on gene expression of SREBP-2 by immunoblot analysis. We found that protein expression levels of both pSREBP-2 and mSREBP-2 were increased in Huh7 cells transiently expressing the NS4B protein as compared with the vector control (Fig. 2*D*). We have further shown that the protein expression level of pSREBP-2 was also increased in clone 9 NS4B stable cells (Fig. 2*E*) as well as clones 3 and 10 stable cells (data not shown).

HCV Replicon Cells Increase the Transcriptional Activities and Gene Expressions of SREBP-1 and SREBP-2—To further investigate whether transcriptional and translational activities of SREBP-1 and SREBP-2 were regulated by viral protein in the context of HCV RNA replication, we transfected either FAS-Luc plasmid or SRE-Luc plasmid in IFN-cured and HCV replicon cells, and reporter activities were analyzed. As shown in Fig. 3, *A* and *B*, transcriptional activities of both SREBP-1 and SREBP-2 were increased in the replicon cells as compared with the IFN-cured cells. Furthermore, protein expression levels of FAS, pSREBP-1, mSREBP-1, pSREBP-2, and mSREBP-2 were significantly increased in HCV subgenomic replicon cells as compared with the IFN-cured cells (Fig. 3*C*). We further confirmed that *pBP1c550-Luc* reporter activity was also increased ~3-fold in replicon cells as compared with the IFN-cured cells (Fig. 3*D*).

HCV NS4B Protein Increases the mRNA Levels of Lipogenic Genes—Because FAS, stearoyl-CoA desaturase (SCD), and acetyl-CoA carboxylase (ACC) are target genes of SREBP-1 (20), we further examined whether mRNA levels of lipogenic genes were modulated by the HCV NS4B protein. For this purpose, total RNAs isolated from vector stable, NS4B-Myc stable, IFN-cured, and HCV subgenomic replicon cells were compared for transcriptional levels of SREBP-1, FAS, SCD, ACC using cDNAs. As demonstrated in Fig. 4*A*, both SREBP-1 and its target gene (FAS, SCD, and ACC) expressions were elevated in NS4B stable cells as compared with vector stable cells. This result was further confirmed in the context of HCV RNA replication of replicon cells (Fig. 4*B*). These data indicated that NS4B-mediated increases of transcriptional and translational

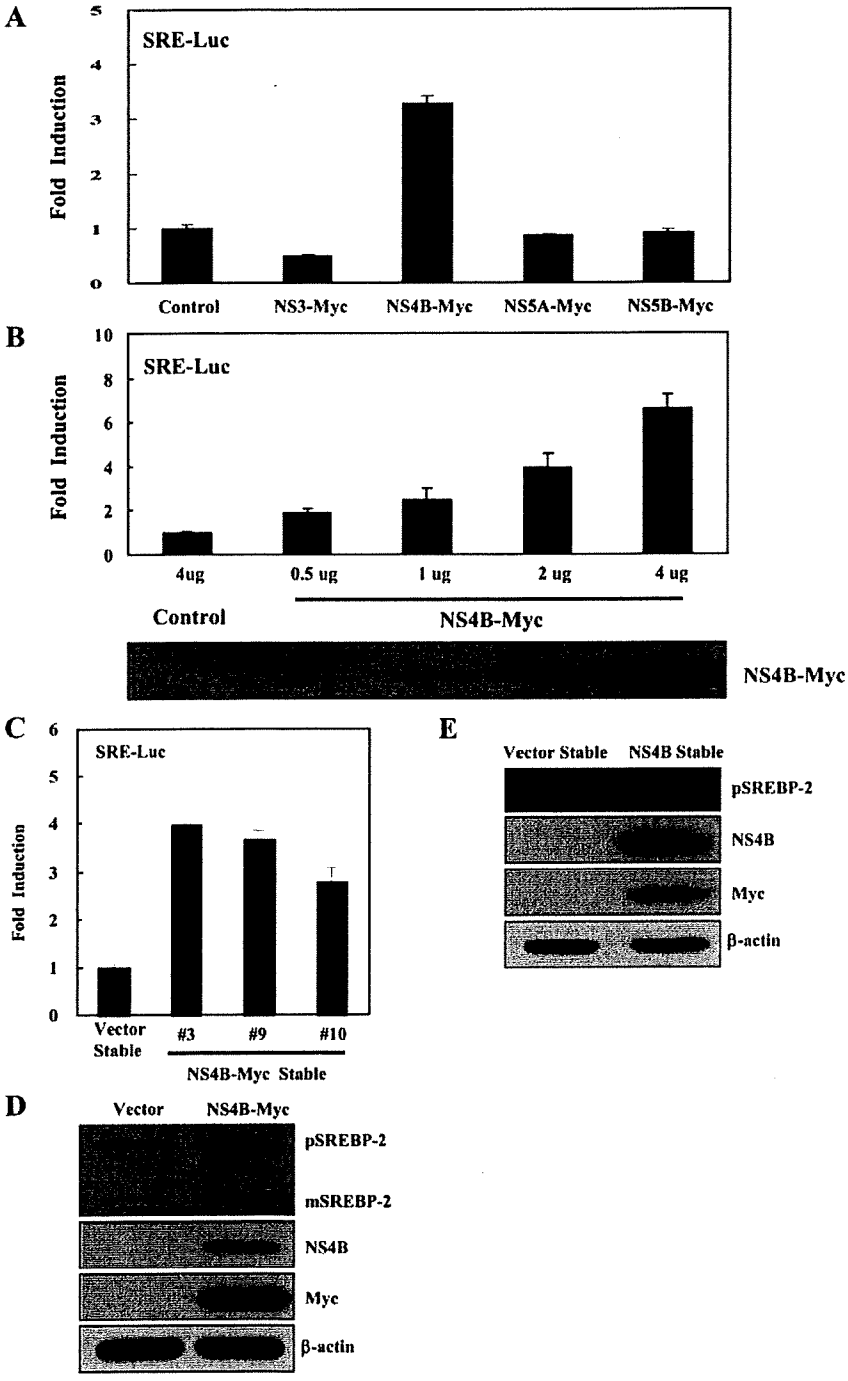


FIGURE 2. **HCV NS4B protein increases the transcriptional activity of SREBP-2.** *A*, Huh7 cells were cotransfected with SRE-Luc reporter plasmid together with the indicated expression plasmids. At 36 h after transfection, cells were harvested, and luciferase activities were determined. The amount of DNA in each transfection was kept constant by adding an appropriate amount of pEF6-Myc empty vector. Data represent the mean of two independent experiments. *B*, HCV NS4B increases the transcriptional activity of SREBP-2 in a dose-dependent manner. Huh7 cells were cotransfected with SRE-Luc reporter plasmid together with increasing amounts of Myc-tagged NS4B expression plasmid. At 36 h after transfection, cells were harvested, and then luciferase activities were determined (*upper panel*). Equal amounts of cell lysates were subjected to immunoblotting with anti-Myc monoclonal antibody (*lower panel*). *C*, both vector and NS4B-Myc stable cells were transfected with SRE-Luc reporter plasmid. At 36 h after transfection, cells were harvested and then luciferase activities were determined. *D*, Huh7 cells were transfected with either vector control or NS4B-Myc expression plasmid. At 36 h after transfection, total cell lysates were immunoblotted with the indicated antibodies. *E*, total cell lysates harvested from both vector stable and NS4B-Myc stable cells were immunoblotted with the indicated antibodies. Protein expression of β-actin was used as a loading control for the same amount of cell lysates.

activities of SREBP-1 were due to up-regulation of the RNA level of SREBP-1.

