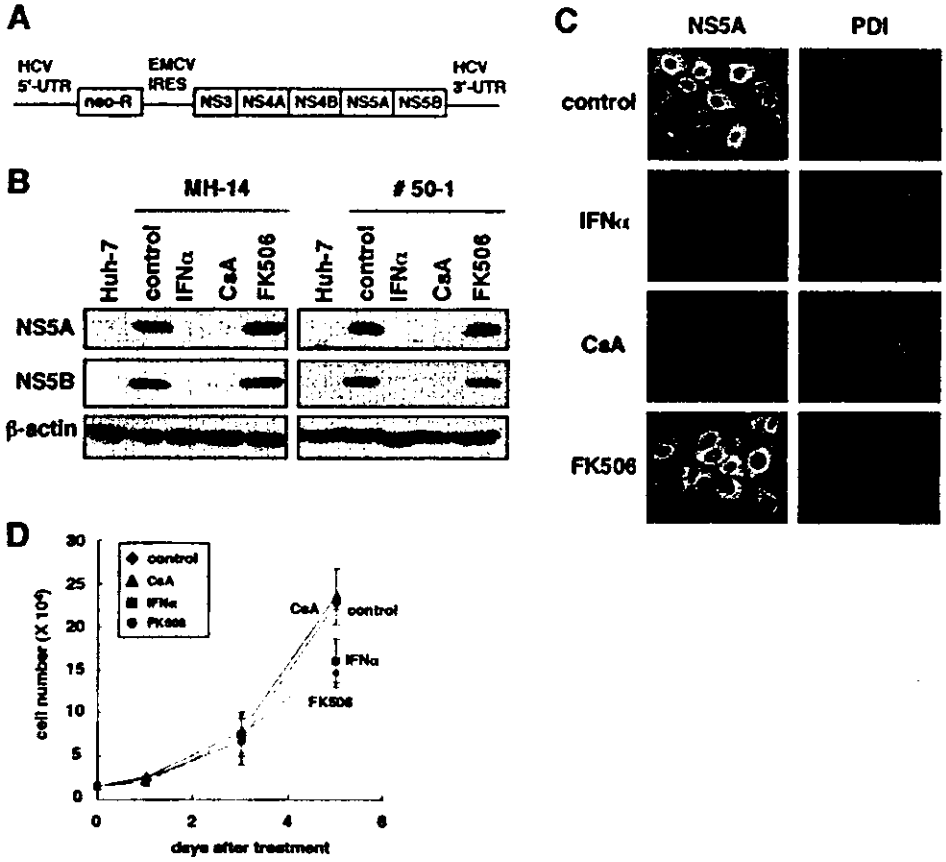

Fig. 1. CsA decreased HCV protein production in HCV replicon cells. (A) Schematic representation of the HCV subgenomic replicon used in this study. The replicon is composed of the HCV genome 5′-untranslated region (UTR) (**solid line**), the neomycin phosphotransferase gene (neo-R) (**open box**), the encephalomyocarditis virus internal ribosomal entry site (EMCV IRES) (**solid line**), the coding region of NS3 to NS5B of HCV (NS3, NS4A, NS4B, NS5A, and NS5B) (**open boxes**), and the HCV genome 3′-untranslated region (**solid line**). (B) Immunoblot analysis for NS5A (**upper panels**), NS5B (**middle panels**), and β-actin protein as an internal control (**lower panels**) was performed using cell lysate from MH-14 (**left panels**) and #50-1 cells (**right panels**) treated with each agent shown above the panels for 7 days. Control, no treatment; IFN-α, 100 U/mL IFN-α; CsA, 1 μg/mL CsA; FK506, 1 μg/mL FK506. Cell lysate from the parental Huh-7 cells was also analyzed as a negative control (Huh-7). (C) Indirect immunofluorescence analysis for NS5A (**left panels**) and protein disulfide isomerase (PDI) protein as an internal control (**right panels**) was performed using MH-14 cells treated without (control) or with 100 U/mL IFN-α (IFN-α), 1 μg/mL CsA (CsA), or 1 μg/mL FK506 (FK506) for 7 days. (D) Effect of CsA on growth of MH-14 cells. MH-14 cells (1×10^4) were propagated for 16 hours and then treated with each agent for 1, 3, and 5 days. Cell numbers were counted and plotted against the time of treatment. The data represent the means of cell numbers in 3 independent experiments. The standard deviation values are shown. ◆, no treatment; ▲, 1 μg/mL CsA; ■, 100 U/mL IFN-α; ●, 1 μg/mL FK506.

Immunoblot Analysis. Immunoblot analysis was performed as described previously.[8] The primary antibodies used in this experiment were anti-NS5A, anti-NS5B, and anti–β-actin (Sigma, St. Louis, MO) antibodies.

Indirect Immunofluorescence Analysis. Indirect immunofluorescence analysis was performed as described previously.[8] The primary antibodies used in this experiment were anti-NS5A and anti–protein disulfide isomerase (StressGen, Victoria, Canada) antibodies.

Reverse-Transcription Polymerase Chain Reaction Analysis. Total RNA of cultured cells was isolated with Sepasol-RNA I Super (nacalai tesque, Kyoto, Japan) as recommended by the manufacturer. Reverse-transcription polymerase chain reaction (RT-PCR) analysis was performed using a one-step RNA PCR kit (Takara, Kyoto, Japan) according to the manufacturer's directions. The primers used for detection of messenger RNAs for 2′,5′-oligoadenylate synthetase and double-strand RNA-dependent protein kinase were 5′-CCGTGAAGTTTGAGGTCCAG-3′, 5′-GACTAATTCCAAGACCGTCCG-3′ and 5′-TGGCCGCTAAACTTGCATATC-3′, and 5′-GCGAGTGTGCTGGTCACTAAAG-3′, respectively.

Northern Blot Analysis. Northern blot analysis was performed as described previously.[6] The probe complementary to the NS5B sequence used in this experiment was described previously.[6]

Real-Time RT-PCR Analysis. The 5′-untranslated region of HCV genome RNA was quantified using the ABI PRISM 7700 sequence detector (Applied Biosys-

tems, Foster City, CA) as described previously.[9] The forward and reverse primers used in this experiment were 5′-CGGGAGAGCCATAGTGG-3′ and 5′-AGTACCACAAGGCCTTTCG-3′, respectively. The fluorogenic probe was 5′-CTGCGGAACCGGTGAGTACAC-3′. As an internal control, ribosomal RNA was also quantified using TaqMan ribosomal RNA control reagents (Applied Biosystems).

In Vitro *HCV Infection Experiment.* The *in vitro* HCV infection experiment was performed essentially as described previously.[10,11] PH5CH8 cells (1×10^5) were infected with the plasma 1B-2 (equivalent to 10^4 to 10^5 HCV RNA copies), which was prepared from an HCV-positive blood donor.[11] At 24 hours postinoculation, the cells were washed 3 times with phosphate-buffered saline and maintained with fresh medium.

Transfection and Reporter Assay. Transfection into MH-14 and H9 cells was performed using FuGENE 6 (Roche, Indianapolis, IN) and Lipofectamine 2000 transfection reagent (Invitrogen, San Diego, CA), respectively, according to the manufacturer's protocol. The reporter assay was performed as described previously.[8] The reporter plasmids used in this study were pNFAT-Luc, pAP1-Luc, pNFκB-Luc (PathDetect Reporter System; Stratagene, La Jolla, CA), and pRL-TK (dual-luciferase reporter assay system; Promega, Madison, WI).

Results

CsA Treatment Decreased HCV Protein Levels in HCV Replicon Cells. We examined the effects of various types of compounds on the replication of the HCV genome using MH-14 cells, in which the HCV subgenomic replicon as shown in Fig. 1A autonomously replicated (Miyanari et al., manuscript in submission). Consequently, we observed a suppressive effect of immunosuppressant CsA on HCV genome replication as described below. As shown in Fig. 1B, treatment with 1 μg/mL CsA as well as 100 U/mL IFN-α, which was used as a positive control, for 7 days decreased the amount of HCV NS5A and NS5B proteins to levels undetectable by immunoblot analysis (Fig. 1B, left panels). Indirect immunofluorescence analysis showed that NS5A protein production was reduced in all of the cells treated with 1 μg/mL CsA, whereas the level of protein disulfide isomerase, which is an endoplasmic reticulum marker, as an internal control was not altered under this condition (Fig. 1C). This reduction of HCV proteins was not observed on treatment with another well-known immunosuppressant, FK506 (1 μg/mL)[12] (Fig. 1B and C). Treatment with CsA, FK506, or IFN-α had little influence on cell growth (Fig. 1D), indicating that the CsA activity to reduce HCV protein levels was not due to a cytotoxic effect. The similar effect of CsA was observed using #50-1 cells (Fig. 1B, right panels, and data not shown), which is another cell clone carrying the HCV subgenomic replicon.[6] The above data suggested that CsA has the potential to decrease HCV protein expression in HCV replicon cells.

CsA Treatment Decreased Replicon RNA Levels in HCV Replicon Cells. We then investigated whether CsA affects replicon RNA levels in MH-14 cells. We analyzed replicon RNA in MH-14 cells treated without or with CsA, FK506, or IFN-α for 7 days by Northern blot analysis. As shown in Fig. 2A, treatment with 1 μg/mL CsA decreased the amount of replicon RNA to an undetectable level. Treatment with 100 U/mL IFN-α produced a similar result, whereas treatment with FK506 did not show a remarkable effect on the replicon RNA level, which is consistent with the result of the effect on HCV protein levels shown in Fig. 1B and C. To quantitate the effect of CsA treatment more accurately, we performed real time RT-PCR analysis for measuring HCV RNA levels. As shown in Fig. 2B, treatment with CsA decreased the replicon RNA titer to about 1/500 of the original in a dose-dependent manner; in addition, treatment with IFN-α lowered the replicon RNA titer to about 1/400 (Fig. 2B). Treatment with FK506 did not affect the replicon RNA titer at any dose used in this study (Fig. 2B).

We examined the time-course alteration of the replicon RNA titer after treatment with CsA and IFN-α. Treatment with 1 μg/mL CsA decreased the titer gradually and the level of HCV RNA was reduced to about 1/300 of the original on the seventh day (Fig. 2C). In the case of cotreatment with CsA and IFN-α, we observed a further reduction at any time point examined (third, fifth, and seventh day) compared with the single treatment with either CsA or IFN-α; that is, the replicon RNA level in MH-14 cells treated with both CsA and IFN-α for 7 days was about 1/5 that in the cells treated with IFN-α alone (Fig. 2C).

