

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), sug-

gesting 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

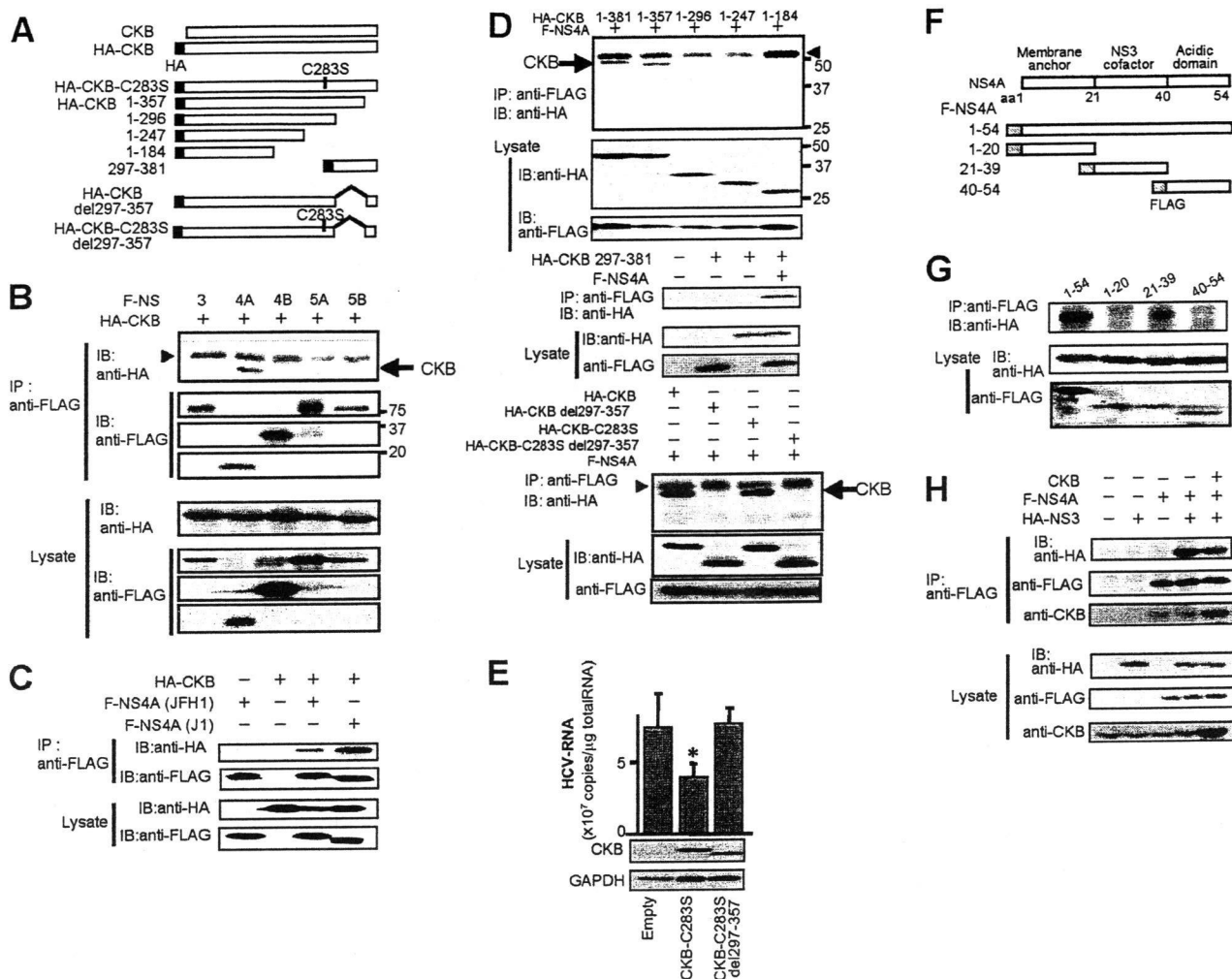


FIG. 3. CKB interacts with HCV NS4A. (A) Structures of CKB constructs used in the present study. A full-length wild-type CKB without an epitope tag (CKB) or with an N-terminal HA tag (HA-CKB), HA-CKB with deletions (aa 1 to 357, aa 1 to 296, aa 1 to 247, aa 1 to 184, and aa 297 to 381 and del297-357), 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

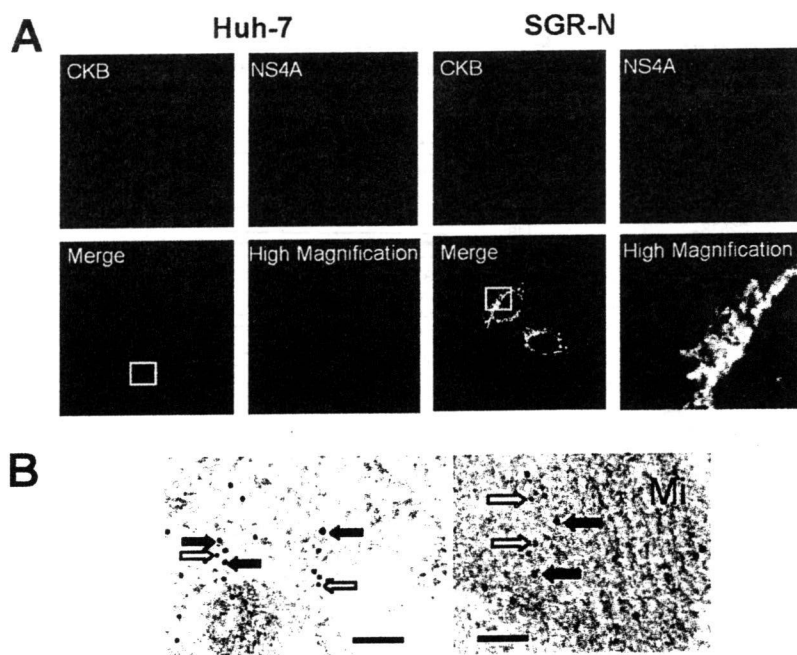


FIG. 4. Colocalization of CKB with HCV NS4A. (A) Indirect immunofluorescence analysis. The primary antibodies used were anti-CKB goat PAb (red) and anti-NS4A MAb (green). Merged images of red and green signals are shown. High-magnification panels are enlarged images of white squares in the merge panels. (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.