HCV NS4B Protein Increases the Transcriptional Activity of Exogenous Mature SREBP-1 and Induces Lipid Accumulation—To further investigate whether NS4B protein was able to modulate transcriptional activity of SREBP-1, both vector and NS4B-Myc stable cells were cotransfected with FAS-Luc reporter and either mature Flag-SREBP-1a (1–490 amino acids) or mature HA-SREBP-1c (1–447 amino acids) expression plasmids. Because both SREBP-1a and SREBP-1c are mature forms, these can directly translocate to the nucleus and activate transcription of target genes. Indeed, both SREBPs (mature form) activated FAS transcriptional activities in vector stable cells, and SREBP (mature form)-mediated FAS activations were significantly increased by NS4B protein (Fig. 5*A*). Because NS4B increases endogenous SREBP activation (Fig. 5*A*, *lane 1 versus lane 4* in *upper panel*), SREBP (mature form)-mediated FAS activations (Fig. 5*A*, *lanes 2* and *3 versus lanes 5* and *6*) showing that NS4B increases the transcriptional activity of SREBPs are additional evidence. These data suggest that NS4B stimulates SREBPs transcriptionally. Because we used NS4B protein-activated lipogenic signaling, we then asked whether lipid was accumulated by the NS4B protein. For this purpose, Huh7 cells transfected with either NS4B-Myc or vector plasmid were treated with BODIPY 493/503 for staining of lipid. Indeed, BODIPY signals were greatly increased in cells expressing NS4B as compared with vector control cells (Fig. 5*B*, *left panel*). This was further confirmed by quantification analysis (Fig. 5*B*, *right panel*). We have confirmed that the total amounts of lipid were increased in NS4B stable cells, and in HCV subgenomic replicon cells as compared with vector stable and IFN-cured cells, respectively (data not shown). To further confirm whether transcriptional activity of SREBP-1 was

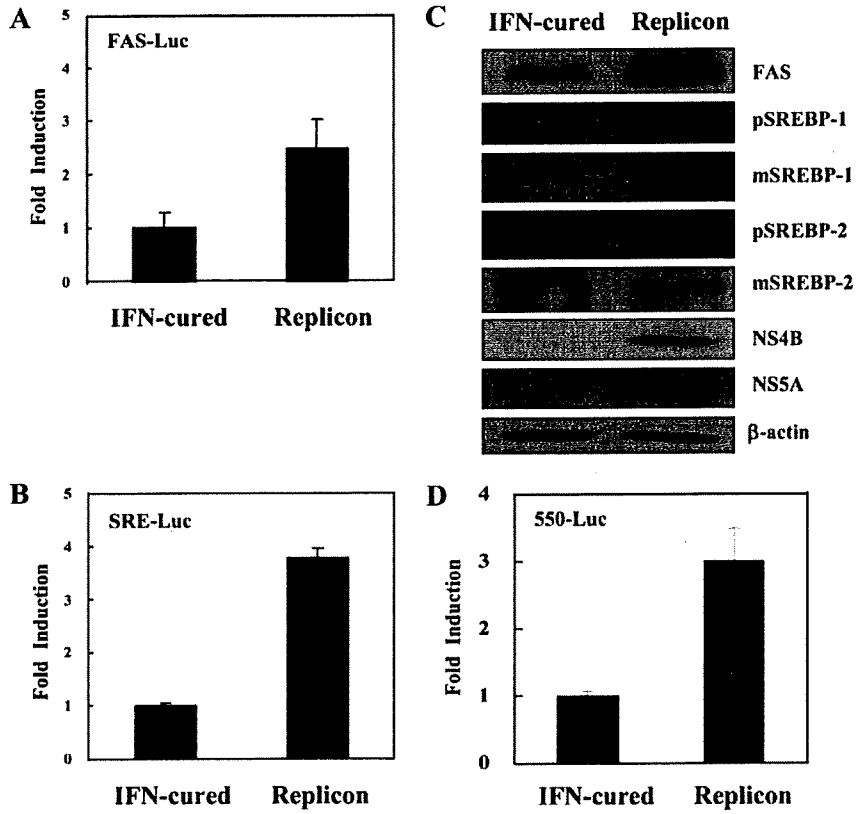


FIGURE 3. **HCV subgenomic replicon increases both SREBP-1 and SREBP-2 activities.** Both IFN-cured and HCV subgenomic replicon cells were transfected with either FAS-Luc (*A*) or SRE-Luc (*B*) reporter plasmid. At 36 h after transfection, cells were harvested and then luciferase activities were determined. Data represent the mean of two independent experiments. *C*, total cell lysates harvested from IFN-cured and HCV subgenomic replicon cells were immunoblotted with the indicated antibodies. Protein expression of β-actin was used as a loading control for the same amount of cell lysates. *D*, both IFN-cured and HCV subgenomic replicon cells were transfected with 550-Luc reporter plasmid. At 36 h after transfection, cells were harvested, and luciferase activities were determined. Data represent the mean of two independent experiments.

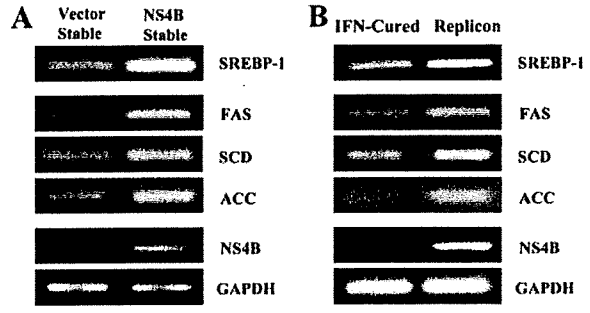


FIGURE 4. **HCV NS4B protein up-regulates RNA levels of both SREBP-1 and its target genes.** *A* and *B*, total RNAs were isolated from vector stable, NS4B-Myc stable, IFN-cured, and HCV subgenomic replicon cells. The cDNAs were synthesized by avian myeloblastosis virus reverse transcriptase from 1 μg of total RNAs. To estimate transcriptional levels of SREBP-1 and its target genes (*FAS*, *SCD*, and *ACC*), cDNAs were subjected to PCR amplification using gene-specific primers, respectively. The amplified DNA was analyzed by agarose gel electrophoresis.

also increased in HCV-infected cells, we used the infectious JFH-1 strain of HCV (29). At 3 days after infection with HCV, Huh7 cells were transfected with the FAS-Luc reporter plasmid, harvested at 24 h after transfection, and then luciferase activities were determined (Fig. 5*C*). HCV infection was confirmed by immunostaining cells with NS5A antibody (data not shown). Fig. 5*C* showed that the promoter activity of the FAS gene was increased ~3-fold in HCV-infected cells as compared with mock-infected cells. Furthermore, the protein level of FAS was also increased in HCV-infected cells (Fig. 5*D*). We have further shown that more lipids were accumulated in HCV-infected cells as compared with un-infected cells (Fig. 5*E*, *left panel*). This was verified by quantification analysis (Fig. 5*E*, *right panel*). These results strongly suggest that the HCV NS4B protein may mediate hepatic lipid accumulation via activation of SREBPs.