CsA Inhibited HCV Multiplication in Infected Cultured Hepatocytes. As an *in vitro* infection experiment system of HCV, there have been some reports using a human nonneoplastic hepatocyte cell line, PH5CH cells.[10,11,13-15] HCV reportedly multiplied in PH5CH8 cells that were infected with plasma from HCV-infected patients and was released into culture medium. HCV RNA from infected PH5CH8 cells was also confirmed to be replicatable in Huh-7 cells.[16] Then, using this *in vitro* infection system, we investigated whether CsA also functions to suppress HCV replication. PH5CH8 cells were treated with HCV-positive plasma; subsequently, the HCV RNA genome titer at various time points after in-

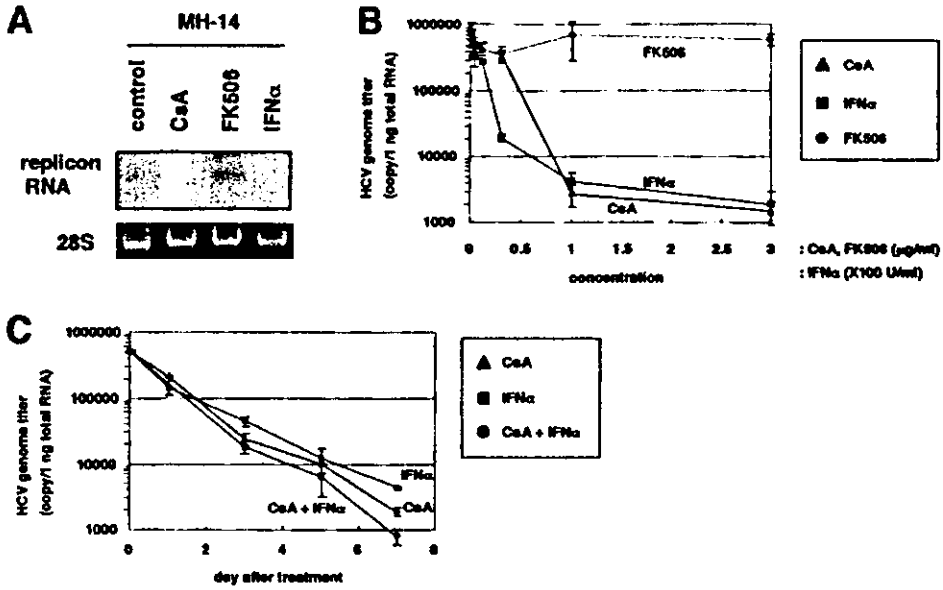

Fig. 2. CsA decreased HCV replicon RNA levels in HCV replicon cells. (A) Total RNA from the cells in the same condition as shown in Fig. 1B was recovered, and HCV replicon RNA was detected by Northern blot analysis (**upper panel**). As an internal control, the ethidium bromide staining pattern of 28S ribosomal RNA is also shown (**lower panel**). (B) HCV replicon RNA level was quantified by real-time RT-PCR analysis using total RNA from MH-14 cells treated with various concentrations of CsA, FK506, and IFN-α for 7 days. The amount of HCV replicon RNA per 1 ng of total RNA was plotted against the concentration of CsA (μg/mL) (▲), FK506 (μg/mL) (○), or IFN-α (×100 U/mL) (■) shown below the x-axis. The data represent the means of HCV replicon RNA titer in 3 independent experiments. The standard deviation values are shown. (C) HCV replicon RNA was quantified as described in B using total RNA from MH-14 cells incubated for 1, 3, 5, or 7 days with 1 μg/mL CsA (▲), 100 U/mL IFN-α (■), and a combination of 1 μg/mL CsA and 100 U/mL IFN-α (●). The amount of HCV replicon RNA per 1 ng of total RNA was plotted against the time of treatment.

oculation was quantified by real-time RT-PCR analysis. As shown in Fig. 3, the HCV RNA genome titer on the fifth day after inoculation in the cells was increased about 10-fold compared with that on the first day. However, a significant increase of the HCV RNA genome titer at these time points was not observed in the cells treated continuously with CsA or IFN-α (Fig. 3). This result suggested that CsA inhibited the replication of HCV infected in cultured hepatocytes.

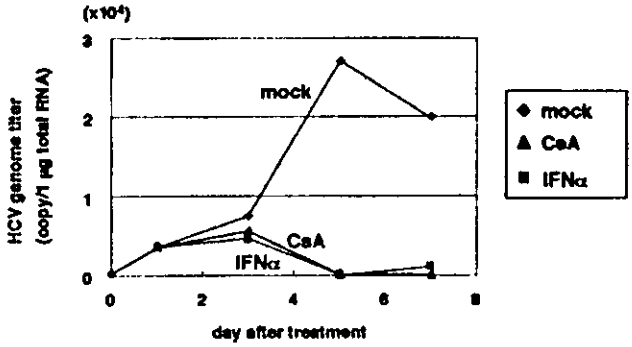

Fig. 3. CsA inhibited HCV multiplication in PH5CH8 cells infected with HCV-positive plasma. PH5CH8 cells were infected with HCV-positive plasma for 24 hours. After being washed with phosphate-buffered saline 3 times, the cells were cultured with fresh medium supplemented without (◆) or with 1 μg/mL CsA (▲) or 100 U/mL IFN-α (■). HCV genome RNA per 1 μg total RNA was detected by real-time RT-PCR analysis.

Anti-HCV Activity of CsA Was Not Mediated by IFN-α–Induced Signal Transduction Pathway. Because the efficacy, dose dependency, and time-course alteration of anti-HCV effect of CsA was almost the same as that of IFN-α, the possibility was raised that CsA exerted anti-HCV activity via the same mechanism as IFN-α. To investigate this possibility, we analyzed the expression of IFN-α downstream genes in HCV replicon cells. RT-PCR analysis showed that the messenger RNA for 2′,5′-oligoadenylate synthetase and double-strand RNA-dependent protein kinase, which are both IFN-α downstream antiviral proteins,[17] was induced by IFN-α but not by CsA (Fig. 4). These results suggested that CsA exerted anti-HCV activity without the induction of known IFN-α downstream genes. Consequently, it was suggested that CsA exerted its anti-HCV effect independent of the IFN-α signal transduction pathway.

CsA Exerted Anti-HCV Activity Independent of Its Immunosuppressive Function. CsA is known to bind to cellular target molecules, cyclophilins (CyP), to inactivate their peptidyl-prolyl *cis/trans* isomerase function.[7] However, this peptidyl-prolyl *cis/trans* isomerase inhibition is not involved in the CsA-induced immunosuppression. The CsA/CyP complex can also associate with another cellular protein, calcineurin (CN), which belongs to the phosphatase superfamily. This binding abrogates its

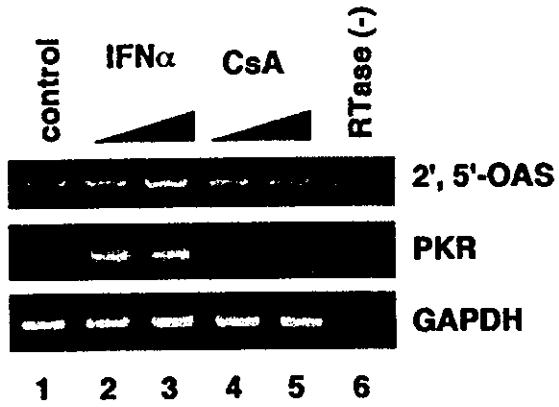

Fig. 4. CsA exerted anti-HCV activity independent of the signal transduction pathway activated by IFN-α. MH-14 cells were treated without (**lane 1**) or with 100 (**lane 2**) or 300 U/mL IFN-α (**lane 3**) and 1 (**lane 4**) or 3 μg/mL CsA (**lane 5**) for 3 days. The messenger RNAs of 2′,5′-oligoadenylate synthetase (2′,5′-OAS) (**upper panel**), double-strand RNA-dependent protein kinase (PKR) (**middle panel**), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (**lower panel**) were detected by RT-PCR analysis. RTase (−) (**lane 6**) is an experimental control reacted without reverse transcriptase.

phosphatase activity, resulting in silencing of transcription factor NF-AT. The inhibition of the CN/NF-AT pathway is the essential mechanism for CsA-mediated immunosuppression.[7] To clarify the mechanism of anti-HCV function of CsA in cultured hepatocytes, we investigated whether CN/NF-AT inhibition capacity of CsA is related to anti-HCV activity or not. We examined transcriptional activity of NF-AT in MH-14 cells treated with CsA by reporter assay. As shown in Fig. 5A and D, although CsA treatment suppressed NF-AT activity in the T-lymphocyte cell line (H9 cells), as reported previously, CsA had no effect on NF-AT activity in MH-14 cells. In T lymphocytes, it has also been reported that CsA blocks other signaling pathways such as JNK, p38, and nuclear factor κB, possibly via CN inhibition.[7] However, our reporter assay suggests that CsA also does not influence these signaling pathways of hepatocytes in contrast to T lymphocytes (Fig. 5B and C). From the above results, it was suggested that CN-inducible signaling path-

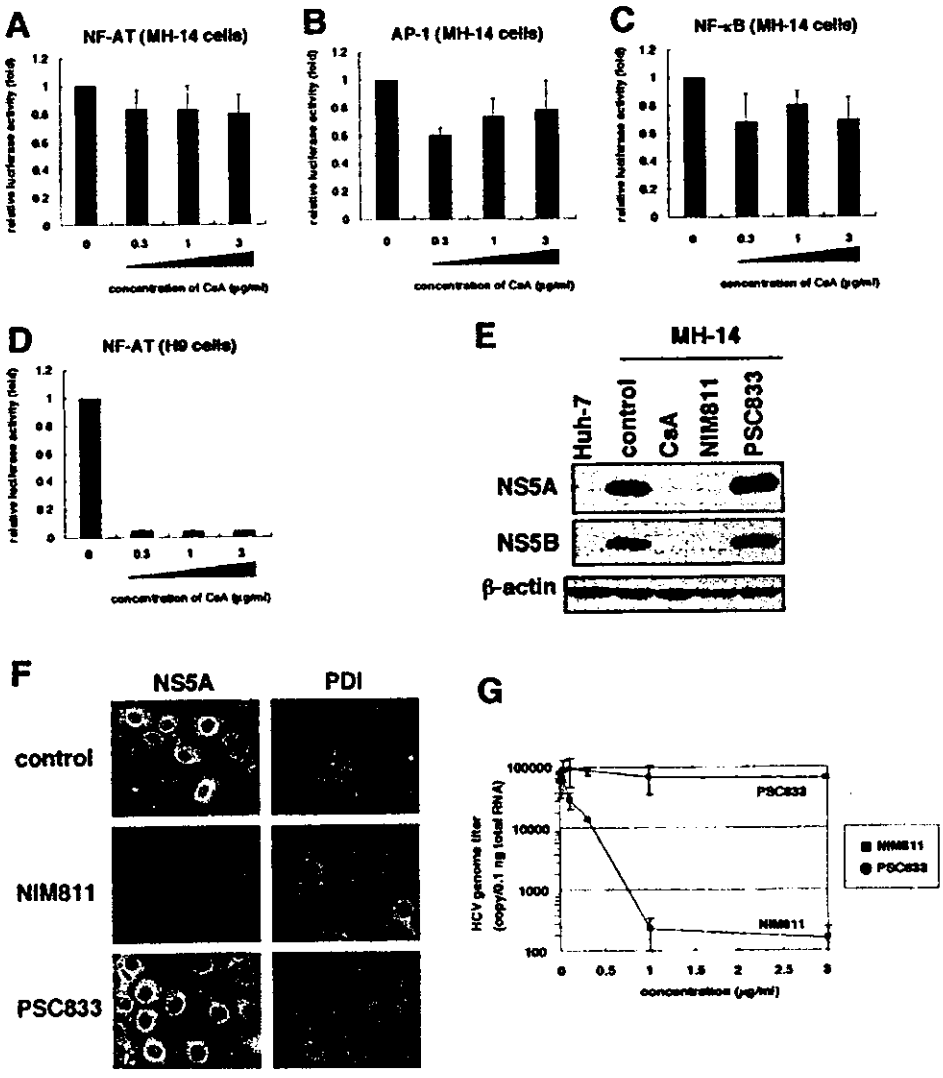

Fig. 5. Anti-HCV activity of CsA is independent of its immunosuppressive function. (A-C) MH-14 cells or (D) H9 cells were transfected with either (A and D) pNFAT-Luc, (B) pAP1-Luc, or (C) pNFκB-Luc and pRL-TK reporter plasmids as described in Materials and Methods followed by treatment with the indicated dose (0, 0.3, 1, or 3 μg/mL) of CsA. At 48 hours after transfection, luciferase activities of whole cell lysates were measured. The data represent the means of the relative luciferase activities in 3 independent experiments. The standard deviation values are shown. (E) Immunoblot analysis, (F) indirect immunofluorescence analysis, and (G) real-time RT-PCR analysis was performed using MH-14 cells treated with NIM811 or PSC833. Experimental condition and presentation of data is essentially the same as described in Figs. 1B, 1C, and 2B, respectively. Control in E and F, no treatment; CsA in E, 1 μg/mL CsA; NIM811 in E and F, 1 μg/mL NIM811; PSC833 in E and F, 1 μg/mL PSC833.

ways including the NF-AT pathway were not related with the anti-HCV activity of CsA in hepatocytes. This conclusion was further supported by the following results using CsA derivatives.[18] NIM811 is a nonimmunosuppressive CsA analogue that can bind to CyP but lacks the capacity of inhibition of CN and their downstream NF-AT pathway. PSC833 is a CsA derivative having no capacity to associate with both CyP and the CN/NF-AT pathway. As shown in Fig. 5E to G, NIM811 treatment can still decrease HCV protein and replicon RNA levels in MH-14 cells. However, PSC833 did not affect the level of HCV protein and replicon RNA titer (Fig. 5E-G). The above results indicate that immunosuppressive function but not CyP suppression capacity of CsA is dispensable for its anti-HCV activity in hepatocytes.