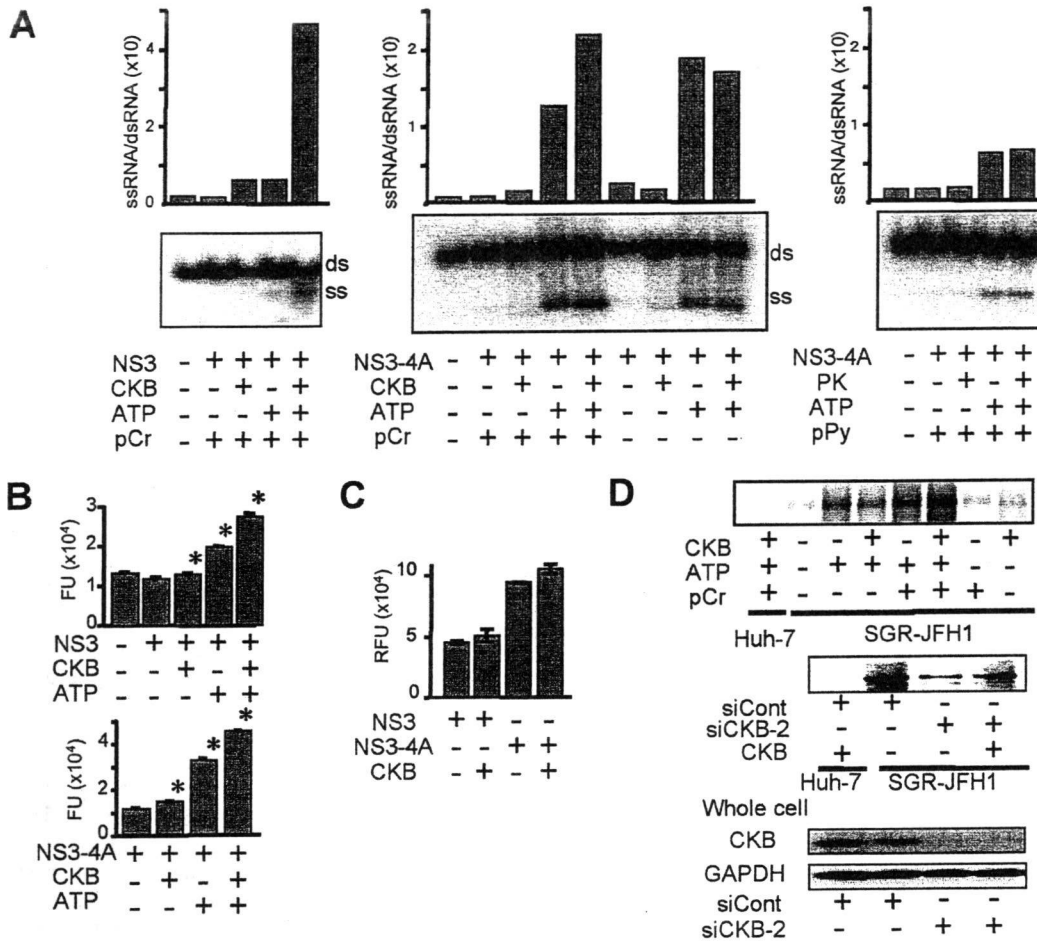


FIG. 5. CKB enhances NS3-4A helicase and HCV replicase activities. (A) In vitro RNA helicase activity of NS3-4A or NS3 was determined by detecting unwound single-strand RNA (ss) derived from the partially dsRNA substrate (ds). Band intensities corresponding to unwound products and those to dsRNA substrates 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. 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 pos-

itive 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.

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

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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 PPAR γ (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).

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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.