AKT Activation Is Required for NS4B-mediated SREBP Activation—It has previously been reported that activation of AKT kinase-induced gene expression of SREBP-1 (31), and hepatic overexpression of AKT resulted in steatosis (32). To determine whether AKT was involved in NS4B-mediated activation of SREBP-1, we investigated expression levels of both total AKT and phospho-AKT (p-AKT) proteins in vector stable and NS4B stable cells. We found that *p*-AKT protein levels were significantly higher in all three NS4B stable cells than vector stable cells (Fig. 6*A*). This was also evident in Huh7 cells transiently expressing the NS4B protein (Fig. 6*B*). We further confirmed that *p*-AKT levels were higher in HCV subgenomic replicon cells than IFN-cured cells (data not shown). We then investigated whether SREBP-1 activation in cells expressing NS4B was mediated through the phosphatidylinositol 3-kinase/AKT signaling pathway. For this purpose, both vector and NS4B stable cells transfected with either FAS-Luc or SRE-Luc reporters were either left untreated or treated with LY294002 (phosphatidylinositol 3-kinase inhibitor), and then luciferase activities were determined. As shown in Fig. 6*C*, NS4B-mediated promoter activations of FAS (*upper panel*) and HMG-CoA synthase (*lower panel*) genes were significantly reduced by LY294002. Furthermore, NS4B-mediated increases of FAS and pSREBP-1 protein levels were also significantly decreased by LY294002 (Fig. 6*D*). These results indicate that NS4B-induced SREBP activation was mediated through the AKT signaling pathway.

HCV NS4B Protein Synergistically Elevates the Core-mediated SREBP-1 Transcriptional Activation—We have previously reported that HCV core induced hepatic lipid accumulation by activating SREBP1 and PPARγ (19). We therefore asked whether the NS4B protein has synergistic effects on HCV core-

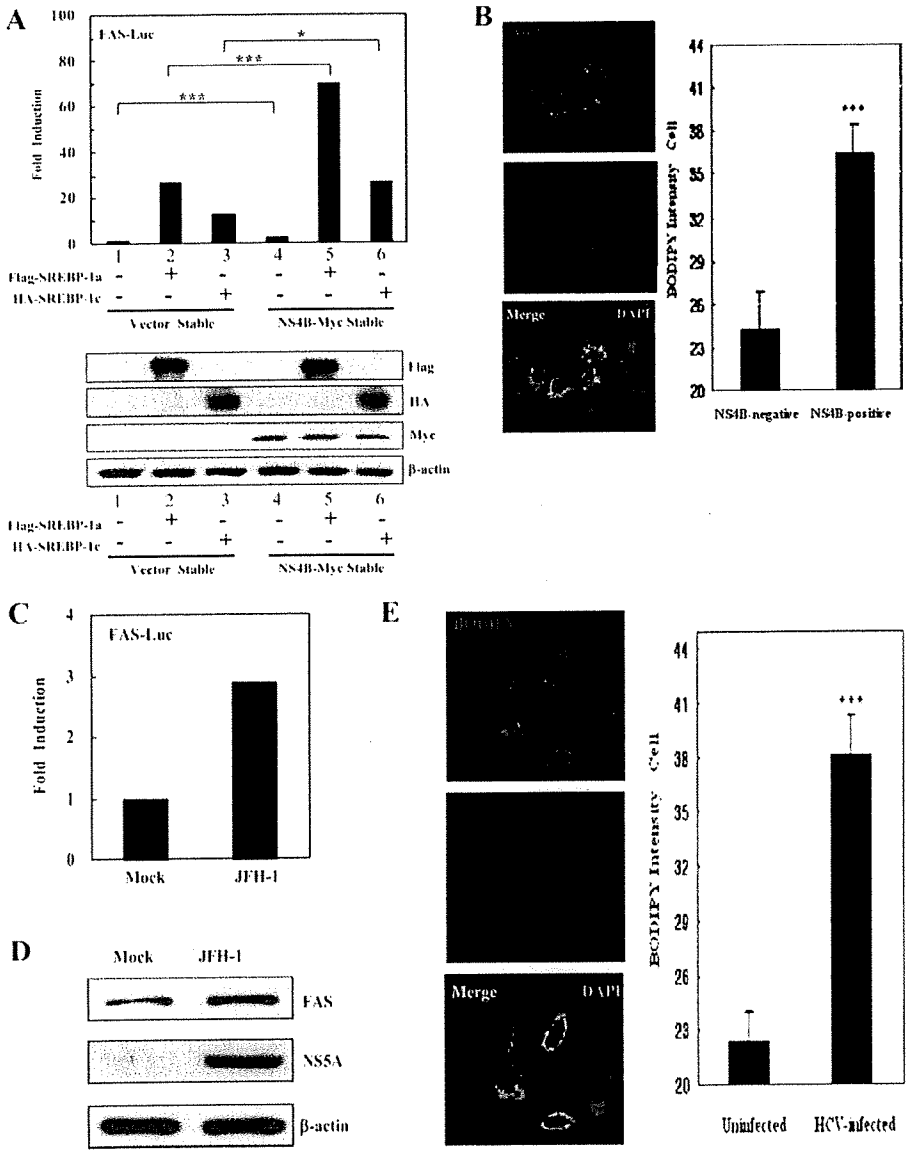


FIGURE 5. **HCV NS4B protein induces lipid accumulation through activation of SREBPs.** *A*, both vector and NS4B-Myc stable cells were cotransfected with FAS-Luc reporter and Flag-SREBP-1a (1–490 amino acids) or HA-SREBP-1c (1–447 amino acids) expression plasmids. At 36 h after transfection, cells were harvested and then luciferase activities were determined (*top panel*). Data represent the mean of two independent experiments. ***, $p < 0.001$, vector stable *versus* NS4B stable cells transfected with Flag-SREBP-1a. *, $p < 0.05$, vector stable *versus* NS4B stable cells transfected with HA-SREBP-1c. Equal amounts of cell lysates were subjected to immunoblotting with anti-FLAG, anti-HA, anti-Myc, and anti-β-actin monoclonal antibody (*bottom panel*). *B*, Huh7 cells were transfected with NS4B-Myc expression plasmid. At 36 h after transfection, cells were fixed and incubated with anti-Myc monoclonal antibody for 2 h. After being washed with PBS, cells were further incubated with TRITC-conjugated goat anti-mouse IgG and BODIPY 493/503 (1 μM, Invitrogen) for 1 h. Samples were analyzed for immunofluorescence staining using the LSM 510 laser confocal microscopy system and BODIPY intensity was quantified. Each *bar* represents the average intensity of BODIPY staining. ***, $p < 0.001$, NS4B-negative cells *versus* NS4B-positive cells. *C*, Huh7 cells were either mock-infected or infected with HCV JFH-1. At 3 days after infection, cells were transfected with FAS-Luc reporter plasmid. At 24 h after transfection, cells were harvested and then luciferase activities were determined. *D*, at 3 days after infection, total cell lysates were immunoblotted with either anti-FAS antibody (*top panel*) or anti-NS5A antibody (*middle panel*). Protein expression of β-actin was used as a loading control for the same amount of cell lysates (*bottom panel*). *E*, at 3 days after infection, cells were fixed and incubated with anti-NS5A polyclonal antibody for 2 h. After being washed with PBS, cells were further incubated with TRITC-conjugated goat anti-mouse IgG and BODIPY 493/503 (1 μM, Invitrogen) for 1 h. Immunofluorescence staining was performed as described in *B*. ***, $p < 0.001$, mock-infected cells *versus* HCV-infected cells. Cells were counterstained with 4′,6-diamidino-2-phenylindole (*DAPI*) to label nuclei.

mediated transcriptional activation of SREBP-1. For this purpose, Huh7 cells were cotransfected with FAS-Luc reporter plasmid and either NS4B or core, or both NS4B and core. Fig. 7*A* showed that FAS-Luc reporter activity was increased by either NS4B or core alone. Interestingly, promoter activity of the FAS gene was more elevated by HCV core protein than NS4B protein. As shown in Fig. 7*A*, FAS luciferase activity was significantly increased by cotransfection of Huh7 cells with NS4B and core expressing plasmids (*lane 4*). Likewise, the protein level of FAS was increased in cells expressing either NS4B or core protein (Fig. 7*B*). Furthermore, the protein level of FAS was more elevated in Huh7 cells cotransfected with NS4B and core as compared with either core or NS4B alone (Fig. 7*B*, *lane 4*). These data indicate that HCV NS4B protein synergistically activates the transcriptional activity of HCV core-mediated SREBP-1.