Discussion

In this study, we showed that CsA has anti-HCV potential *in vitro*, both in HCV replicon cells and in an HCV-infected cell line. Because the cell culture system used in this experiment is free from the acquired immune system, our observation indicated that CsA had potential for anti-HCV effects that are independent of its function as an immunosuppressant via T lymphocytes. Scattered reports have noted the effectiveness of CsA treatment in patients with HCV.[19-21] Notably, it was recently reported that combination therapy of IFN-α and CsA was more effective in HCV than IFN-α monotherapy.[22] In the setting of liver transplantation for hepatitis C, during which CsA is given as an immunosuppressant, there have been conflicting reports regarding the efficacy of CsA.[23-26] One explanation of the discrepancy may be that CsA has a strong immunosuppressive function via its action on T lymphocytes, which may allow HCV-infected cells to escape from the immune system and result in viral replication.[7] Therefore, CsA treatment may result in opposite effects, including anti-HCV activity in hepatocytes and immunosuppressive activity via T lymphocytes. It seems this bifunctional character of CsA would make the evaluation of CsA treatment in patients with hepatitis C difficult. In another study, it was shown that NIM811, a nonimmunosuppressive CsA analogue,[18] still has an anti-HCV activity *in vitro*. The *in vivo* effect of NIM811 on HCV replication is still unknown and is the subject of future investigations. However, because of the advantage of NIM811 exhibiting no immunosuppressive effect, this CsA derivative is expected to show a more preferable *in vivo* effect than CsA. Further *in vivo* analysis may present a way to develop a new anti-HCV agent.

CsA was reported to possess antiviral activities against human immunodeficiency virus type I,[27,28] herpes simplex virus,[29] and vaccinia virus.[30] Regarding human immunodeficiency virus type I, in particular, it was suggested that CsA suppresses viral infection through inhibition of the function of CyP, which plays an important role in the life cycle of human immunodeficiency virus type I.[31] The precise molecular mechanism of anti-HCV activity of CsA remains to be clarified. Additional studies to analyze this mechanism may show not only a novel target for anti-HCV therapy but also cellular factors regulating HCV replication that are still unknown.

Acknowledgment: CsA derivatives, NIM811 and PSC833, were kindly provided by Novartis (Basel, Switzerland).

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MOLECULAR AND CELLULAR BIOLOGY, Nov. 2003, p. 7498–7509 Vol. 23, No. 21
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.21.7498–7509.2003

Modulation of Retinoid Signaling by a Cytoplasmic Viral Protein via Sequestration of Sp110b, a Potent Transcriptional Corepressor of Retinoic Acid Receptor, from the Nucleus

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Received 17 March 2003/Returned for modification 29 April 2003/Accepted 24 July 2003

Hepatitis C virus (HCV) core protein (core) plays a significant role in the development of chronic liver diseases caused by HCV infection. We have discovered that the core sensitized all-*trans*-retinoic acid (ATRA)-induced cell death in MCF-7 cells. Activation of retinoic acid receptor alpha (RARα)-mediated transcription by the core was also seen in all the cell lines tested. By use of a yeast two-hybrid system, we identified Sp110b as a candidate for a core-interacting cellular factor. Although the function of Sp110b has remained unknown, we observed that Sp110b interacts with RARα and suppresses RARα-mediated transcription. These data suggest that Sp110b is a transcriptional cofactor negatively regulating RARα-mediated transcription. RNA interference-mediated reduction of endogenous Sp110b levels depressed the ability of the core to activate RARα-mediated transcription, suggesting an essential role for Sp110b in this pathway. The normal nuclear subcellular localization of Sp110b was altered by molecular interaction with the core to the cytoplasmic surface of the endoplasmic reticulum. This evidence suggests a model in which the core sequesters Sp110b from the nucleus and inactivates its corepressor function to activate RARα-mediated transcription. These findings likely describe a novel system in which a cytoplasmic viral protein regulates host cell transcription.

Hepatitis C virus (HCV) is a causative agent of liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (1, 8, 20). HCV core protein (core), one of the viral structural proteins (18), is a multifunctional protein regulating several defined cellular events. Core gene transgenic mice develop hepatic steatosis and HCC (23, 31, 32). Also, expression of the core protein has the potential to transform several cultured rodent cells (40, 51) and has been observed to modulate apoptotic responses of the cells (28, 41, 43). From these observations, core protein function is thought to be closely related to the molecular basis of HCV-related diseases. Despite the efforts of many researchers, the precise mechanisms governing the phenomena caused by core expression have remained unknown.

Other viral proteins, such as adenovirus E1A and E1B (44), human T-cell leukemia virus type 1 (HTLV-1) Tax (29), and human papillomavirus (HPV) E6 and E7 (33), also mediate a variety of functions in the cells. A variety of novel cellular mechanisms and proteins have been disclosed through the investigation of these viral proteins. Therefore, understanding the molecular mechanisms governing the phenomena induced by the core, such as tumor development, cellular transformation, and modulation of apoptotic responses, may also disclose a novel cellular signaling pathway. In this study, we investigated the molecular mechanism underlying modulation of apoptosis by core expression.

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Of the various apoptotic stimuli tested, we determined that all-*trans*-retinoic acid (ATRA)-induced cell death was sensitized by core expression. This sensitization correlated with the activation of ATRA-induced transcription and the enhancement of downstream proapoptotic gene expression. To investigate the mechanism by which the core activates ATRA-induced transcription, we screened for cellular factors interacting with the core by the yeast two-hybrid system. This search identified Sp110b, whose function is unknown, as a core-interacting protein. Here we demonstrate that Sp110b is a potent transcriptional corepressor of retinoic acid receptor alpha (RARα), playing a critical role in core-mediated activation of RAR signaling.

Until now, several viral proteins, such as adenovirus E1A (2, 26) and E1B (24), human immunodeficiency virus type 1 (HIV-1) Tat (3), HTLV-1 Tax (4, 46, 53), and HPV E6 (38) and E7 (37), have been reported to regulate cellular transcriptional activity via interactions with transcriptional cofactors such as CBP/p300, PCAF, histone deacetylase complex, and SRC-1. These viral proteins are primarily located in the nucleus, directly modulating the function of transcriptional cofactors. In this study, however, we determined that HCV core interacted with Sp110b on the cytoplasmic surface of the endoplasmic reticulum (ER) to modulate retinoid-dependent nuclear receptor signaling. It is suggested that the core sequesters Sp110b away from the nucleus and inactivates the corepressor function of Sp110b, resulting in the activation of ATRA-induced transcription and sensitization to ATRA-mediated cell death. This is the first model explaining the mechanism of transcriptional regulation by the core. Furthermore, this seems

to be a novel system of host transcriptional regulation by a cytoplasmic viral protein through the altered localization of a cellular transcriptional cofactor from the nucleus.

MATERIALS AND METHODS

Plasmid constructs. The pcDNA-myc, pCMV-FLAG, and pCA-FLAG vectors were obtained by inserting the Myc, FLAG, and FLAG tag coding sequences into the *Bam*HI-*Xho*I sites of pcDNA3 (Invitrogen), the *Eco*RI-*Hin*dIII sites of pKS(+)/CMV (28), and the *Eco*RI site of pCAGGS (a gift from J. Miyazaki, Osaka University Medical School) (34), respectively. Sp110 and Sp110b cDNAs covering the entire open reading frame regions were obtained by reverse transcriptase PCR (RT-PCR) from a human liver total-RNA (Clontech) template by using primers 5′-GTTGAATTCATGTTCACCATGACAAGAG-3′ and 5′-GTTCTCGAGTCAAGGAAGAGTCCAG-3′ for Sp110 and primers 5′-GTTGAATTCATGTTCACCATGACAAGAG-3′ and 5′-GTTCTCGAGTTATTCTTGGAGGACAG-3′ for Sp110b. To obtain pcDNA-Sp110, pCA-Sp110, pcDNA-Sp110b, and pCMV-Sp110b, the Sp110 or Sp110b PCR product was subcloned into the *Eco*RI-*Xho*I site of pcDNA-myc, pCA-FLAG, or pCMV-FLAG. The series of plasmids expressing deletion mutants of Sp110 and Sp110b, pcDNA-Sp110(1-276), pcDNA-Sp110(277-453), pcDNA-Sp110(454-689), pcDNA-Sp110 (277-388), pcDNA-Sp110(389-689), pcDNA-Sp110b(389-453) (CBR fragment), pcDNA-Sp110b(389-539), and pcDNA-Sp110b(1-276 + 454-539), was constructed by inserting PCR fragments generated by using appropriate synthetic oligonucleotides as primers and either pcDNA-Sp110 or pcDNA-Sp110b as the template.

The generation of pCMV-core has been described previously (28). pcDNA-core was obtained by inserting a *Bam*HI-*Eco*RI pCMV-core fragment encoding the core protein into the *Bam*HI-*Eco*RI site of pcDNA3. pCMV-core(6162M) was made by oligonucleotide-directed mutagenesis using oligonucleotides 5′-CCTCGTGGAGGGCTACAACCTATCCC-3′ and 5′-GGGATAGGTTGTAGCCCTCCACGAGG-3′ as primers. pGEX-Sp110 and pGEX-Sp110b, encoding a fusion of glutathione *S*-transferase (GST) and either Sp110 or Sp110b, respectively, were created by insertion of the *Eco*RI-*Xho*I Sp110 or Sp110b fragment, respectively, into the *Eco*RI-*Xho*I site of the pGEX-6P1 vector (Clontech). To obtain pGEX-core(1-80), the DNA fragment generated by PCR using oligonucleotides 5′-GTTGGATCCATGAGCACAAATCC-3′ and 5′-GTTCTCGAGTCACCCGGGCTGAGCCC-3′ as primers and pcDNA-core as a template was inserted into the *Bam*HI-*Xho*I site of pGEX-6P1. All expression plasmids encoding other core derivatives were made as described previously (54).

pRARE-Luc, a reporter plasmid containing three copies of the RARα binding element (RARE), was obtained by inserting the *Mlu*I-*Xho*I PCR fragment prepared with primers 5′-GTTACGCGTGTTCACCGAAAGTTCACCGAGAGTTCACCGAAAGTTCACAGCCA-3′ and 5′-GTTCTCGAGGTGAACTTTCGGTGAACTGGCTGTGAACTTTCGGTGAACTCTCG-3′ into the *Mlu*I-*Xho*I site of p-55BLuc (kindly provided by T. Kiyono, National Cancer Center Research Institute, Tokyo, Japan) (21). pSG5-RARα, encoding RARα, was kindly provided by P. Chambon of the Institut de Genetique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM/ULP/College de France, and N. Kato at the Institute of Molecular and Cellular Biosciences, The University of Tokyo.

Cell culture and transfection. MCF-7, COS-7, 293T, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% fetal bovine serum and L-glutamine. Plasmid transfection of cells was performed as described previously (54).

Establishment of HCV core-producing cell lines. After transfection with pLXSH-core or pLXSH (51), MCF-7 cells were selected in the culture medium containing 250 μg of hygromycin B (Wako)/ml to establish several clones of stable transfectants containing these plasmids.

Immunoblot analysis. Immunoblot analysis was performed essentially as described previously (54). The antibodies used in these experiments were specific for HCV core (515S; a gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science), α-tubulin (Ab-1; Calbiochem), FLAG (M2; Sigma), and SC-35 (Sigma).

Reporter assay. The pRARE-Luc reporter plasmid, containing three copies of RARE upstream of a basal transcription promoter region, was used for the reporter assay as described previously (28).

Yeast two-hybrid screening. The region of the core containing amino acids (aa) 1 to 80, subcloned into the pHybLex/Zeo vector (Invitrogen), was used to screen a human prostate cDNA library (Clontech) according to the Hybrid Hunter protocol (Invitrogen). A total of 5×10^6 transformants were selected based on histidine prototrophy and β-galactosidase activity.