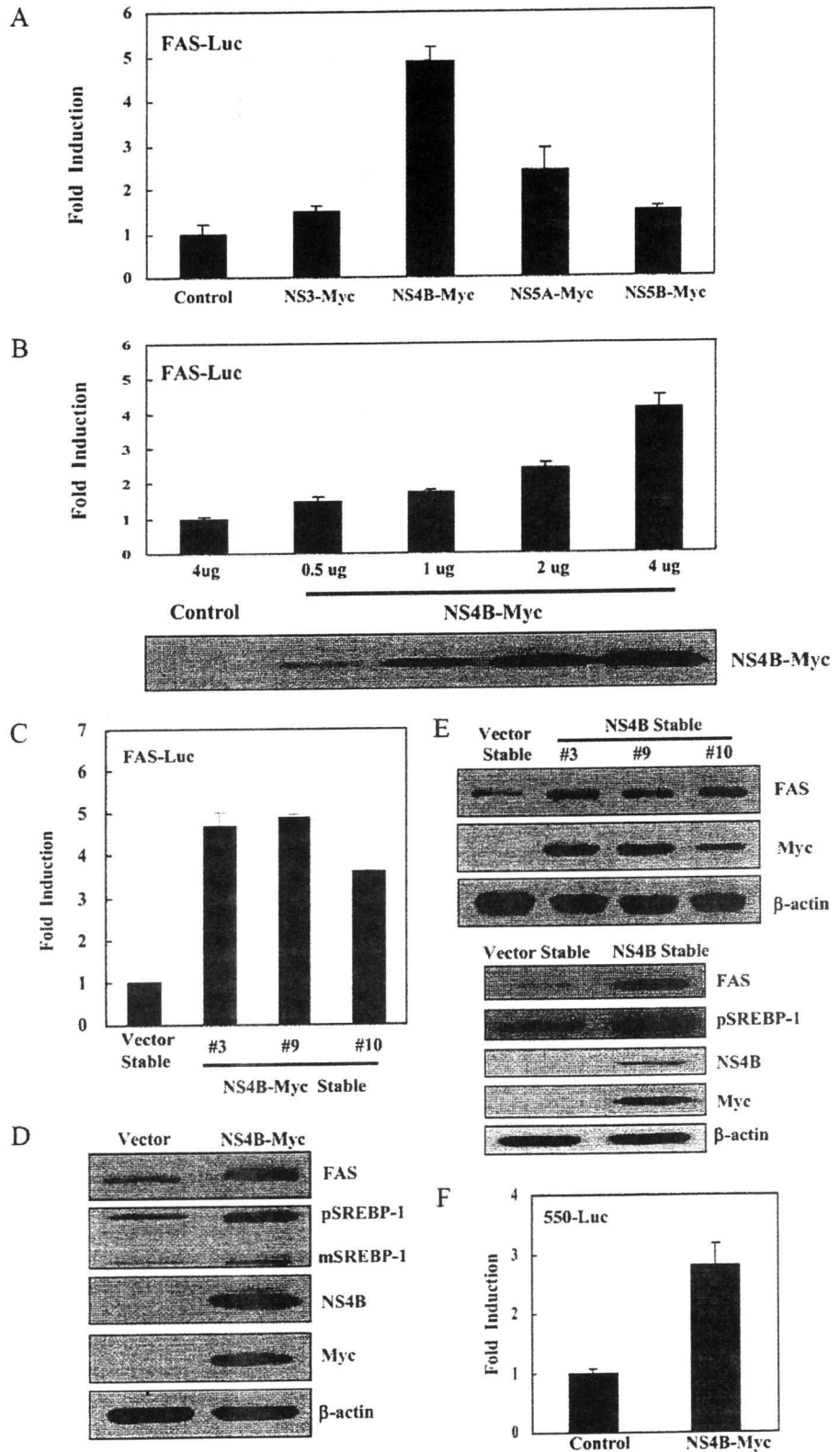
HCV NS4B Stimulates SREBP Signaling

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, $\sim 5 \times 10^5$ 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 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$ 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 anti-

body (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 \times 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 $^{\circ}\text{C}$. Cells were incubated in 5% bovine serum albumin for 20 min at 37 $^{\circ}\text{C}$ and then incubated with anti-Myc (Santa Cruz) monoclonal antibody for 2 h at 37 $^{\circ}\text{C}$. 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 $^{\circ}\text{C}$. 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-ACGG-CAGCCCCTGTAAACGACCACTGTGA-3 and SREBP-1-r, 5-TGCCAAGATGGTTCGCCCACTCACCAGG-3; FAS-f, 5-GAAACTGCAGGAGCTGTC-3 and FAS-r, 5-CACGGAGT-TGAGGCGGAT-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-cgcggatccatgGCCTCACAACCTTCCT-3 and NS4B-r, 5-ggcg-aattccaTCCGCTGATGAAAT-3; GAPDH-f, 5-GCTCTCC-AGAACATCATCCCTGCC-3 and GAPDH-r, 5-CGTTGT-CATACCAGGAAATGAGCTT-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.

HCV NS4B Stimulates SREBP Signaling

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 \pm 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. 1A). However, both NS3 and NS5B proteins do not increase the promoter activity of the FAS gene although the NS5A protein increases \sim 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. 1B). 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. 1C, 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. 1D, 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. 1E, upper panel). In addition, the protein expression level of pSREBP-1 was also increased in the number 3 NS4B stable cell line (Fig. 1E, 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 *pBPlc550-Luc* (28), the luciferase gene containing 550-bp fragments of the mouse SRBPP-1c promoter (550-Luc), and NS4B expression plasmid. As expected, luciferase activity was increased \sim 3-fold in cells expressing NS4B as compared with the control vector (Fig. 1F).

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 pSyn-SRE-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 \sim 3-fold the promoter activity of the HMG-CoA synthase gene as compared with the vector control (Fig. 2A). 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. 2B). 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. 2C, 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. 2D). We have further shown that the protein expression level of pSREBP-2 was also increased in clone 9 NS4B stable cells (Fig. 2E) 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. 3C). We further confirmed that *pBPlc550-Luc* reporter activity was also increased \sim 3-fold in replicon cells as compared with the IFN-cured cells (Fig. 3D).

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. 4A, 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. 4B). These data indicated that NS4B-mediated increases of transcriptional and translational