DISCUSSION

HCV infection is strongly associated with hepatic steatosis (3) and steatosis occurs in 40 to 86% of patients with chronic hepatitis C (14). It has been previously reported that the HCV core protein induced steatosis in *in vitro* and transgenic mice studies (17, 33, 34). Interestingly, steatosis is more frequent in patients infected with HCV genotype 3 although not all patients with genotype 3 infection have steatosis (15, 16). It has been suggested that the phenylalanine residue positioned at 164 of the core protein can increase the steatosis (35) and polymorphism of HCV core protein is also associated with intracellular accumulation of lipid (36). Furthermore, FAS gene is highly expressed in HCV-infected chimpanzees (37). Recent studies have shown that HCV infection enhances the proteolytic processing of SREBPs in hepatic cells (38) and HCV NS2 protein can up-regulate the transcription of SREBP-1c and FAS (39). Nevertheless, the molecular mechanisms underlying lipo-

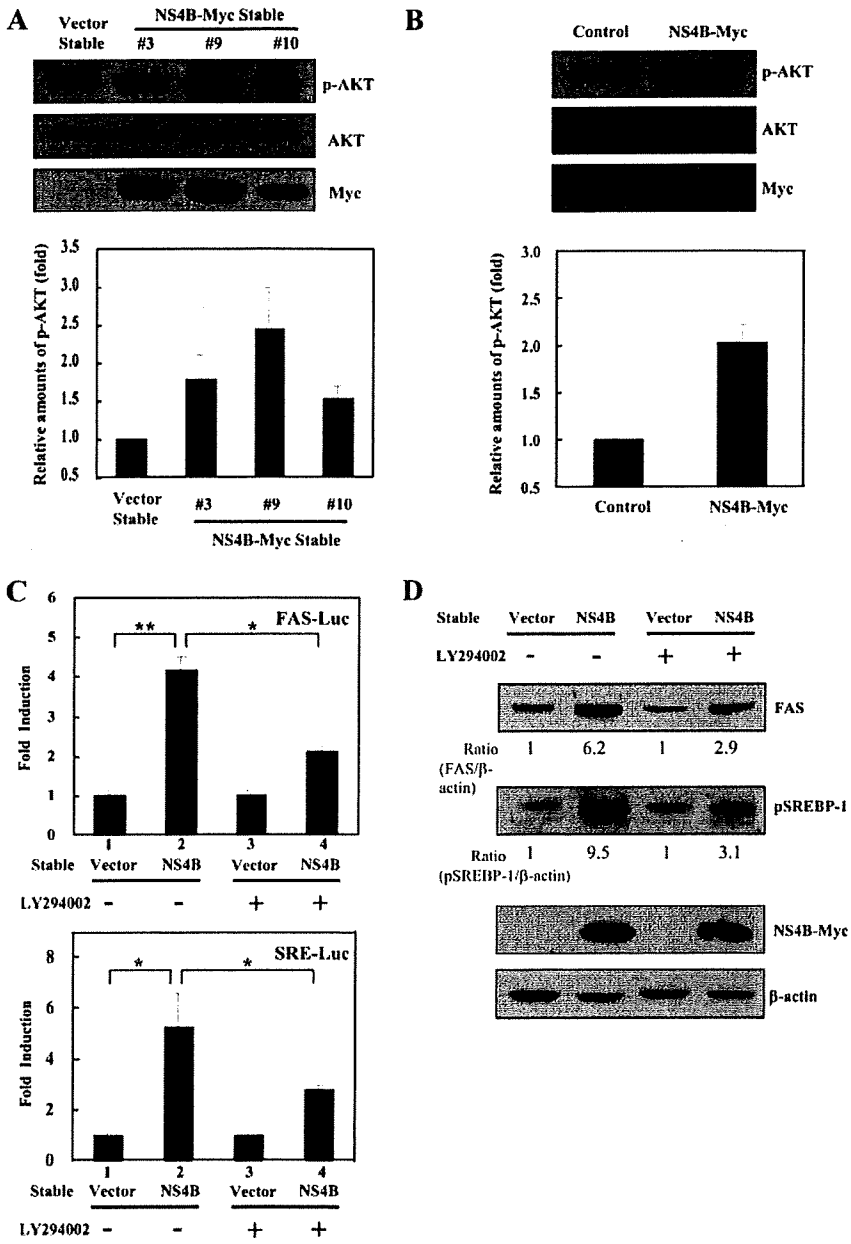


FIGURE 6. **AKT is required for NS4B-mediated SREBP activation.** *A*, total cell lysates harvested from both vector stable and NS4B-Myc stable cells were immunoblotted with the indicated antibodies (*upper panel*). Triplicate experimental data of *p*-AKT levels were quantified (*lower panel*). *B*, Huh7 cells were transiently transfected with either vector or NS4B-Myc expression plasmid. At 24 h after transfection, cells were harvested and immunoblotted with the indicated antibodies (*upper panel*). Data from triplicate experiments were quantified and each *bar* represents the average intensity of *p*-AKT level (*lower panel*). *C*, both vector stable and NS4B-Myc stable cells were transfected with either FAS-Luc (*upper panel*) or SRE-Luc (*lower panel*) reporter plasmid. At 24 h after transfection, cells were either left untreated (Me_2SO) or treated with LY294002 (5 μM) for an additional 12 h. Cells were harvested and then luciferase activities were determined. Data represent the mean of two independent experiments. *D*, total cellular extracts used in *C* were immunoblotted with the indicated antibodies. Quantification of the band intensity was determined by using a calibrated GS-800 densitometer.

genic signaling by either HCV or cellular factors are not fully understood.

In the present study, we set out to investigate the possible effects of HCV nonstructural proteins on lipogenic gene activities. Our results demonstrated that transcriptional activities of SREBPs were increased by NS4B protein but not by other nonstructural proteins in Huh7 cells. Both mRNA and protein levels of the FAS gene were also up-regulated by NS4B. Furthermore, NS4B in the context of the HCV subgenomic replicon activated the SREBPs and increased both mRNA and protein levels of the FAS gene. We have further shown that HCV NS4B increased the protein levels of both precursor and mature forms of SREBP-1 and SREBP-2. These results indicate that the NS4B protein enhances SREBP-1 and SREBP-2 protein expression at the transcriptional level.

It has been previously reported that HCV RNA replication requires the fatty acid biosynthetic pathway (40–42). In addition, a putative amphipathic helix within the N-terminal 26 residues of NS4B mediates membrane association, and these residues are critical for HCV replication in cell culture (10). In this study, we demonstrate that the HCV NS4B protein increases the FAS and SREBP activities via AKT signaling pathway. Therefore, we tempt to speculate that HCV NS4B-mediated lipogenesis may contribute to efficient HCV replication.