GST pulldown assay. GST and the GST-Sp110, GST-Sp110b, and GST-core (1-80) fusion proteins, encoded by pGEX-6P1, pGEX-Sp110, pGEX-Sp110b, and pGEX-core(1-80), respectively, were produced in BL21 cells (Amersham Biosciences) following treatment with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After preparation of proteins by using glutathione-Sepharose resin (Amersham Biosciences), proteins bound to the resin were incubated with in vitro-translated, radiolabeled proteins at 4°C for 1 h. ^{35}S-radiolabeled proteins were prepared with the TNT T7 quick coupled transcription-translation system (Promega) using 0.25 μg of expression plasmid and L-[^{35}S]methionine (Amersham Biosciences), according to the manufacturer's protocol. After five washes in binding buffer (containing 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride for the experiment for which results are shown in Fig. 3B and 5D, and containing those reagents supplemented with 0.5% NP-40, 0.5% sodium deoxycholate, and 0.05% sodium dodecyl sulfate (SDS) for the experiment for which results are shown in Fig. 3A), resin-bound radiolabeled proteins were fractionated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography.

Immunoprecipitation. Cells were lysed in immunoprecipitation buffer, containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.5% NP-40 for the experiment for which results are shown in Fig. 3C. For Fig. 5E, the buffer was composed of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% NP-40, 0.1% sodium deoxycholate, and 0.01% SDS. After centrifugation, the supernatant was incubated with an anti-FLAG antibody (M2; Sigma), an anti-hemagglutinin (HA) antibody (3F10; Roche), or normal mouse immunoglobulin G (IgG; Zymed Laboratories) for at least 1 h. Immune complexes were recovered by adsorption to protein G-Sepharose resin (Amersham Biosciences). After four washes in immunoprecipitation buffer, the immunoprecipitates were analyzed by immunoblot analysis.

Northern blot analysis. Total RNA, isolated from human tissue (Clontech), was analyzed by Northern blot hybridization using ULTRAhyb (Ambion) according to the manufacturer's protocol. Probes were prepared by PCR in the presence of [^{32}P]dCTP (Amersham Biosciences) by using *Hin*dIII-*Xba*I-digested pCMV-Sp110b as a template and 5′-TGAGGCTGTCCCTCTTGG-3′ as a primer.

RT-PCR. The relative expression of Sp110 and Sp110b mRNAs was evaluated by semiquantitative RT-PCR with a one-step RNA PCR kit (Takara) by using one sense primer (5′-GTTGAATTCATGCTCCAAGTGGTGGATAAG-3′) and two antisense primers (5′-GTTCTCGAGTCAAGGAAGAGTCCAG-3′ and 5′-GTTCTCGAGTTATTCTTGGAGGACAG-3′). Sp110 and Sp110b RNAs synthesized from pcDNA-Sp110 and pcDNA-Sp110b in vitro by using the MEGAscript T7 kit (Ambion) were used as standards. The total RNA of cultured cells was isolated with Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. The primers, 5′-GCATGGTCAACTGCAACGATG-3′ and 5′-GGGCGCATCGTACTTGGTG-3′, were used for detection of tissue transglutaminase (tTGase) mRNA.

DNA-protein complex immunoprecipitation assay. 293T cells treated with 1 μM ATRA were transfected with expression plasmids together with either the reporter plasmid pRARE-Luc, containing RARE, or p-55BLuc, which lacks RARE, as a negative control. After cross-linking with formaldehyde for 15 min, cells were lysed, sonicated, and subjected to immunoprecipitation using anti-FLAG, anti-HA, or normal mouse IgG. Recovered immunocomplexes were incubated at 65°C for 16 h and then digested with proteinase K for 2 h. DNA was extracted from the immunocomplexes with phenol and precipitated with ethanol. The primers used for detection of DNA were 5′-CACTGCATTCTAGTTGTGG-3′ and 5′-ACCAACAGTACCGGAATG-3′.

Production of an antibody against Sp110 and Sp110b. A rabbit polyclonal antiserum against Sp110 and Sp110b was generated by immunization with a synthesized peptide (STPSDKKGKKRKRC, corresponding to aa 270 to 283 of Sp110 and Sp110b) conjugated to keyhole limpet hemocyanin (MBL, Nagoya, Japan).

RNA interference (RNAi) technique. A 21-nucleotide small interfering RNA (siRNA) duplex (5′-AAGCUUCAAACGUGUUGGUGC-3′) containing 3′ dTdT overhanging sequences was synthesized (Dharmacon). siRNA transfection was performed using the Oligofectamine reagent (Invitrogen) according to the manufacturer's protocol.

Indirect immunofluorescence analysis. Indirect immunofluorescence analysis was performed as described previously (54). Cells were permeabilized with 0.05% Triton X-100 and treated with the primary anti-FLAG (M2; Sigma), anti-Myc (9E10; Santa Cruz), anti-core (515S), or anti-RARα (C-20; Santa Cruz) antibody and the rabbit antiserum against Sp110 and Sp110b. The secondary antibodies conjugated to Alexa 488 and 568 series (Molecular Probes) were used to visualize primary antibody staining. Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI).

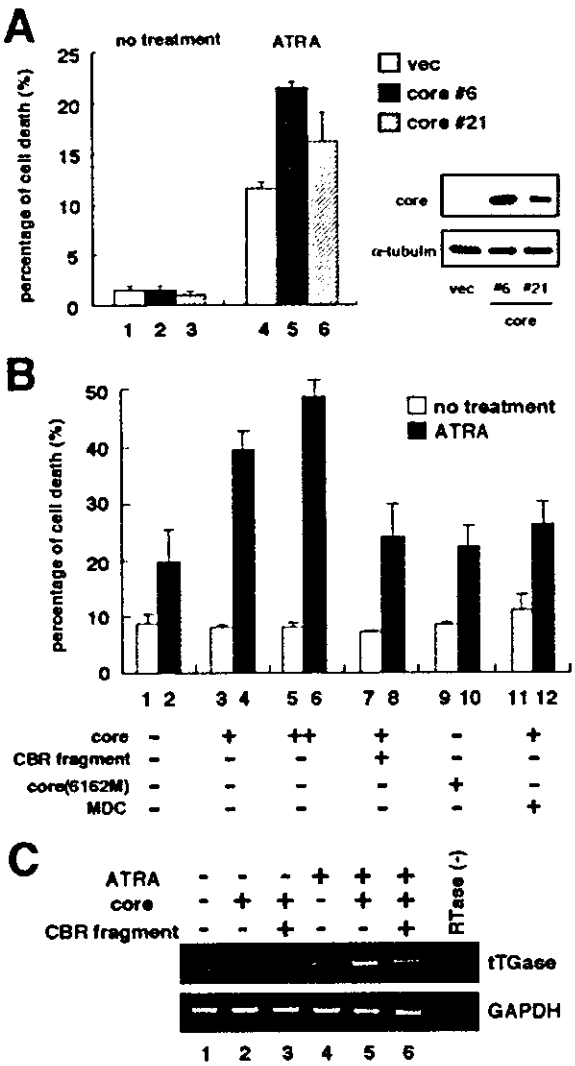

FIG. 1. HCV core sensitized ATRA-induced cell death. (A) Production levels of the core in each clone of MCF-7 cells stably transfected with pLXSH-core (MCF-7-core 6 and -core 21) or the empty vector pLXSH (MCF-7-vec) were analyzed by immunoblot analysis with an anti-core (right, upper panel) and an anti-α-tubulin (right, lower panel) antibody as an internal control. MCF-7-core 6, MCF-7-core 21, and MCF-7-vec cells (5×10^4 each) were incubated in the absence (no treatment) or presence (ATRA) of 1 μM ATRA for 96 h. Living and dead cells were quantitated by staining with trypan blue. The average percentage of cell death from three independent experiments is presented. Open (bars 1 and 4), solid (bars 2 and 5), and hatched (bars 3 and 6) bars, MCF-7-vec, -core 6, and -core 21, respectively. (B) MCF-7 cells (5×10^4) transfected with 2.5 μg of pMACS-K^k and each expression plasmid given below were treated with (solid bars) or without (open bars) 1 μM ATRA for 96 h after the concentration of transfected cells by using the MACSelect system (see Materials and Methods). For bars 11 and 12, 50 μM monodansylcadaverine (MDC) was added simultaneously with ATRA. The percentage of cell death was estimated as described for panel A. Bars 1 and 2, empty vector; bars 3, 4, 11, and 12, pCMV-core (3 μg); bars 5 and 6, pCMV-core (6 μg); bars 7 and 8, pCMV-core (3 μg) and pCMV-Sp110b(389-453) (CBR fragment; 4.5 μg); bars 9 and 10, pCMV-core(6162M) (3 μg). (C) Enhancement of ATRA-induced tTGase expression by the core. MCF-7 cells (2×10^5) transfected with pMACS-K^k and each expression plasmid given below were treated for 48 h with (lanes 4 to 6) or without (lanes 1 to 3) 1 μM ATRA after cell concentration as described for panel B. Following the extraction of total RNA from these cells, mRNA levels of tTGase (upper panel) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (lower panel) were semiquantified by RT-PCR as described in Materials and Methods. RTase(−), experimental control treated like other samples, but without reverse transcriptase. Lanes: 1 and 4, empty vector; 2 and 5, pCMV-core; 3 and 6, pCMV-core and pCMV-Sp110b(389-453) (CBR fragment).

Fractionation of cell extracts. Cells transfected with various plasmids were harvested and homogenized in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM EDTA. After centrifugation (MX-150; Tomy) at 3,900 rpm for 7 min, postnuclear supernatants were further centrifuged at 7,000 rpm for 5 min. The resulting supernatant was subjected to ultracentrifugation (himac CS120FX; Hitachi) at 50,000 rpm for 1 h. The pellet and supernatant fractions were collected as microsomal membranes and cytosolic fractions, respectively.

Evaluation of cell death. Dead cell numbers were evaluated as described previously (28). The cells were stained with trypan blue dye; the percentage of cell death was measured by scoring at least 500 cells per experiment.

Concentration of cells transiently transfected with the expression plasmids. The specific concentration of transiently transfected cells from heterogeneous cell populations was determined with the MACSelect system (Miltenyi Biotec) as described previously (28).

RESULTS

HCV core sensitizes ATRA-induced cell death. To investigate the effect of the core on sensitivity to apoptosis induced by a variety of stimuli, we established MCF-7 cells constitutively producing the core. Examination of the response of these cells to a variety of apoptotic stimuli determined that ATRA-induced cell death was enhanced in core-producing cells (Fig. 1A). Similar results were obtained for MCF-7 cells transiently producing the core protein (Fig. 1B, bars 1 to 6). This enhancement of apoptotic induction was accompanied by an enhancement of the expression of tTGase (Fig. 1C, lanes 4 and 5), which is downstream of ATRA and involved in ATRA-mediated apoptosis (19, 35, 36). It was confirmed that suppression of tTGase function by a tTGase inhibitor, monodansylcadaverine (36), reduced ATRA-induced cell death in core-producing MCF-7 cells (Fig. 1B, bars 3, 4, 11, and 12). Thus, the core appears to sensitize cells to ATRA-mediated cell death, accompanied by an enhancement of proapoptotic tTGase gene induction.