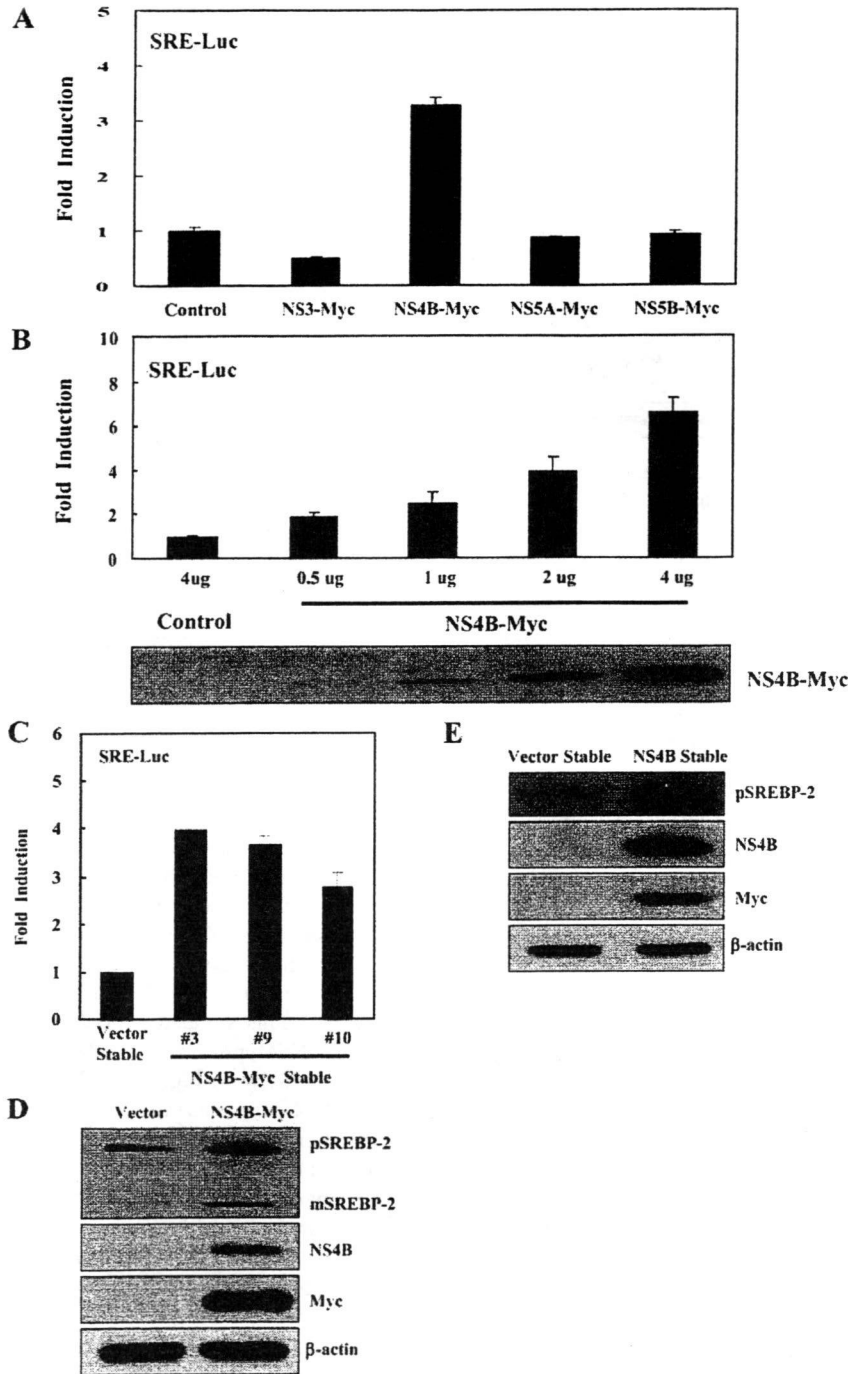


FIGURE 2. HCV NS4B protein increases the transcriptional activity of SREBP-2. *A*, Huh7 cells were cotransfected with SRE-Luc reporter plasmid together with the indicated expression plasmids. At 36 h after transfection, cells were harvested, and luciferase activities were determined. The amount of DNA 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. 5A). Because NS4B increases endogenous SREBP activation (Fig. 5A, lane 1 versus lane 4 in upper panel), SREBP (mature form)-mediated FAS activations (Fig. 5A, 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. 5B, left panel). This was further confirmed by quantification analysis (Fig. 5B, 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

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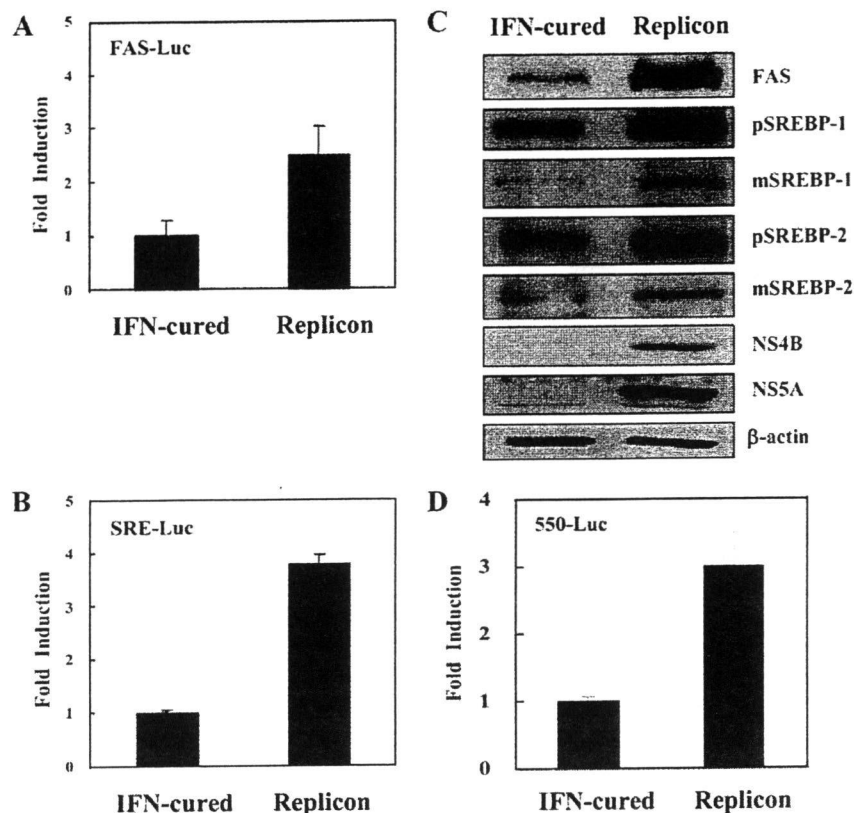


FIGURE 3. HCV subgenomic replicon increases both SREBP-1 and SREBP-2 activities. Both IFN-cured and HCV subgenomic replicon cells were transfected with either FAS-Luc (A) or SRE-Luc (B) reporter plasmid. At 36 h after transfection, cells were harvested and then luciferase activities were determined. 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.