Several studies have shown that activations of PPAR by HCV core protein and hepatitis B virus X protein were required for hepatic accumulation of lipid (19, 27, 43). Because PPARα regulates constitutive transcription of genes encoding fatty acid metabolizing enzymes (44), and PPARγ is a master regulator of genes involved in fatty acid and glucose metabolism (45), further studies are required to determine the role of NS4B in PPAR regulation.

Recently, it has been reported that HCV infection enhanced the proteolytic processing of SREBPs in hepatic cells (38). In the present study, we have demonstrated that HCV infection increased the transcriptional activity of SREBP-1 and the protein level of FAS, and induced lipid accumulation in Huh7 cells. However, HCV infection had only a 3-fold effect on FAS expression. This effect is relatively modest as compared with the effect of HCV core and the NS4B co-expression system (23-fold). In fact, there is a genotype difference in steatosis induction (46). Because we

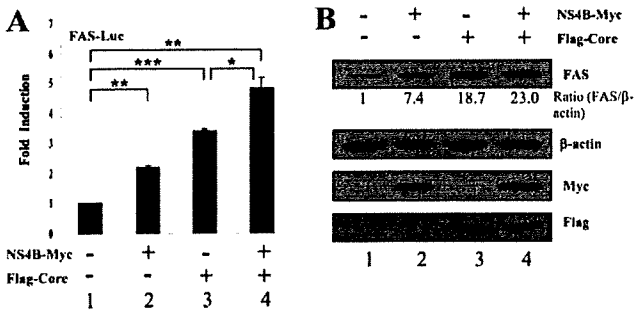


FIGURE 7. **HCV core-mediated transcriptional activation of SREBP-1 was synergistically elevated by NS4B protein.** *A*, Huh7 cells were transfected with vector, NS4B-Myc, FLAG-core, individually or cotransfected with NS4B-Myc and FLAG-core in the presence of FAS-Luc reporter plasmid. At 36 h after transfection, cells were harvested, and luciferase activities were determined. Data represent the mean of two independent experiments. *, $p < 0.05$, core *versus* HCV NS4B/core. **, $p < 0.01$, vector *versus* HCV NS4B. ***, $p < 0.001$, vector *versus* HCV core. *B*, total cellular extracts used in *A* were immunoblotted with the indicated antibodies. Quantification of the band intensity was determined by using a calibrated GS-800 densitometer.

used HCV genotype 2, the only available infectious HCV clone (JFH1) in the cell culture system (29), the fold discrepancy in FAS expression between two systems may be partly due to genotype difference, as we compared NS4B derived from either genotype 1b or genotype 2a (JFH-1) for its effects on transcriptional activation of SREBP-1 and found that NS4B protein derived from genotype 1b increased to a higher level of transcriptional activation of SREBP-1 than genotype 2a (data not shown). Alternatively, fold difference in FAS expression in two systems may be due to viral protein expression levels because only 20–30% of total cells are observed to be infected with the JFH-1 cell culture system. On the other hand, HCV core and NS4B proteins were overexpressed by the cotransfection experiment.

Because HCV core protein is involved in lipogenesis (19, 38), we investigated the co-expression effects of NS4B and core proteins on lipogenic signaling. Indeed, the HCV NS4B protein synergistically elevated the transcriptional activity of HCV core-mediated SREBP-1. We noticed that the effect of NS4B on SREBP activation was relatively modest. However, this effect was significant enough to increase both RNA and protein levels of lipogenic genes. This increase in turn resulted in an induction of lipid accumulation in NS4B expressing cells. Indeed, BODIPY staining data showed the similar level of lipid content between HCV NS4B-transfected cells (Fig. 5*B*) and HCV-infected cells (Fig. 5*E*), implying that NS4B alone substantially contributes to the accumulation of lipid. It has been reported that hepatic steatosis is an important factor of the progression of fibrosis (14, 47) and hepatocellular carcinoma (48) in patients with chronic HCV infection. In this regard, we speculate that NS4B-mediated lipid accumulation may contribute to the development of hepatocellular carcinoma.

In conclusion, we have demonstrated that HCV NS4B stimulated the expression of SREBPs and FAS protein and this was accomplished by activation of the AKT signaling pathway. However, how AKT activation affects SREBP gene expression is still not fully understood. Currently, both AKT (49) and liver X receptor (50) are known to be involved in SREBP activation. Nevertheless, how these proteins regulate SREBP is not understood. Therefore, further research is required to understand how NS4B may mediate its effect on SREBP. Collectively, our data imply that NS4B-mediated up-regulation of SREBPs may be associated with HCV-induced steatosis. These results provide a novel mechanism of liver pathogenesis associated with HCV-mediated lipogenesis.

REFERENCES

1. Lauer, G. M., and Walker, B. D. (2001) *N. Engl. J. Med.* **345,** 41–52
2. 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., and Kuo, G. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6547–6549
3. Ramalho, F. (2003) *Antiviral Res.* **60,** 125–127
4. Simmonds, P. (2004) *J. Gen. Virol.* **85,** 3173–3188
5. Lindenbach, B. D., and Rice, C. M. (2005) *Nature* **436,** 933–938
6. Bartenschlager, R., Lohmann, V., Wilkinson, T., and Koch, J. O. (1995) *J. Virol.* **69,** 7519–7528
7. Hügle, T., Fehrmann, F., Bieck, E., Kohara, M., Kräusslich, H.-G., Rice, C. M., Blum, H. E., and Moradpour, D. (2001) *Virology* **284,** 70–81
8. Lundin, M., Lindstrom, H., Grönwall, C., and Persson, M. A. (2006) *J. Gen. Virol.* **87,** 3263–3272
9. Egger, D., Wölk, B., Gosert, R., Bianchi, L., Blum, H. E., Moradpour, D., and Bienz, K. (2002) *J. Virol.* **76,** 5974–5984
10. Elazar, M., Liu, P., Rice, C. M., and Glenn, J. S. (2004) *J. Virol.* **78,** 11393–11400
11. Park, J.-S., Yang, J. M., and Min, M.-K. (2000) *Biochem. Biophys. Res. Commun.* **267,** 581–587
12. Einav, S., Sklan, E. H., Moon, H. M., Gehrig, E., Liu, P., Hao, Y., Lowe, A. W., and Glenn, J. S. (2008) *Hepatology* **47,** 827–835
13. Negro, F. (2006) *World J. Gastroenterol.* **12,** 6756–6765
14. Asselah, T., Rubbia-Brandt, L., Marcellin, P., and Negro, F. (2006) *Gut* **55,** 123–130
15. Mihm, S., Fayyazi, A., Hartmann, H., and Ramadori, G. (1997) *Hepatology* **25,** 735–739
16. Rubbia-Brandt, L., Fabris, P., Paganin, S., Leandro, G., Male, P.-J., Giostra, E., Carlotto, A., Bozzola, L., Smedile, A., and Negro, F. (2004) *Gut* **53,** 406–412
17. Perlemuter, G., Sabile, A., Letteron, P., Vona, G., Topilco, A., Chrétien, Y., Koike, K., Pessayre, D., Chapman, J., Barba, G., and Bréchot, C. (2002) *FASEB J.* **16,** 185–194
18. Dharancy, S., Malapel, M., Perlemuter, G., Roskams, T., Cheng, Y., Dubuquoy, L., Podevin, P., Conti, F., Canva, V., Philippe, D., Gambiez, L., Mathurin, P., Paris, J. C., Schoonjans, K., Calmus, Y., Pol, S., Auwerx, J., and Desreumaux, P. (2005) *Gastroenterology* **128,** 334–342
19. Kim, K. H., Hong, S. P., Kim, K., Park, M. J., Kim, K. J., and Cheong, J. (2007) *Biochem. Biophys. Res. Commun.* **355,** 883–888
20. Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002) *J. Clin. Investig.* **109,** 1125–1131
21. Brown, M. S., and Goldstein, J. L. (1997) *Cell* **89,** 331–340
22. Cho, Y. G., Yoon, J. W., Jang, K. L., Kim, C. M., and Sung, Y. C. (1993) *Mol. Cells* **3,** 195–202
23. Choi, S.-H., Park, K.-J., Ahn, B.-Y., Jung, G., Lai, M. M., and Hwang, S. B. (2006) *Mol. Cell. Biol.* **26,** 3048–3059
24. Toth, J. I., Datta, S., Athanikar, J. N., Freedman, L. P., and Osborne, T. F. (2004) *Mol. Cell. Biol.* **24,** 8288–8300
25. Bennett, M. K., Lopez, J. M., Sanchez, H. B., and Osborne, T. F. (1995) *J. Biol. Chem.* **270,** 25578–25583
26. Dooley, K. A., Millinder, S., and Osborne, T. F. (1998) *J. Biol. Chem.* **273,** 1349–1356
27. Kim, K. H., Shin, H.-J., Kim, K., Choi, H. M., Rhee, S. H., Moon, H.-B., Kim, H. H., Yang, U. S., Yu, D.-Y., and Cheong, J. (2007) *Gastroenterology* **132,** 1955–1967
28. Yoshikawa, T., Shimano, H., Amemiya-Kudo, M., Yahagi, N., Hasty, A. H., Matsuzaka, T., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Kimura, S., Ishibashi, S., and Yamada, N. (2001) *Mol. Cell. Biol.* **21,** 2991–3000
29. Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z.,