HCV core protein activates RAR-mediated transcription. ATRA-induced apoptosis and tTGase expression are reportedly mediated by the transcriptional activity of RAR, the nuclear receptor for ATRA (11, 19, 36). Therefore, we next examined the effect of core expression on the activity of RAR-mediated transcription by a reporter assay. We used the RAR-responsive reporter plasmid pRARE-Luc, which includes a basal promoter with three copies of RARE upstream of the luciferase gene, to assess the RARα-mediated transcriptional activity of the cells producing the core either transiently (Fig. 2A) or constitutively (Fig. 2B). Luciferase activity increased in a manner dependent on core concentration. Similar results were obtained with additional cell lines, including HeLa, Huh-7, and MCF-7 cells, producing the core transiently (data not shown). Both the whole HCV polyprotein and the core alone activated luciferase activity (data not shown). In Huh-7 cells carrying an HCV full-genome replicon, established in our laboratory (unpublished data), RARα activity increased approximately threefold over the levels observed in parental Huh-7 cells (data not shown). Since the luciferase activity

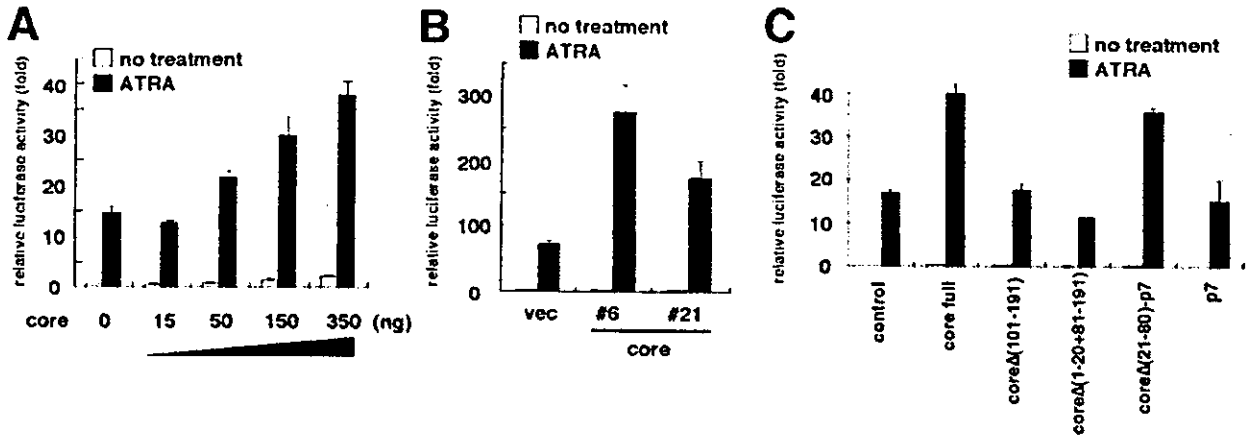

FIG. 2. Activation of RARα-mediated transcription in cells expressing the core. (A) COS-7 cells were transfected with 25 ng of pRARE-Luc, pCMV-core at the doses indicated, and the empty vector for a total amount of 400 ng of plasmids. Cells were then treated with (solid bars) or without (open bars) 1 μM ATRA. At 24 h posttransfection, luciferase activities of whole-cell lysates were measured. Data are means of the relative luciferase activities in three independent experiments. Error bars, standard deviations. (B) MCF-7-core 6, MCF-7-core 21, and MCF-7-vec cells were transfected with 25 ng of the pRARE-Luc reporter plasmid. (C) Identification of the region within the core essential for activation of RARα-mediated transcription. COS-7 cells were transfected with 25 ng of pRARE-Luc and 350 ng of a series of truncated core expression plasmids [core full, pCMV-core; coreΔ(101-191), pCMV-coreΔ(101-191); coreΔ(1-20 + 81-191), pCMV-coreΔ(1-20 + 81-191); coreΔ(21-80)-p7, pCMV-coreΔ(21-80)-p7] or control plasmids (control, pKS+/CMV; p7, pCMV-p7). Experimental conditions and presentation of data for panels B and C were the same as those described for panel A.

driven by a reporter plasmid lacking RARE was not affected by core expression (data not shown), these results suggested that the core protein activates RARα-mediated transcription independently of cell type.

To identify the region of the core protein responsible for RARα activation, we performed a detailed deletion analysis as described previously (54). We prepared carboxy-terminal deletion constructs fused with HCV p7 containing a signal sequence to allow localization to the ER. We confirmed that these fused proteins reside on ER membranes, as seen with the wild-type core (27, 54) (data not shown). Cells producing the core constructs lacking the amino-terminal 100 aa [coreΔ(101-191)] or aa 21 to 80 [coreΔ(1-20 + 81-191)] demonstrated reporter activities similar to those of negative-control cells transfected with the empty vector (Fig. 2C). Production of the region from aa 21 to aa 80 [coreΔ(21-80)-p7] upregulated reporter activity significantly from the levels observed in negative-control cells and cells producing p7 alone. These data indicated that the region from aa 21 to aa 80 of the core [coreΔ(21-80)] is responsible for the activation of RARα-mediated transcription.

Identification of cellular factors interacting with HCV core by yeast two-hybrid screening. To clarify the molecular mechanisms governing transcriptional activation of RARα by the core protein, we tried to identify cellular factors interacting with coreΔ(21-80) by yeast two-hybrid screening. Using the amino-terminal 80 aa of the core (core80) fused to the LexA DNA binding region as bait, we screened a human prostate cDNA library for interacting proteins. Six independent candidate clones for binding with the bait were selected from 5×10^6 transformants. Sequencing analysis revealed that one of the candidate clones encoded Sp110b. A splicing variant of this protein, Sp110, reportedly activates RARα-mediated transcription. A schematic diagram of the molecular structures of Sp110 and Sp110b is shown in Fig. 3B (left).

Sp110b interacts with HCV core through its central region. To confirm an interaction between the core and Sp110b, we performed a GST pulldown assay in vitro. Recombinant Sp110 and Sp110b fused to GST (GST-Sp110 and GST-Sp110b) were incubated with in vitro-translated wild-type ^{35}S-labeled core. The ^{35}S-labeled core was copurified with Sp110b but not with Sp110 (Fig. 3A, top panel). In vitro-translated Sp110b was efficiently pulled down with GST-core80, but only a small amount of Sp110 could be observed in the pulled-down fraction (Fig. 3Ba and b). These results suggested that the core interacts more efficiently with Sp110b than with Sp110. The efficient interaction of the core with Sp110b appeared to be specific, as no association was observed with Sp100, which exhibits significant homology to Sp110 and Sp110b (Fig. 3Bk).

We used deletion analysis to determine the region of Sp110b interacting with the core. The in vitro-synthesized fragment of Sp110b from aa 277 to 453, but not fragments of Sp110 from aa 1 to 276 or aa 454 to 689, was copurified with GST-core80 (Fig. 3Bc to e). Sp110b lacking the region from aa 277 to 453 could not be copurified with GST-core80 (Fig. 3Bj), suggesting that the region of Sp110b from aa 277 to 453 is both necessary and sufficient for the interaction with core80. Dissection of this region determined that the region of Sp110b from aa 389 to 453, but not that from aa 277 to 388, was essential for this interaction (Fig. 3Bf and g). Therefore, we have named this region the core-binding region (CBR). Although the CBR is shared by Sp110b and Sp110, the affinity of Sp110 to the core was low in vitro, as described above. Similar results were obtained when the C-terminal fragments of Sp110b and Sp110, which include the CBR, were examined in this assay system (Fig. 3Bh and i). From these results, it appears to be possible that the C-terminal region of Sp110, containing the plant homeobox domain (PHD) and the bromodomain, has an inhibitory effect on core association. Analysis of additional deletion mutants showed that the entire C-terminal domain harboring

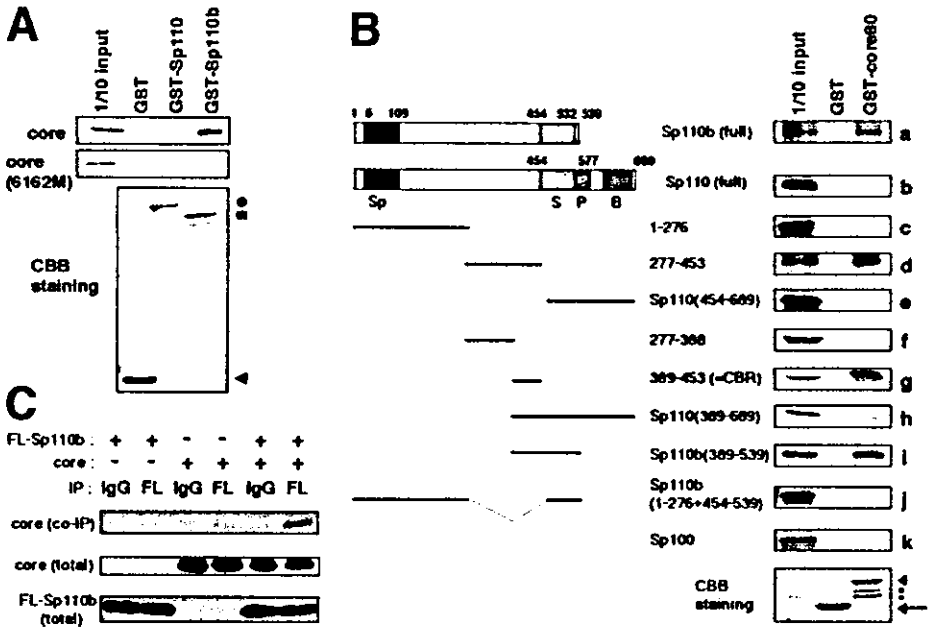

FIG. 3. Interaction of the core with Sp110b. (A) The ^{35}S-labeled in vitro transcription-translation product of full-length core (top panel) or the core(6162M) mutant (center panel) was incubated with recombinant Sp110 or Sp110b fused to GST (GST-Sp110 or GST-Sp110b) or with GST as a negative control. The GST pulldown assay was performed as described in Materials and Methods. 1/10 input, signal for 1/10 the amount of ^{35}S-labeled product used in the pulldown assay. Coomassie brilliant blue (CBB) staining patterns of pulled-down proteins are shown in the bottom panel. Arrowhead, circle, and square indicate the bands corresponding to GST, GST-Sp110, and GST-Sp110b, respectively. (B) Mapping of the region interacting with the core in Sp110b by deletion analysis. (Left) Schematic representations of the full-length and truncated mutants of Sp110 and Sp110b are shown. Numbers above the diagrams indicate the amino acid positions from the amino terminus of Sp110 or Sp110b. The Sp100-like domain (Sp), SAND domain (S), PHD (P), and bromodomain (B) are indicated. (Right) Designations of the GST fusion protein and ^{35}S-labeled derivatives of Sp110 and Sp110b are given above and to the left of the autoradiograms (a through k), respectively. GST-core80, GST fused with the region of the core from aa 1 to 80. CBB staining patterns of pulled-down proteins are shown in the bottom panel. Arrow and arrowhead indicate bands corresponding to GST and GST-core80, respectively. Two dots indicate apparent degradation products of GST-core80. (C) Interaction between the core and Sp110b produced in the cells. Lysates from COS-7 cells transfected with 1 μg of pCMV-Sp110b and/or pCMV-core were used for coimmunoprecipitation, followed by immunoblot analysis. The combinations of plasmids used for the transfection are indicated at the top. "IP" designates the antibodies used for immunoprecipitation, either the anti-FLAG antibody (FL) or normal mouse IgG (used as a negative control). Coimmunoprecipitated core with FLAG-tagged Sp110b was detected with an anti-core antibody (top panel). Center and bottom panels show results of experiments in which the core and FLAG-tagged Sp110b in total-cell lysates from transfectants were detected by anti-core and anti-FLAG antibodies, respectively.

both the PHD and the bromodomain, rather than either domain alone, was likely to be responsible for blocking the interaction with the core (data not shown).

To further confirm the interaction of Sp110b with the core, we performed a coimmunoprecipitation assay. The core was detected in immune complexes pulled down with an anti-FLAG antibody, but not with normal mouse IgG, from the lysates of COS-7 cells producing both FLAG-tagged Sp110b and the core simultaneously (Fig. 3C). Cumulatively, these results indicate that Sp110b interacts specifically with the core.

Sp110b is expressed ubiquitously in human tissues and more abundantly than Sp110. Sp110b expression in human tissues was examined by Northern blot analysis using the ^{32}P-labeled 5′-terminal cDNA region of Sp110b as a probe. A 1.9-kb band was detected in all the human tissues investigated (Fig. 4A). From the information that the Sp110 and Sp110b mRNAs are approximately 2.3 and 1.9 kb, respectively, this band seemed to correspond to Sp110b mRNA. The 2.3-kb band of Sp110 mRNA was not observed in this experiment. To compare the expression levels of Sp110 and Sp110b mRNAs in these specimens, a semiquantitative analysis was conducted by RT-PCR. The band for Sp110 mRNA was detected in total RNAs from human spleens and Jurkat cells, a human T-lymphoma cell line (Fig. 4B, lanes 11 and 14). Little or no signal was observed in other tissues and cell lines (Fig. 4B, lanes 10 to 15). We observed, however, that Sp110b mRNA was more abundant than Sp110 mRNA in all the tissues and cell lines investigated. In vitro-synthesized Sp110 and Sp110b mRNAs were mixed at variable ratios as templates in this system (Fig. 4B, lanes 1 to 9); the expression of Sp110b mRNA was estimated to be about 10- and 3- to 5-fold higher than that of Sp110 mRNA in nonleukocytes and leukocytes, respectively. These results were consistent with data in which Sp110b protein but not Sp110 protein was detected in lysates of HeLa (Fig. 6A) and Huh-7 (data not shown) cells by using an antibody recognizing both Sp110 and Sp110b protein. Thus, Sp110b mRNA is expressed ubiquitously in all the human tissues examined, more abundantly than Sp110.