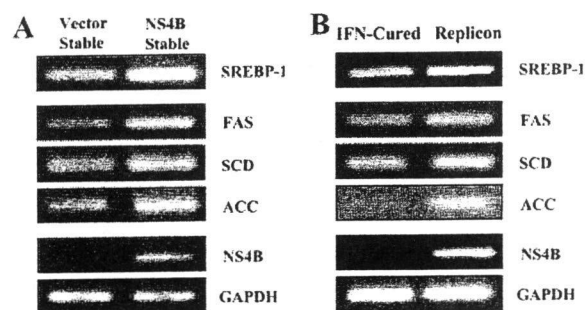


FIGURE 4. HCV NS4B protein up-regulates RNA levels of both SREBP-1 and its target genes. A and B, total RNAs were isolated from vector stable, NS4B-Myc stable, IFN-cured, and HCV subgenomic replicon cells. The cDNAs were synthesized by avian myeloblastosis virus reverse transcriptase from 1 μ g of total 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. 5C). HCV infection was con-

firmed by immunostaining cells with NS5A antibody (data not shown). Fig. 5C showed that the promoter activity of the FAS gene was increased \sim 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. 5D). We have further shown that more lipids were accumulated in HCV-infected cells as compared with un-infected cells (Fig. 5E, left panel). This was verified by quantification analysis (Fig. 5E, 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. 6A). This was also evident in Huh7 cells transiently expressing the NS4B protein (Fig. 6B). 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. 6C, 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. 6D). 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 PPAR γ (19). We therefore asked whether the NS4B protein has synergistic effects on HCV core-

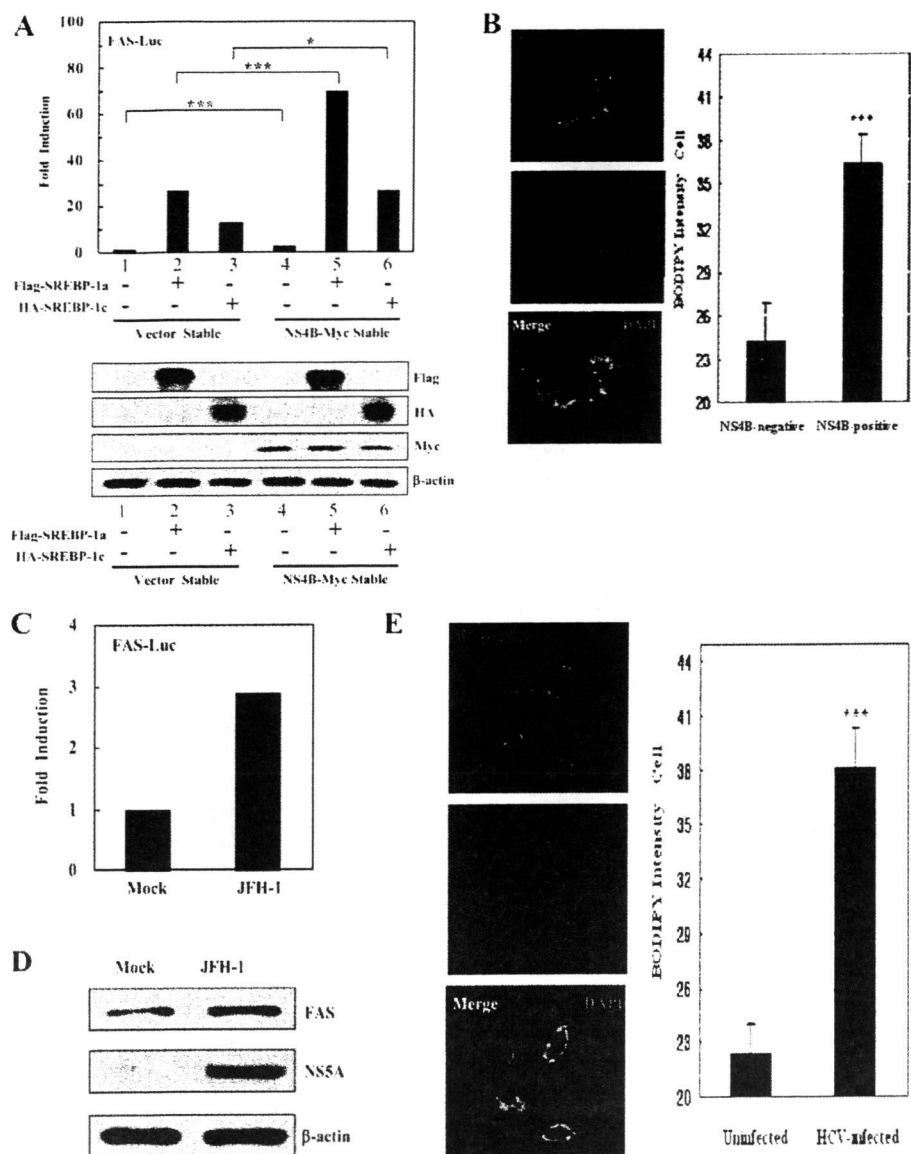


FIGURE 5. HCV NS4B protein induces lipid accumulation through activation of SREBPs. *A*, both vector and NS4B-Myc stable cells were cotransfected with FAS-Luc reporter and Flag-SREBP-1a (1–490 amino acids) or HA-SREBP-1c (1–447 amino acids) expression plasmids. At 36 h after transfection, cells were 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 μ M, 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 μ M, 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. 7A 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. 7A, 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. 7B). 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. 7B, *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-