Murthy, K., Habermann, A., Kräusslich, H.-G., Mizokami, M., Bartenschlager, R., and Liang, T. J. (2005) *Nat. Med.* **11,** 791–796
30. Choi, S.-H., Jeong, S.-H., and Hwang, S. B. (2007) *Gastroenterology* **132,** 343–357
31. Fleischmann, M., and Iynedjian, P. B. (2000) *Biochem. J.* **349,** 13–17
32. Ono, H., Shimano, H., Katagiri, H., Yahagi, N., Sakoda, H., Onishi, Y., Anai, M., Ogihara, T., Fujishiro, M., Viana, A. Y., Fukushima, Y., Abe, M., Shojima, N., Kikuchi, M., Yamada, N., Oka, Y., and Asano, T. (2003) *Diabetes* **52,** 2905–2913
33. Barba, G., Harper, F., Harada, T., Kohara, M., Goulinet, S., Matsuura, Y., Eder, G., Schaff, Z., Chapman, M. J., Miyamura, T., and Bréchot, C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 1200–1205
34. Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Matsuura, Y., Miyamura, T., and Koike, K. (1997) *J. Gen. Virol.* **78,** 1527–1531
35. Jhaveri, R., McHutchison, J., Patel, K., Qiang, G., and Diehl, A. M. (2008) *J. Infect. Dis.* **197,** 283–291
36. Hourioux, C., Patient, R., Morin, A., Blanchard, E., Moreau, A., Trassard, S., Giraudeau, B., and Roingeard, P. (2007) *Gut* **56,** 1302–1308
37. Su, A. I., Pezacki, J. P., Wodicka, L., Brideau, A. D., Supekova, L., Thimme, R., Wieland, S., Bukh, J., Purcell, R. H., Schultz, P. G., and Chisari, F. V. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 15669–15674
38. Waris, G., Felmlee, D. J., Negro, F., and Siddiqui, A. (2007) *J. Virol.* **81,** 8122–8130
39. Oem, J.-K., Jackel-Cram, C., Li, Y.-P., Zhou, Y., Zhong, J., Shimano, H., Babiuk, L. A., and Liu, Q. (2008) *J. Gen. Virol.* **89,** 1225–1230
40. Ye, J., Wang, C., Sumpter, R., Jr., Brown, M. S., Goldstein, J. L., and Gale, M., Jr. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 15865–15870
41. Kapadia, S. B., and Chisari, F. V. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 2561–2566
42. Kim, S. S., Peng, L. F., Lin, W., Choe, W.-H., Sakamoto, N., Kato, N., Ikeda, M., Schreiber, S. L., and Chung, R. T. (2007) *Gastroenterology* **132,** 311–320
43. Tanaka, N., Moriya, K., Kiyosawa, K., Koike, K., Gonzalez, F. J., and Aoyama, T. (2008) *J. Clin. Investig.* **118,** 683–694
44. Aoyama, T., Peters, J. M., Iritani, N., Nakajima, T., Furihata, K., Hashimoto, T., and Gonzalez, F. J. (1998) *J. Biol. Chem.* **273,** 5678–5684
45. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) *Cell* **79,** 1147–1156
46. Abid, K., Pazienza, V., de Gottardi, A., Rubbia-Brandt, L., Conne, B., Pugnale, P., Rossi, C., Mangia, A., and Negro, F. (2005) *Hepatology* **42,** 744–751
47. Fartoux, L., Chazouillères, O., Wendum, D., Poupon, R., and Serfaty, L. (2005) *Hepatology* **41,** 82–87
48. Ohata, K., Hamasaki, K., Toriyama, K., Matsumoto, K., Saeki, A., Yanagi, K., Abiru, S., Nakagawa, Y., Shigeno, M., Miyazoe, S., Ichikawa, T., Ishikawa, H., Nakao, K., and Eguchi, K. (2003) *Cancer* **97,** 3036–3043
49. Furuta, E., Pai, S. K., Zhan, R., Bandyopadhyay, S., Watabe, M., Mo, Y. Y., Hirota, S., Hosobe, S., Tsukada, T., Miura, K., Kamada, S., Saito, K., Iizumi, M., Liu, W., Ericsson, J., and Watabe, K. (2008) *Cancer Res.* **68,** 1003–1011
50. Higuchi, N., Kato, M., Shundo, Y., Tajiri, H., Tanaka, M., Yamashita, N., Kohjima, M., Kotoh, K., Nakamuta, M., Takayanagi, R., and Enjoji, M. (2008) *Hepatol. Res.* **38,** 1122–1129

Biochemical and Biophysical Research Communications 379 (2009) 330–334

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

3D cultured immortalized human hepatocytes useful to develop drugs for blood-borne HCV

Hussein Hassan Aly [a], Kunitada Shimotohno [b], Makoto Hijikata [a,c,*]

[a] Laboratory of Human Tumor Viruses, The Institute for Virus Research, Kyoto University, Department of Viral Oncology, 53 Kawaharacho, Shogoin, Sakyoku, Kyoto 606-8507, Japan
[b] Center for Human Metabolomic Systems Biology, Keio University, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan
[c] Laboratory of Viral Oncology, Graduate School of Biostudies, Kyoto University, Konoecho, Yoshida, Sakyoku, Kyoto 606-8501, Japan

ARTICLE INFO

Article history:
Received 5 December 2008
Available online 25 December 2008

Keywords:
Hepatitis C virus
Infection
Replication
3D culture
PPAR
Immortalized hepatocytes
Blood-borne HCV

ABSTRACT

Due to the high polymorphism of natural hepatitis C virus (HCV) variants, existing recombinant HCV replication models have failed to be effective in developing effective anti-HCV agents. In the current study, we describe an *in vitro* system that supports the infection and replication of natural HCV from patient blood using an immortalized primary human hepatocyte cell line cultured in a three-dimensional (3D) culture system. Comparison of the gene expression profile of cells cultured in the 3D system to those cultured in the existing 2D system demonstrated an up-regulation of several genes activated by peroxisome proliferator-activated receptor alpha (PPARα) signaling. Furthermore, using PPARα agonists and antagonists, we also analyzed the effect of PPARα signaling on the modulation of HCV replication using this system. The 3D *in vitro* system described in this study provides significant insight into the search for novel anti-HCV strategies that are specific to various strains of HCV.