Sp110b is a potent transcriptional corepressor of RAR. Although Sp110 is reported to activate RARα-mediated transcription (6), the molecular function of Sp110b remains unknown. To characterize the function of Sp110b, we examined the effect of this protein on RARα-mediated transcription by a reporter assay. As reported previously (6), ectopic expression

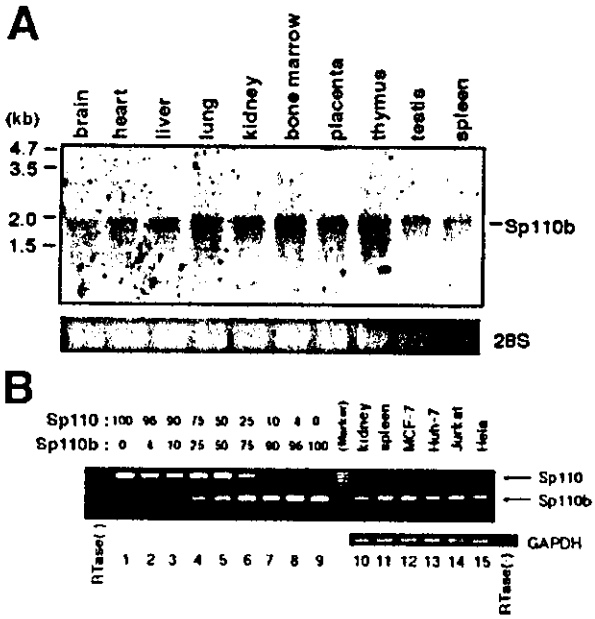

FIG. 4. Expression of Sp110 and Sp110b mRNAs in human tissues and cell lines. (A) Northern blot analysis detecting Sp110 and Sp110b mRNAs in human tissues. (Upper panel) Ten-microgram portions of total RNAs from human brain, heart, liver, lung, kidney, bone marrow, placenta, thymus, testis, and spleen were analyzed by using a ^{32}P-labeled Sp110b fragment as a probe. Positions of molecular size markers are indicated on the left. (Lower panel). Ethidium bromide staining of 28S rRNA served as a loading control. (B) Semiquantitative RT-PCR analysis of Sp110 and Sp110b mRNAs in human tissues and cell lines. (Upper panel) cDNA fragments of Sp110 and Sp110b mRNAs were simultaneously amplified by RT-PCR using a common sense primer and two antisense primers specific to Sp110 and Sp110b mRNAs, respectively. To evaluate the quantifying ability of this system, mixtures of in vitro-synthesized Sp110 and Sp110b RNAs at various concentrations (given above the gel, in femtograms) were used as templates (lanes 1 to 9). Total RNAs from human kidneys (lane 10) and spleens (lane 11) and from MCF-7 (lane 12), Huh-7 (lane 13), Jurkat (lane 14), and HeLa (lane 15) cells were examined. (Lower panel) As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was detected by a similar protocol (lanes 10 to 15). RTase(−), experimental control treated like other samples, but without reverse transcriptase.

of Sp110 augmented reporter activity in a dose-dependent manner (Fig. 5A). In contrast, luciferase activity was drastically reduced in cells producing Sp110b in a dose-dependent manner, irrespective of ectopic RARα production (Fig. 5B and C). Reporter activity from the reporter plasmid lacking RARE was not affected by ectopically produced Sp110 or Sp110b (data not shown). Similar results were obtained in all the cell lines examined, including Huh-7, MCF-7, and HeLa cells (data not shown). Moreover, we observed the downregulation of RAR-responsive genes, including tTGase and transforming growth factor β2 (TGF-β2), in cells producing Sp110b (data not shown). These data suggest that Sp110b suppresses RARα function. To investigate the function of endogenous Sp110b, we introduced an siRNA, Sp110(b)-siRNA, to specifically knock down the expression of Sp110 and Sp110b in HeLa cells in which Sp110b was expressed but Sp110 protein was not detected (Fig. 6A). Endogenous Sp110b levels were dramatically reduced, while α-tubulin levels remained the same (Fig. 6A). Under these conditions, RARα-mediated transcription was activated two- to threefold over that in cells transfected with a randomized siRNA (Fig. 6B, bars 1 and 3). These findings indicated that Sp110b suppresses the transcriptional activity of RARα without altering RARα production levels (Fig. 5C, lower panel).

The existence of a liganded nuclear receptor binding motif (LXXLL) (17) in Sp110 and Sp110b (aa 525 to 529; LGELL) raised the possibility that Sp110 and Sp110b may interact with the nuclear receptor RARα in the presence of RARα ligands. We investigated this possibility using a GST pulldown assay. RARα was copurified with both GST-Sp110 and GST-Sp110b in the presence, but not in the absence, of ATRA (Fig. 5D, top and center panels), indicating that RARα interacts with Sp110 and Sp110b in vitro in a ligand-dependent manner. The interaction between Sp110b and RARα in the presence of ATRA was confirmed by a coimmunoprecipitation assay with 293T cells (Fig. 5E). Further analysis on whether Sp110b, together with RARα, associates with the target promoter was performed by a DNA-protein complex immunoprecipitation assay. A RARE-containing promoter was immunoprecipitated with either Sp110b or RARα in the presence of ATRA (Fig. 5F). These data suggest that, in conjunction with RARα, Sp110b associates with the target promoter containing RARE in the presence of ATRA. In addition, Sp110 and Sp110b were observed in the nuclei of cells in a dense granular staining pattern (Fig. 7A, panels 2, 9, and 30), as previously reported for Sp110 (6). RARα was also located primarily in the nucleus (Fig. 7A, panels 3 to 5 and 10 to 12), as reported previously (7).

These results suggest that Sp110b is a transcriptional corepressor downregulating RARα activity in the nucleus.

The ability of HCV core to activate RARα was reduced by RNAi elimination of endogenous Sp110b. The accumulated evidence suggests that Sp110b, a potent transcriptional corepressor of RARα, may play a role in the activation of RARα-mediated transcription by the core. We therefore examined the capacity of the core to activate RARα upon elimination of endogenous Sp110b protein in HeLa cells by the RNAi technique. When endogenous Sp110b protein was knocked down by siRNA treatment (Fig. 6A), the effect of core expression on RARα-mediated transcription (about 1.30-fold activation) was significantly reduced from levels observed in cells treated with a control siRNA (about 2.69-fold activation) (Fig. 6B). This result suggests that endogenous Sp110b plays an important role in the activation of RARα-mediated transcription by the core protein.

HCV core changes the subcellular localization of Sp110b from the nucleus to the cytoplasmic surface of the ER. Our finding that Sp110b interacts with the core and plays an important role in RARα activation by the core presented a paradoxical problem in that Sp110b was likely to be a nuclear factor but the core was located mainly around the perinuclear region in the cytoplasm (Fig. 7A, panel 1). To investigate this question, we examined the subcellular localization of each protein by indirect immunofluorescence analysis. Upon coproduction of the core protein, the localization of Sp110b changed dramatically from the nucleus to the perinuclear region of the cytoplasm, where the core was located (Fig. 7A, panels 9 and 13 to 15). In contrast, Sp110 was observed in the nucleus, irrespective of core production (Fig. 7A, panels 2 and 6 to 8). Similar results were obtained in 293T cells (data not shown).