HCV NS4B Stimulates SREBP Signaling

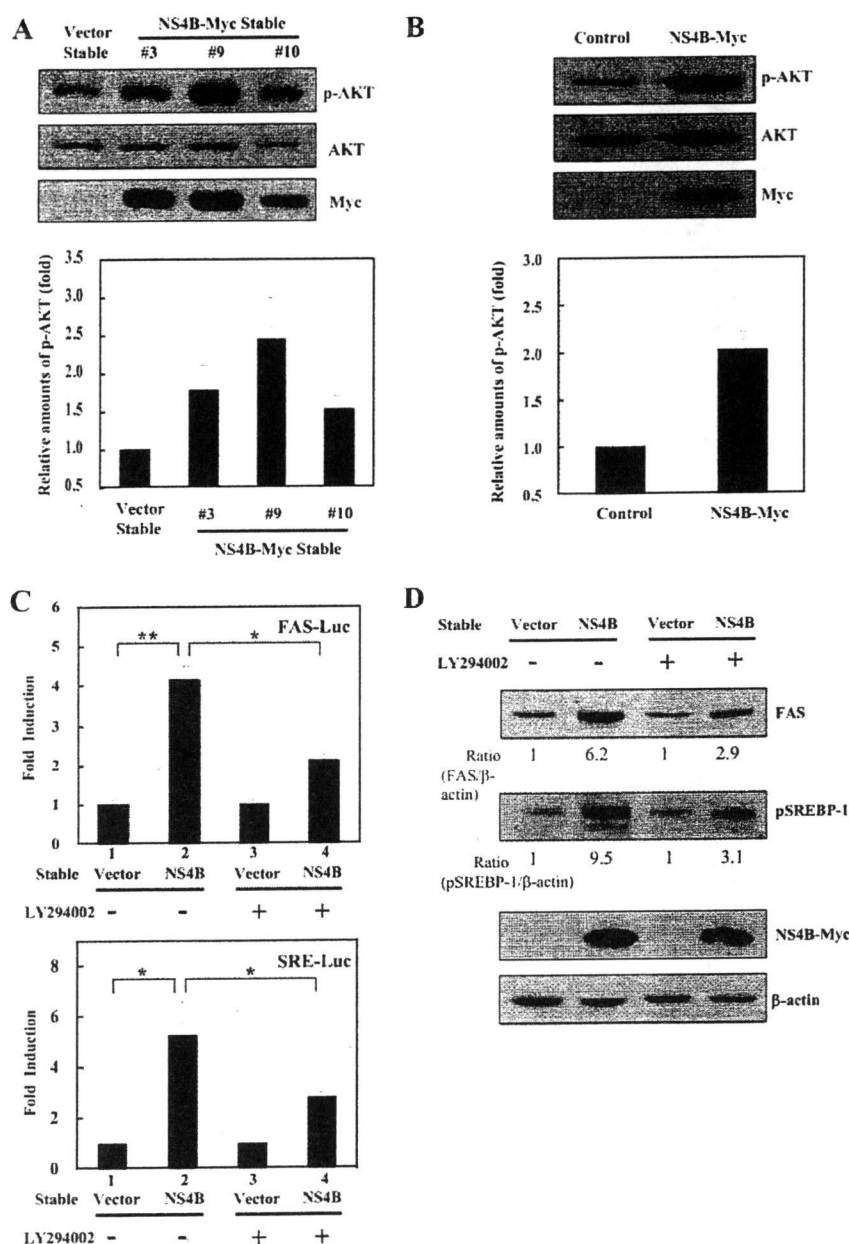


FIGURE 6. AKT is required for NS4B-mediated SREBP activation. *A*, total cell lysates harvested from both vector stable and NS4B-Myc stable cells were immunoblotted with the indicated antibodies (upper panel). Triplicate experimental data of p-AKT levels were quantified (lower panel). *B*, Huh7 cells 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 ($5 \mu\text{M}$) 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 PPAR α regulates constitutive transcription of genes encoding fatty acid metabolizing enzymes (44), and PPAR γ 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

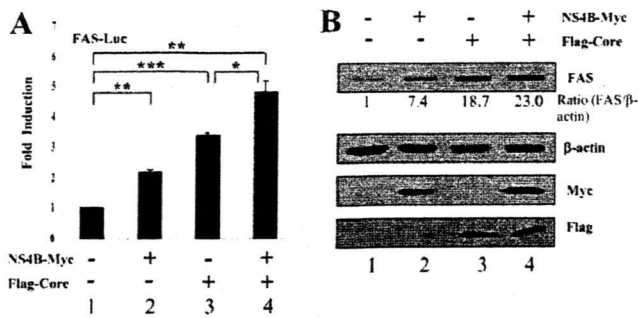


FIGURE 7. HCV core-mediated transcriptional activation of SREBP-1 was synergistically elevated by NS4B protein. A, Huh7 cells were transfected with vector, NS4B-Myc, FLAG-core, individually or cotransfected with NS4B-Myc and FLAG-core in the presence of FAS-Luc reporter plasmid. At 36 h after transfection, 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. 5B) and HCV-infected cells (Fig. 5E), 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 under-

stood. 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.

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Expert Opinion

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2. Natural history of HBV infection
3. Treatment of HBV infection
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6. Approaches to overcome limited therapeutic effects of antiviral drugs in chronic HBV infection
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Treatment of hepatitis B virus-infected patients: utility of therapeutic recommendations in developing countries

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Background: The treatment of individuals infected with hepatitis B virus (HBV) is a complex issue in practical settings, despite the explosion of new and effective antiviral agents. **Objective:** To assess the scope and limitations of ongoing treatment guidelines against HBV from a global perspective. **Methods:** Present therapeutic guidelines against HBV have been discussed with emphasis on their value in developing countries that harbor about 90% of the total number of global patients who are infected with HBV. **Results/Conclusion:** Treatment of HBV-infected patients should be appropriately followed up and healthcare delivery systems should be able to combat treatment-induced adverse side effects. Current therapeutic guidelines should be optimized based on the socio-economic conditions of developing countries.