Infection with Hepatitis C virus (HCV) is a serious health problem worldwide and leads to high rates of liver cirrhosis and hepatocellular carcinoma [1]. Given that the standard HCV therapy remains insufficient for the successful treatment of many patients [2], the development of more effective and less toxic anti-HCV agents is required. *In vitro* systems like the HCV replicon-bearing cells and the infectious particle-producing JFH1 system, has contributed to the discovery of new targets for anti-HCV therapy. However, these recombinant HCV genomes only proliferate in sublines of HuH-7 cells, which do not permit infection or proliferation of blood-borne HCV. Due to the high polymorphism of natural HCV, data from recombinant HCV systems could be evaluated by studying the therapeutic response of a variety of naturally occurring HCVs. However, the current systems available for such study remain insufficient due to the low infection and replication efficiency of the natural HCV strains.

More recently, production and secretion of infectious HCV particles has been reported in two independent three-dimensional (3D) cell culture systems, termed the radial-flow bioreactor (3D/RFB) and the thermoreversible gelatin polymer (3D/TGP) systems. These results were not observed in monolayer cultures [3], suggesting that hepatocytes cultured in 3D more closely resemble liver cells *in vivo* [4] and thus support HCV proliferation. In addition, analysis of gene expression levels in 3D cultured cells revealed that the newly established immortalized human hepatocyte (HuS-E/2 cells) gene profile was altered to more closely resemble that of human liver tissue when the cells were cultured in 3D/TGP [5].

In the current study, we cultured HuS-E/2 cells in 3D/TGP and demonstrated efficient proliferation of natural HCV. Furthermore, gene expression analysis of these cells demonstrated the activation of the peroxisome proliferators-activated receptor α (PPARα) signaling pathway, suggesting an important role for this pathway in the replication of natural HCV. Thus, the *in vitro* system described appears to be a useful tool for the study of HCV infection and proliferation as well as for the development of effective anti-viral agents against various natural HCVs.

Materials and methods

Cell culture. Immortalized human hepatocytes (HuS-E/2) and LucNeo#2 replicon cells [6] were cultured as previously described [5,7]. For the 3D-TGP culture system, 1×10^5 HuS-E/2 cells were cultured in 1 ml Mebiol gel (Mebiol Inc., Kanagawa, Japan)/well in 12-well plates. Five hundred microliters of fresh medium was overlaid on the solidified gel, and was changed every 2 days. Cell

* Corresponding author. Address: Laboratory of Human Tumor Viruses, The Institute for Virus, Kyoto University, Department of Viral Oncology, 53 Kawaharacho, Shogoin, Sakyoku, Kyoto 606-8507, Japan. Fax: +81 75 751 3998.
E-mail address: mhijikat@virus.kyoto-u.ac.jp (M. Hijikata).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.12.054

extraction from the gel was done at the designated time points according to the manufacturer's protocol.

RNA extraction, reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR (Q-PCR). At the designated time points, total cellular RNA was extracted and 1 μg of total RNA was used as a template for RT-PCR and for the quantitative detection of HCV-RNA using real-time RT-PCR (Q-PCR) as previously described [10].

HCV infection experiment. HCV infection experiments were carried out using sera from patients infected with HCV. Infection in 2D culture was undertaken as previously described [5]. For 3D/TGP cultured cells, the gel was solidified, and 50 μl HCV-containing patient serum with a titer of 1×10^6 HCV-RNA/ml was added to the culture and mixed. The culture was continued until the cells were extracted. Following extraction from 3D-TGP, cells were centrifuged and washed three times thoroughly with PBS. RNA was then extracted from the cells as described above. HCV infection into HuS-E/2 cells was also examined in the presence of anti-E2 mouse monoclonal antibody (917) as outlined previously [8].

Treatment of cells with PPARα signaling agonists and antagonists. Fenofibrate or MK886 (Sigma–Aldrich, USA) were added to the culture medium of HuS-E/2 (2D-HuS-E/2) cells from day 0 of HCV infection; or the culture medium of LucNeo#2 replicon harboring cells. The cells were then cultured to the designated time point.

Microarray analysis. Gene expression profiles of 3D/TGP cultured HuS-E/2 cells were obtained by microarray analysis (3D-Genes Human 25, Toray, Tokyo, Japan) and compared to those of cells cultured in 2D.

Results

3D/TGP cultures enhance HCV proliferation in HuS-E/2 cells

Infection and proliferation of the HCV genotype 1b (HCV-RC5) derived from the serum of patient RC5 in HuS-E/2 cells cultured in 3D/TGP (3D/TGP-HuS-E/2 cells) was investigated and compared with that of HuS-E/2 cells cultured in 2D (2D-Hus-E/2). As outlined in Fig. 1A, the HCV-RNA levels in the 3D/TGP-HuS-E/2 cells were significantly higher at all of the time points examined following infection than in the 2D-HuS/E2 cells, suggesting that the 3D/TGP system greatly enhances the proliferation of naturally occurring HCV in HuS-E/2 cells. Similar results were also obtained for sera from additional patients (data not shown). To examine whether the infection is viral envelope-receptor mediated, the infection experiments using serum treated with anti-HCV-E2 antibody (α-E2) or with anti-tubulin (negative control) was also performed. Pre-incubation of the serum with α-E2 significantly reduced the total amount of HCV-RNA in the cells upon infection (Fig. 1B). This result suggested that the infection of natural HCV into 3D/TGP-HuS-E/2 cells was HCV-E2-dependent.

Inhibition of natural HCV replication in HuS-E/2 cells by Interferon

In order to test the effects of anti-viral agents on natural HCV replication in 3D/TGP HuS-E/2 cells, 50–100 U/ml of IFNα was added to the medium overlaying the HCV-RC5 infected 3D/TGP-HuS-E/2 cells. The two treatment concentrations resulted in the inhibition of HCV-RNA replication in 3D-HuS-E/2 cells by