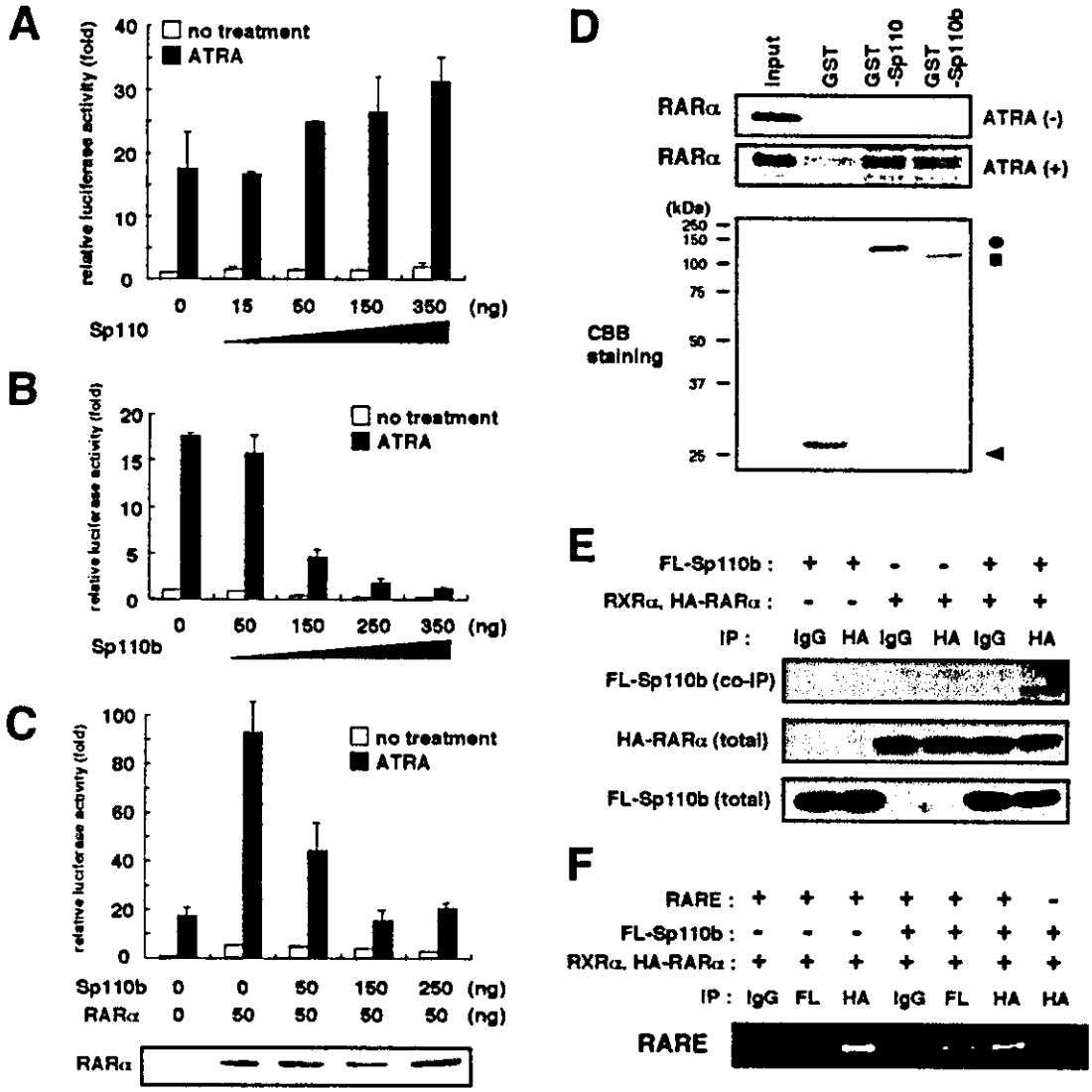

FIG. 5. Sp110b is a potent transcriptional corepressor of RARα. (A through C) Activation and suppression of RARα-mediated transcription by Sp110 and Sp110b, respectively. COS-7 cells were transfected with 25 ng of pRARE-Luc with pCA-Sp110 (A), pCMV-Sp110b (B), or pCMV-Sp110b with pSG5-RARα (C) at the indicated doses for a total of 400 ng of plasmids by adjusting with the empty vector. Additional conditions were as described in the legend to Fig. 2A. RARα protein production levels for each condition were examined by immunoblot analysis (C, lower panel). (D) Interaction of Sp110 and Sp110b with RARα. A GST pulldown assay was performed as described in Materials and Methods. ^{35}S-labeled RARα was incubated with GST-Sp110, GST-Sp110b, or GST in the absence [ATRA(−)] or presence [ATRA (+)] of 1 μM ATRA. The Coomassie brilliant blue (CBB) staining pattern of pulled-down products is shown in the bottom panel. Positions of molecular size markers are given on the left. Arrowhead, circle, and square indicate bands corresponding to GST, GST-Sp110, and GST-Sp110b, respectively. (E) Co-immunoprecipitation assay detecting the interaction between Sp110 and RARα. Lysates from ATRA-treated 293T cells overproducing FLAG-tagged Sp110b (FL-Sp110b) and/or RXRα with HA-tagged RARα (HA-RARα) were used for coimmunoprecipitation followed by immunoblot analysis. Data are presented essentially as described in the legend to Fig. 3C. IgG, normal mouse IgG; HA, anti-HA antibody. FL-Sp110b coimmunoprecipitated (co-IP) with HA-RARα was detected by using an anti-FLAG antibody (upper panel). Center and lower panels show HA-RARα and FL-Sp110b in total-cell lysates detected by anti-HA and anti-FLAG antibodies, respectively. (F) DNA-protein complex immunoprecipitation assay detecting the RARα-Sp110b-RARE complex. 293T cells overproducing either RXRα with HA-RARα or FL-Sp110b, or both, and carrying either pRARE-Luc (RARE +) or the p-55BLuc reporter plasmid lacking RARE (RARE −), were treated with ATRA. Formaldehyde-cross-linked DNA-protein complexes were then immunoprecipitated with anti-HA (HA), anti-FLAG (FL), or normal mouse IgG (IgG). The DNA extracted from the respective immunoprecipitates was amplified by PCR as described in Materials and Methods.

The subcellular localization of other well-known nuclear proteins—p53 (12), poly(ADP-ribose) polymerase (45), and RARα (7)—was not influenced by core expression, in a manner similar to that observed for Sp110 (data not shown). These results indicate that the core specifically alters the subcellular localization of Sp110b. We further confirmed this phenomenon in HeLa cells by observation of the alteration of the location of endogenous Sp110b by the core. In the absence of core expression, a nuclear staining pattern was seen (Fig. 7A, panel 30) as described above. However, perinuclear localization was observed in certain cells producing the core, similar to the pattern seen for exogenous Sp110b (Fig. 7A, panels 31 to 33). Since

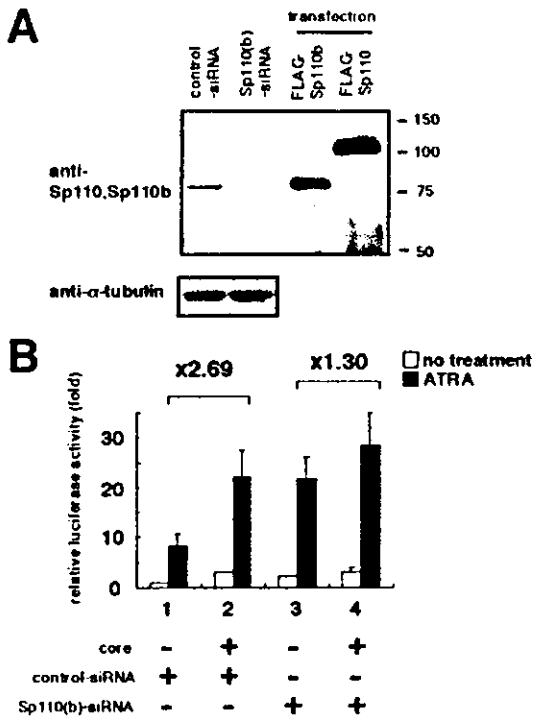

FIG. 6. The RARα activation capacity of HCV core was reduced by RNAi elimination of endogenous Sp110b. (A) RNAi elimination of endogenous Sp110b protein in HeLa cells. Extracts from cells treated with 300 U of gamma interferon/ml after transfection with either an siRNA specific for Sp110 and Sp110b [Sp110(b)-siRNA] or a randomized siRNA (control-siRNA) was analyzed with an antibody recognizing both Sp110 and Sp110b (upper panel) or anti-α-tubulin (lower panel). As a positive control, small amounts of extracts from cells overproducing FLAG-tagged Sp110 or Sp110b were also analyzed. Positions of molecular size markers (in kilodaltons) are given on the right. (B) HeLa cells were transfected with control-siRNA (bars 1 and 2) or Sp110(b)-siRNA (bars 3 and 4). At 24 h posttransfection, plasmid transfection with 25 ng of pRARE-Luc and 300 ng of either pKS+/CMV (bars 1 and 3) or pCMV-core (bars 2 and 4) was carried out. An additional 24 h later, the cells were harvested; luciferase activity was measured as described in the legend to Fig. 2A. Data are means of the relative luciferase activities in three independent experiments. Comparisons of luciferase activities (bars 1 versus 2 [×2.69] and bars 3 versus 4 [×1.30]) are shown above the graph. The combinations of siRNAs and plasmids used are indicated below the graph.

Sp110b but not Sp110 was detected in HeLa cells by using this antibody (as shown in Fig. 6A), the endogenous protein detected around the perinuclear region in the presence of the core appears to be primarily Sp110b. Thus, core expression altered the subcellular localization of Sp110b.

To investigate the colocalization of Sp110b with the core, we fractionated cell extracts into nuclear, microsomal-membrane, and cytosolic fractions for detection of Sp110b (Fig. 7B). Fraction identities were confirmed by detection of SC-35 (49), the core (27, 54), and α-tubulin for the nuclear, microsomal-membrane, and cytosolic fractions, respectively (Fig. 7B, lower panels). Following coproduction of the core, Sp110b shifted into the microsomal-membrane fraction (Fig. 7B, top panel). Core expression thus altered the subcellular localization of Sp110b from the nucleus to the cytoplasmic surface of the ER, where the core was originally located.

To investigate the molecular mechanisms governing the change in Sp110b subcellular localization by the core, we utilized a core point mutant, core(6162M). This protein, containing substitutions of arginines at positions 61 and 62 for glycine and leucine, respectively, has little affinity for Sp110b (Fig. 3A, center panel). Production of the core(6162M) mutant had no effect on the subcellular localization of Sp110b (Fig. 7A, panels 16 to 19). In addition, Sp110b(1-276 + 454-539), which lacks the CBR and does not associate with the core in vitro (Fig. 3Bj), remained in the nucleus irrespective of core production (Fig. 7A, panels 20 to 23). We also performed an interaction-competition analysis using a peptide fragment containing the CBR of Sp110b (myc-CBR fragment), a fragment capable of colocalizing with the core in the cytoplasm (Fig. 7A, panels 24 to 26). Excess myc-CBR fragment inhibited the colocalization of Sp110b with the core (Fig. 7A, panels 27 to 29), suggesting that molecular interaction of Sp110b with the core is essential for the alteration of Sp110b subcellular localization by core production.

Transcriptional activation of RARα by HCV core was exerted via sequestration of the transcriptional corepressor Sp110b away from the nucleus. The above results raised the possibility that Sp110b, a potent nuclear transcriptional corepressor of RARα, was functionally modulated by the core through sequestration from the nucleus. To investigate this possibility, we examined by a reporter assay whether the core modulates the suppressive effect of Sp110b on RARα activity (Fig. 8A). Sp110b-induced transcriptional suppression was overcome by production of the core in a dose-dependent manner (Fig. 8A). As the expression levels of both ectopically produced Sp110b and RARα were not affected by core production (data not shown), the core likely inactivates the function of Sp110b suppressing RARα-mediated transcription.

To determine if the sequestration of Sp110b from the nucleus is essential for the activation of RARα-mediated transcription by the core, we performed an interaction-competition analysis. The CBR fragment, which itself did not affect the transcriptional activity of RARα in the absence of the core (Fig. 8B, bars 7 and 8), served as a competitor for Sp110b sequestration by the core (Fig. 7A, panels 24 to 29). Coproduction of this CBR fragment with the core reversed the core-induced transcriptional activation in a dose-dependent manner (Fig. 8B, bars 3, 4, and 9 to 14). Moreover, we observed that the core mutant core(6162M), which could not alter the localization of Sp110b, also lacked the ability to augment transcriptional activity (Fig. 8B, bars 5 and 6). From the above results, we concluded that the activation of RARα-mediated transcription by the core results from the sequestration from the nucleus and subsequent inactivation of the transcriptional corepressor Sp110b.

Sequestration of Sp110b from the nucleus by HCV core caused sensitization to ATRA-mediated cell death. We analyzed the relationship of Sp110b sequestration by the core to sensitization to ATRA-induced cell death and enhancement of tTGase gene induction (Fig. 1) by an interaction-competition analysis using the CBR fragment. tTGase mRNA levels, increased by core expression in the presence of ATRA, decreased following coproduction of the CBR fragment (Fig. 1C, upper panel, lanes 4 to 6). When the core and the CBR fragment were simultaneously produced in transiently transfected cells, the observed levels of ATRA-induced cell death were