Keywords: developed countries, developing countries, HBV, treatment guidelines

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1. Introduction

Hepatitis B virus (HBV), a member of the family *Hepadnaviridae*, is a non-cytopathic DNA virus that affects an estimated 2 billion persons worldwide [1]. Infection can be acute and self-limiting or lead to severe and fulminant hepatitis or chronic and persistent infection with complications such as liver cirrhosis (LC), hepatic decompensation, and hepatocellular carcinoma (HCC). About 1 million people die annually due to complications of HBV-related liver diseases [2].

Specific antiviral therapy is not usually recommended for patients with acute HBV infection because most acute hepatitis B (AHB) infections remain subclinical or self-limiting if clinically symptomatic. Fulminant hepatitis due to HBV represents a medical emergency, and such patients are provided with aggressive supportive therapy.

Chronic HBV infection is a state in which HBV replication persists for > 6 months after the initial infection, although assessing the timing of primary infection is often very difficult. For practical purposes, individuals infected with HBV who express hepatitis B surface antigen (HBsAg) in the sera for > 6 months are regarded as being chronic HBV carriers. These patients are characterized by the presence of HBV DNA, HBsAg, with or without other HBV-related antigens or antibodies in the sera and the liver [3]. However, HBsAg might be undetectable in some chronic HBV carriers due to low levels of HBV replication or other factors. Many chronic HBV carriers exhibit essentially no features of clinically apparent liver damage, whereas variable degrees of pathological liver damage are detectable by biopsy in many of these patients. Some chronic HBV carriers develop complications such as LC and HCC, which are more frequent among those with chronic hepatitis B (CHB). However, some apparently inactive HBV carriers can also develop LC and HCC [4]. As infection with HBV is lifelong, assessment of liver

damage at a single time-point in a chronic carrier provides neither information about the real clinical status of these patients nor insights about the possibility of future complications. This is particularly important in developing countries that harbor about 90% of total chronic HBV carriers. Due to poor socio-economic conditions and primitive healthcare delivery systems, many HBV infections remain undetected in these countries. These issues are important from global perspectives for the management of chronic HBV-infected patients.

The extent of liver damage in chronic HBV carriers should be assessed from liver biopsies, but this is not always possible. Accordingly, the extent of liver damage in most patients is determined as serum levels of the liver-derived enzyme, alanine aminotransferase (ALT).

Specific treatment is not presently recommended for patients with chronic HBV infection but without symptoms of clinically apparent liver damage, although such patients harbor serum HBV and HBV-related antigens. Antiviral therapy is recommended for patients with CHB with elevated ALT, and also for patients with complications [5]. Several studies have also recommended that patients with high HBV replication should be treated, irrespective of the extent of liver damage. Indeed, serum concentrations of HBV DNA do not always move in parallel with serum ALT levels [6].

During the mid 1980s, injections of IFN became the first approved therapy for patients with CHB; then the management of CHB was substantially modified over the next three decades. Of the several potentially effective antiviral drugs against HBV that are now available [7], oral antiviral drugs represent a major development. The application of novel oral antiviral agents against chronic HBV-infected individuals has also been accompanied by complex challenges [8-10]. These include a need for globally acceptable treatment guidelines, fixation of therapeutic end points, proper follow-up during medication and also after cessation of treatment, combating side effects, and adjustments to treatment guidelines in the context of the socio-economic conditions and medical infrastructures of developing countries.

This review discusses the scope and limitations of current strategies for treating chronic HBV infection. We comprehensively discuss the natural history of HBV-related disease, therapeutic goals and causes of the limited effectiveness of current therapeutic approaches. We finally provide our opinions regarding the value of current treatment guidelines against HBV from global perspectives, especially in the context of the socio-economic conditions of developing countries.

2. Natural history of HBV infection

2.1 Acute HBV infection

Patients with acute HBV infection express HBV DNA, HBsAg, hepatitis Be antigen (HBeAg), and IgM anti-HBc (IgM class antibody to hepatitis B core antigen [HBcAg]). As HBV-related markers are also detected in patients with chronic HBV infection, a diagnosis of acute HBV infection depends

on the serological detection of IgM class anti-HBc and the absence of a history of previous HBV infection [11]. However, this is an extremely difficult issue in endemic areas of HBV, because previous HBV infection remains unnoticed in most HBV-infected patients. Moreover, IgM class anti-HBc is also detectable in some patients with chronic HBV infection during acute exacerbation of chronic liver diseases. Acutely HBV infection can sometimes be followed by increased serum ALT levels and the clinical appearance of jaundice, or it can escape detection. Little is understood about the factors that determine the course of icteric hepatitis or non-icteric (subclinical) acute HBV infection. Patients with acute HBV infection remain at risk of developing HBV-related complications due to altered immune status or the use of immunosuppressive drugs [12]. However, most patients with resolved acute HBV infection develop antibody to HBsAg (anti-HBs), which protects against HBV. Notably, HBV DNA is often detected in serum decades after the apparently complete resolution of acute HBV infection (indicated by persistent anti-HBs). These factors indicate that all individuals who have been infected with HBV are at risk of developing chronic HBV infection.

2.2 Chronic HBV infection

Infection with HBV is usually self-limiting. Acute HBV infection may pass unnoticed or a self-limiting episode of acute hepatitis might occur [11,12]. On the other hand, some HBV-infected persons cannot control HBV replication and inappropriately respond to the initial HBV infection, which allows chronic infection to develop. This might depend on age and immune competence at the time of infection. Over 90% of neonates, infants and immunosuppressed individuals, as well as about 1 – 5% of immunocompetent adults, develop chronic HBV infection after exposure to HBV [13]. Circumstantial evidence supports the notion that the genotype of the infective HBV plays a role in the pathogenesis of chronic HBV infection, but this remains unconfirmed.