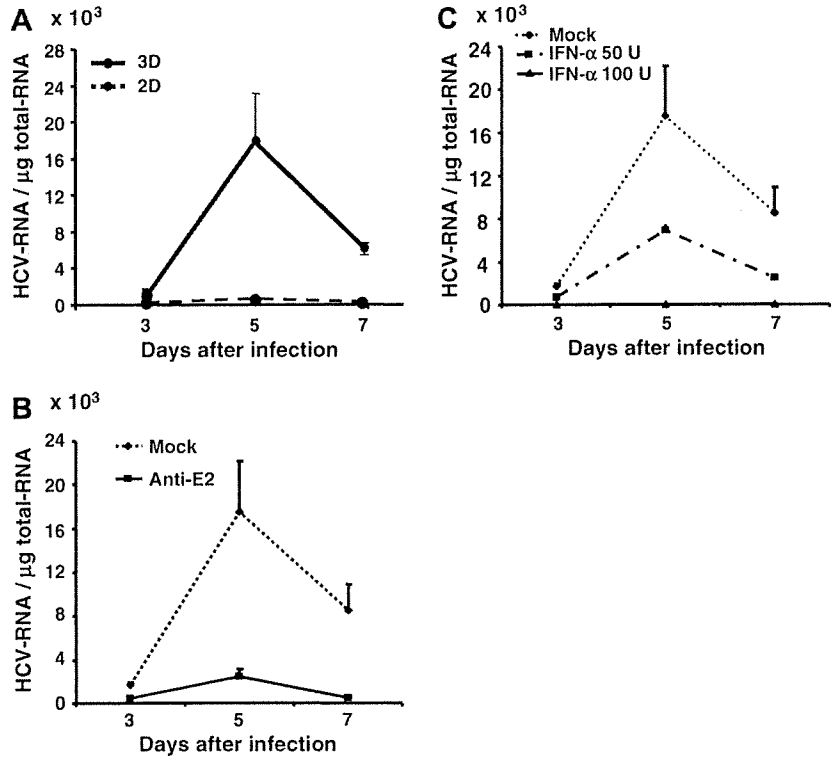


Fig. 1. HCV infection into 3D/TGP-HuS-E/2 cells. (A) 3D/TGP significantly enhanced HCV proliferation in HuS-E/2 cells. HCV patient serum was used to infect a similar number of HuS-E/2 cells cultured in 2D (hashed line) or 3D/TGP (solid line) culture for 24 h. Cells were then harvested and lysed at the indicated time points (3–7 days). The quantity of genomic HCV-RNA per 1 μg total RNA was determined by Q-PCR analysis. (B) Anti-E2 antibodies blocked HCV infection. HCV infection was performed as described in panel A in the presence of Anti-E2 specific or anti-tubulin (control) antibodies. (C) IFNα inhibits HCV replication in 3D/TGP-HuS-E/2 cells. HuS-E/2 cells were infected with HCV and fresh medium supplemented with or without (Mock), 50 U/ml, or 100 U/ml IFNα overlaid on the gel containing the cells and HCV proliferation measured as described above.

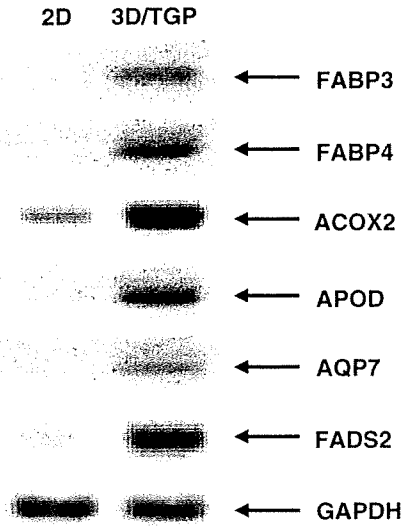


Fig. 2. RT-PCR analysis of the expression of genes identified by microarray. The PPARα regulated genes were increased in 3D/TGP-HuS-E/2 cells (3D-TGP) and their expression levels measured by RT-PCR. 2D represents RNA samples from 2D-HuS -E/2 cells. Twenty cycles of amplification were undertaken for the RT-PCR analysis. GAPDH expression served as an internal control. *Abbreviations:* FABP3, fatty acid binding proteins 3; FABP4, fatty acid binding proteins 4; ACOX2, acyl-coenzyme A oxidase 2; APOD, apolipoprotein D; AQP7, aquaporin 7; FADS2, fatty acid desaturase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

approximately 50–60% and almost completely, respectively, when compared to the replication in cells receiving mock treatment (Fig. 1C). These results demonstrate that the IFNα treatment was effective on HCV derived from RC5 and that 3D/TGP-HuS-E/2 cells may be useful for the screening of anti-HCV drugs for the treatment of natural HCV.

Increased activation of the PPARα signaling pathway in 3D cultured HuS-E/2 cells

Given that 3D/TGP-HuS-E/2 cells demonstrated enhanced proliferation of natural HCV, the gene expression profiles of these cells was compared with that of cells cultured under normal 2D conditions using microarray analysis in order to identify the factors required for the enhanced proliferation. Among the 24,268 genes compared in this analysis, 212 genes demonstrated a greater than four folds index increase in expression in 3D/TGP than standard cultured cells. Cell signaling pathway analysis of these 212 genes showed that six genes, including fatty acid binding proteins 4 and 3 (FABP4 and 3), apolipoprotein D (APOD), aquaporin 7 (AQP7), acyl-coenzyme A oxidase 2 (ACOX2), and fatty acid desaturase 2 (FADS2), were targets of PPARα signaling [9–12]. The increased expression of these genes in the 3D/TGP-HuS-E/2 cells was further confirmed by RT-PCR analysis (Fig. 2). Given that PPARα is an essential factor for normal hepatocyte function [13], these results indicate that 3D/TGP culture enhances the hepatocyte-specific characteristics of HuS-E/2 cells.

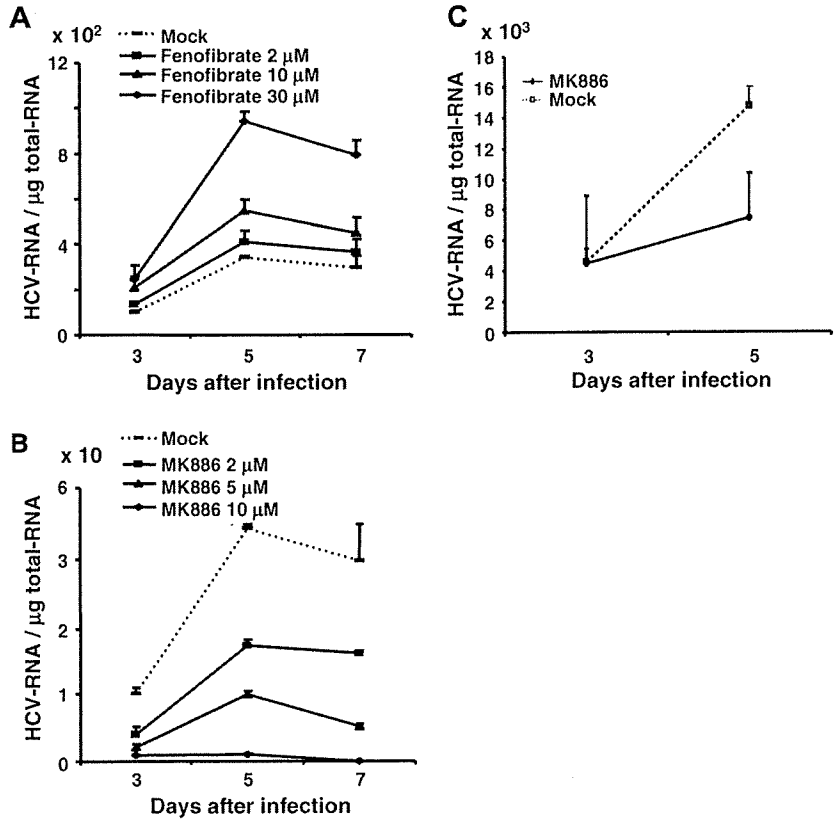


Fig. 3. The effects of PPARα agonists and antagonists on natural HCV proliferation. (A) HuS-E/2 cells were infected with HCV and fresh medium supplemented with or without (Mock) 2, 10, or 30 μM of fenofibrate overlaid on the cells. (B) Medium supplemented with or without (Mock), 2, 5, or 10 μM of MK886 was overlaid on 2D-HuS-E/2 cells infected with HCV. HCV proliferation following treatment was measured by Q-PCR. (C) Medium supplemented with or without (Mock), 10 μM of MK886 was overlaid on 3D/TGP-HuS-E/2 cells infected with HCV. HCV proliferation following treatment was measured by Q-PCR.