The natural history of chronic HBV infection involves a complex interplay between the virus and the host immune system. However, most of these cellular and molecular events remain obscure, and profound individual variations need to be identified. Infected patients express HBsAg in the sera for ≥ 6 months after initial infection, and levels of HBV replication vary. They might also express HBeAg or antibody to HBeAg (anti-HBe).

An understanding of the natural course of different phases of chronic HBV infection is critical in deciding the therapeutic strategy [14,15]. These phases are mainly detected in neonatal or perinatal patients infected with HBV. The initial immune tolerance phase is characterized by high levels of HBV DNA, normal serum ALT, and minimal or no inflammation on liver biopsy. This phase may last for up to four decades and then be followed by an immune clearance phase, the hallmark of which comprises flare-ups of ALT that can be followed by HBeAg negativity, or anti-HBe seroconversion. However,

HBeAg seroconversion does not always occur after ALT flare-ups. Hepatic decompensation has also been identified during this phase. The immune clearance phase can be followed by an 'inactive carrier state'. HBeAg is usually absent and serum HBV DNA is low or undetectable with normal ALT. This state can persist indefinitely, or HBV replication can become reactivated, with features of severe hepatic inflammation. In addition to these, the course of HBV infection can be altered by therapy, especially with nucleotide/nucleoside analogues (NA) [9,11,13]. Immunosuppressive drugs can also induce severe liver damage and increase HBV replication in chronic HBV carriers.

3. Treatment of HBV infection

Specific antiviral therapy is not usually recommended for patients with acute HBV infection. Fulminant hepatitis due to HBV represents a medical emergency, and such patients are provided with aggressive supportive therapy. This review discusses treatment for chronic HBV infection.

3.1 Ideal therapeutic targets

The fundamental aim of treatment is the complete eradication of all forms of HBV from infected hosts [16]. As infection with HBV causes hepatic damage, treatment is also aimed at the complete normalization of liver functions and recovery of liver histology. Complications due to persistent HBV infection should also be completely blocked by treatment. Ideally, treatment for HBV should lead to the complete clearance of all forms of HBV (including cccDNA) from the liver and the serum. Both HBsAg and HBeAg should disappear from the infected hosts, the respective anti-HBs and anti-HBe antibodies should develop, and ALT should be normalized permanently after the end of treatment. The damaged liver should regain its normal lobular hepatic architecture. Treated patients should not develop HBV-related complications such as LC or HCC, and future HBV DNA reactivation or ALT flare-ups should not occur.

3.2 Practical therapeutic targets

The fact is that current drugs or therapeutic regimens do not achieve the ideal therapeutic target in patients with chronic HBV infection. Complete eradication of HBV from an infected host is not presently a realistic goal, because no drugs exist that can destroy all forms of HBV DNA, including cccDNA. In fact, current anti-HBV drugs were discovered through their ability to destroy other viruses [6-8], because antiviral agents based on the complex life cycle of HBV could not be developed. Thus, most drugs can decrease or control the replication of HBV, but they cannot eradicate cccDNA in HBV-infected hosts. In addition, HBV is not cytopathic; the liver of HBV-infected hosts becomes damaged by host immune responses to the HBV. However, little is known about the nature of the host immunity that controls HBV replication and induces liver injury. Although different

types of host immunity play diverse roles in the pathogenesis of HBV infection, insight into these mechanisms remains inadequate [17].

A practical therapeutic target for the treatment of chronic HBV infection should be established based on the condition of each individual. A realistic aim of therapy includes normalization of ALT, persistent inhibition or at least significant suppression of HBV replication, and preventing or delaying the development of LC and HCC. Studies of non-Asian patients have shown that long-term survival benefits are closely linked to the induction of sustained HBeAg seroconversion in HBeAg-positive chronic HBV-infected individuals. Thus, therapeutic targets also include HBeAg negativity or seroconversion to anti-HBe. However, studies in Asian patients have shown that HBeAg seroconversion might not be so important for the final prognosis or for blocking complicating liver diseases. Nevertheless, HBeAg seroconversion represents a primary target of therapy [14,15].

3.3 Assessment of response

The therapeutic response depends on the nature of the therapeutic regimen. A biochemical response is determined by measuring serum ALT concentrations, because sustained normalization of serum ALT indicates a biochemical response [15]. A virological response is based on serum HBV DNA concentrations. An antiviral effect is defined as a reduction in the serum HBV-DNA level of $\geq 1 \log_{10}$ IU/ml from baseline within 3 months of starting therapy [15,18,19]. A histological response is based on hepatic histological changes. Again, responses can be referred to as on-treatment and sustained responses (continued response after cessation of therapy).

3.4 Treatment guidelines for patients with chronic HBV infection

Treatment guidelines for HBV-infected persons have been published by major professional organizations such as the American Association for the Study of Liver Diseases (AASLD), the European Association for the Study of the Liver (EASL), and the Asia-Pacific Association for the Study of the Liver (APASL) [15,20,21]. In addition, liver associations in various countries also develop their own guidelines for treating patients with chronic HBV infection. In some countries, treatment should follow national guidelines to receive government or insurance support.

Treatment is not generally recommended for patients with chronic HBV infection during the immune tolerance phases [21,22], a notion that is somewhat fallacious. In fact, early clinical trials enrolled patients based on the assumption that those with high viral load and liver damage are at risk of developing progressive liver disease. However, studies over the past three decades have indicated that establishing guidelines for treating chronic HBV carriers is an extremely complex task. In addition, generalized treatment guidelines might not be applicable to all types of chronic HBV carriers. For example, HBV has been undetectable by assays that could