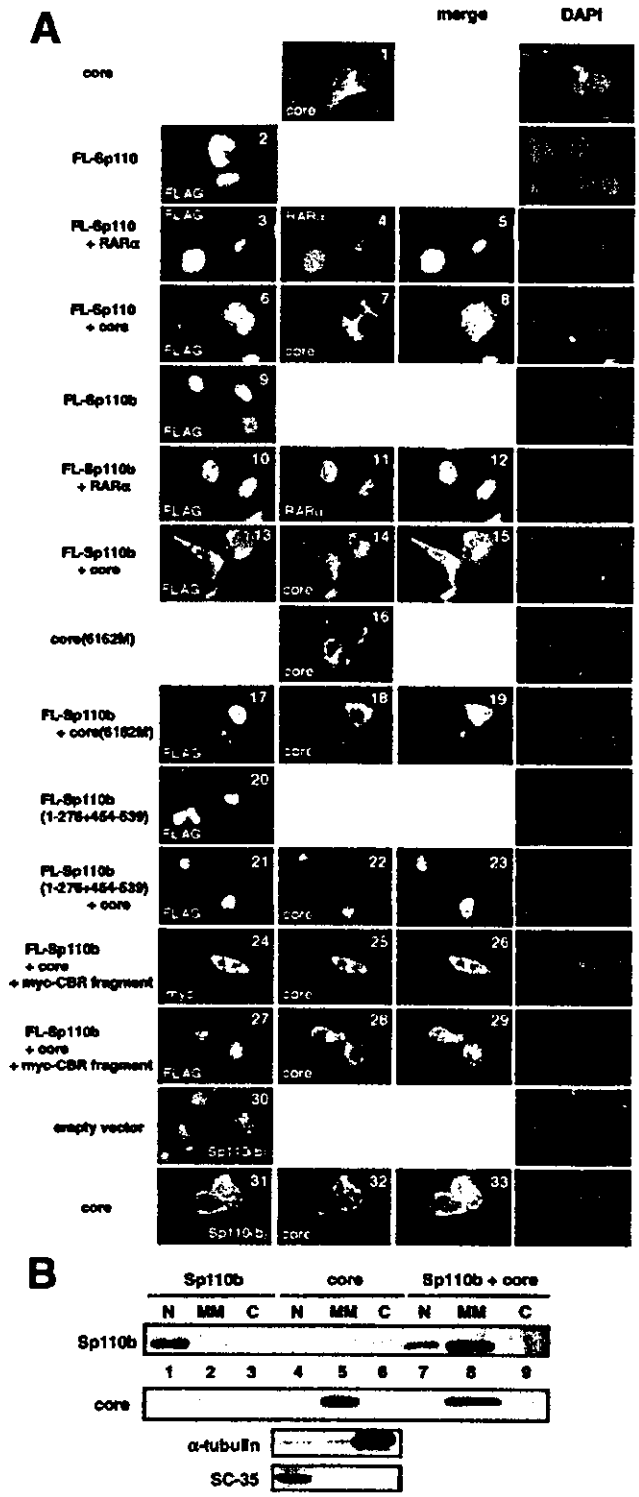

FIG. 7. The subcellular localization of Sp110b was altered from the nucleus to an area around the cytoplasmic surface of ER membranes by core expression. (A) Indirect immunofluorescence analysis was performed on COS-7 cells transfected with pCMV-core (panel 1), pCA-Sp110 (panel 2), pCA-Sp110 with pSG5-RARα (panels 3 to 5), pCA-Sp110 with pCMV-core (panels 6 to 8), pCMV-Sp110b (panel 9), pCMV-Sp110b with pSG5-RARα (panels 10 to 12), pCMV-Sp110b with pCMV-core (panels 13 to 15), pCMV-core(6162 M) (panel 16), pCMV-Sp110b with pCMV-core(6162 M) (panels 17 to 19), pCMV-Sp110b(1-276 + 454-539) (panel 20), pCMV-Sp110b(1-276 + 454-539) with pCMV-core (panels 21 to 23), or pCMV-Sp110b and pCMV-core with pcDNA-Sp110b(389-453) (myc-CBR fragment) (panels 24 to 29) and on HeLa cells transfected with pKS+/CMV (panel 30) or pCMV-core (panels 31 to 33) following treatment with 300 U of gamma interferon/ml to allow the detection of endogenous Sp110b. The primary antibodies used were anti-FLAG (panels 2, 3, 6, 9, 10, 13, 17, 20, 21, and 27) (green), anti-Myc (panel 24) (green), anti-Sp110(b) (panels 30 and 31) (green), anti-core (panels 1, 7, 14, 16, 18, 22, 25, 28, and 32) (red), and anti-RARα (panels 4 and 11) (red). Merged images of green and red signals are shown in panels 5, 8, 12, 15, 19, 23, 26, 29, and 33. DAPI was used to visualize nuclear staining (right panels). (B) Subcellular fractionation was performed on cells transfected with 1 μg of pCMV-Sp110b (lanes 1 to 3), 1 μg of pCMV-core (lanes 4 to 6), and 1 μg each of pCMV-Sp110b and pCMV-core (lanes 7 to 9). The transfectants were homogenized and separated into nuclear (N), microsomal-membrane (MM), and cytosolic (C) fractions by centrifugation as described in Materials and Methods. The FLAG-tagged Sp110b, core, α-tubulin, and SC-35 proteins in those fractions were detected by immunoblot analysis.

similar to those found in cells with no ectopic protein production (Fig. 1B, bars 7 and 8). This result suggested that the CBR fragment reverses the promotion of ATRA-induced cell death mediated by the core. Moreover, the core(6162M) mutant did not enhance cell death induced by ATRA (Fig. 1B, bars 9 and 10).

The data cumulatively suggest that the interaction of HCV core with Sp110b and the subsequent sequestration of Sp110b from the nucleus play a significant role in the sensitization to ATRA-mediated cell death induced by HCV core protein.

DISCUSSION

Sp110b as a transcriptional corepressor of RARα. In this study, we have identified Sp110b as a cellular factor interacting with HCV core. Although the function of Sp110b has remained unknown, we found in this study that Sp110b had the potential to suppress RARα-mediated transcription and that it associated with RARα and the target promoter containing RARE in the presence of ATRA. Moreover, Sp110b mRNA was expressed both ubiquitously and abundantly in human tissues at levels much higher than those of Sp110, a splicing variant of Sp110b. These results suggest that Sp110b is a transcriptional corepressor of RARα, contrasting with the transcriptional coactivating function of Sp110.

The only structural difference between Sp110b and Sp110 is the absence in Sp110b of a C-terminal extension of Sp110, which contains the PHD and the bromodomain (Fig. 3B). Although the function of the PHD is still unknown, the bromodomain is known as a structure for interaction with an acetylated lysine residue (10, 57). This domain has been identified in many proteins involved in transcriptional regulation, likely associating with histone acetyltransferase activity, which is involved in the activation of gene expression (9). From these facts, it seemed reasonable that the bromodomain of Sp110 may be essential for its transcriptional coactivating activity and that Sp110b, lacking this region, would have no potential for transcriptional activation. The suppressive effect of Sp110b on transcription may result from competitive exclusion of other coactivators, such as SRC-1, TIF2/GRIP1, RAC3/ACTR/pCIP/AIB-1, and CBP/p300, via the LXXLL motif from the

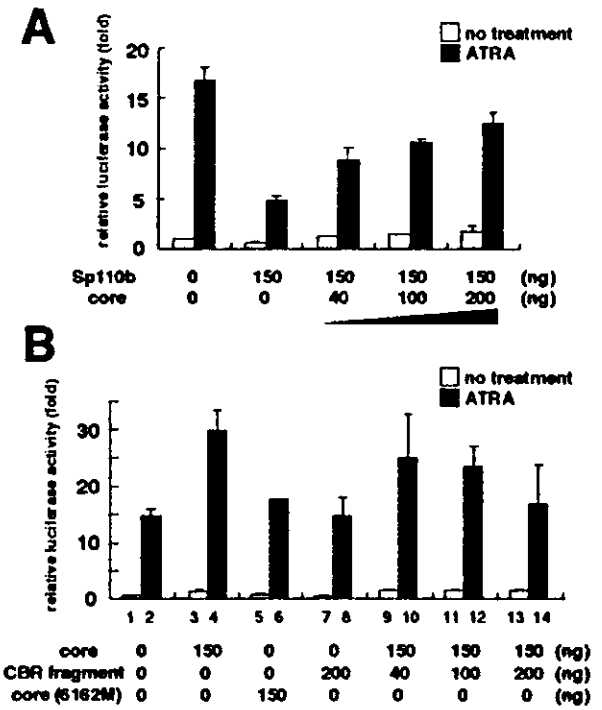

FIG. 8. Sequestration of Sp110b from the nucleus plays a significant role in the activation of RARα-mediated transcription by core expression. (A) A reporter assay was performed using COS-7 cells transfected with a total of 400 ng of plasmids including 25 ng of pRARE-Luc, the effector plasmids pCMV-Sp110b and pCMV-core, and empty vector. Other conditions were the same as those described in the legend to Fig. 2A. The amounts of the effector plasmids (in nanograms) in each experiment are given below the graph. (B) A reporter assay was performed using COS-7 cells transfected with a total of 400 ng of plasmids including 25 ng of pRARE-Luc, the effector plasmids pCMV-core, pCMV-Sp110b(389-453) (CBR fragment), and pCMV-core(6162M), and empty vector. Data are means of the relative luciferase activities from three independent experiments.

RARα-containing transcription complex. Further analysis will be necessary to clarify the molecular mechanisms of transcriptional regulation in which Sp110b participates.

Role of Sp110b in the activation of RARα-mediated transcription by HCV core. The present study suggested that the core activates RARα-mediated transcription by sequestering Sp110b from the nucleus, where Sp110b could exert its suppressive function on RARα-mediated transcription. Sp110b is instead relocalized to the cytoplasm and concentrated at ER membranes (Fig. 9). This phenomenon appears to be independent of cell type, as Sp110b is expressed ubiquitously and can suppress RARα activity in all cell lines examined. In fact, despite the restriction of HCV infection to hepatocytes, the core activated RARα-mediated transcription in all cell lines tested. We also confirmed the physiological relevance of RARα activation by the core in hepatocytes through observations that the core increased mRNA levels of TGF-β2, a gene downstream of RAR, in hepatoma Huh-7 cells (data not shown).

Although the mechanism of Sp110b relocalization by the core remains unknown, some speculations can be put forward. Nascent Sp110b protein, newly synthesized in ribosomes around the ER, is trapped by the core, which resides around the ER, preventing its translocation into the nucleus. Sp110b may also shuttle between the nucleus and the cytoplasm; when Sp110b is in the cytoplasm, Sp110b may be trapped by the core through molecular interaction.

There have been numerous reports that the core modulates the activities of a variety of transcriptional promoters for various genes, including a wide range of transcription factors such as NF-κB (28, 48, 56), AP-1 (48), Elk (14, 16), STAT (55), NF-AT (5), RXR (52), and p53 (25, 42). In some reports, it was speculated that the core, which is mainly located in the cytoplasm, modulates the transcriptional activities of nuclear factors via direct interaction. Previously, however, there been no evidence demonstrating the precise location of this interaction in the cells. In addition, the relationship between the interaction and the transcriptional modulation has remained obscure. To our knowledge, this study is the first to demonstrate the molecular mechanism of transcription factor activity modulation by the core; sequestration of a transcriptional cofactor is

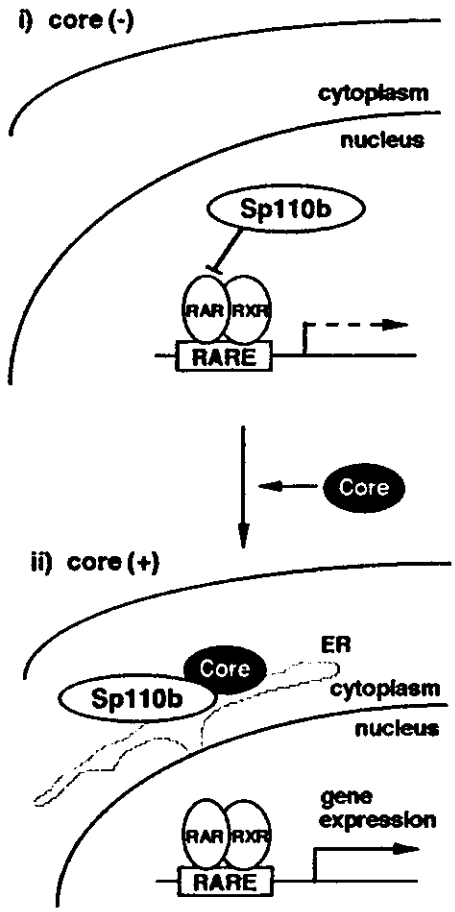

FIG. 9. Schematic representation of the mechanistic model of activation of RARα-mediated transcription by the core. (i) In the absence of the core, Sp110b is located in the nucleus, playing a suppressive role in RARα-mediated transcription. (ii) In the presence of the core, Sp110b is sequestered to ER membranes through interaction with the core, which is located on the cytoplasmic surfaces of ER membranes. This sequestration results in a reduction of the transcriptional suppressive effect of Sp110b in the nucleus. Consequently, ATRA-induced transcription and expression of RARα-responsive genes, such as tTGase, are enhanced.

essential. Recently, the core was reported to interact with RXRα and activate its transcriptional activity, although the mechanism of this activation was unclear (52). While the authors speculated that the core functions as an RXRα cofactor, we demonstrate that the core interacts with a transcriptional cofactor, Sp110b, in the cytoplasm to inhibit its corepressor function. Although experiments analyzing the interaction between Sp110b and RXRα are under way, a similar mechanism of sequestration of Sp110b may be involved in RXRα activation by the core.

Transcriptional cofactors, such as CBP/p300, SRC-1, TIF2/GRIP1, and RAC3/ACTR/pCIP/AIB-1, participate in the regulation of multiple transcription factors (22). These transcriptional cofactors play an important role in the cross talk between different signal transduction pathways; activation of NF-κB represses the transcriptional activation of glucocorticoid receptors, and vice versa, by competition for interaction with CBP and SRC-1, which are required for their activities and are limiting in cells (47). Similar cross talk mechanisms have been reported between AP-1 and androgen receptor (13), NF-κB and p53 (39), c-Myb and GATA-1 (50), and estrogen receptor alpha and NF-κB (15). Constitutive androstane receptor inhibited the activity of estrogen receptor through sequestration of a transcriptional coactivator, GRIP1 (30). Although it remains unknown if Sp110b is involved in the regulation of additional transcription pathways, some of the effects of the core on a variety of transcriptional events may result from the depletion of Sp110b from the nucleus by the core. Further biochemical analysis of Sp110b may shed more light on this point.

ACKNOWLEDGMENTS

We are grateful to P. Chambon, S. Kato, T. Kiyono, and J. Miyazaki for providing plasmids, M. Kohara for the anti-core antibody, and M. Hosaka for Huh-7 cells carrying an HCV full-genome replicon.

This work was supported by grants-in-aid for cancer research and for the second-term comprehensive 10-year strategy for cancer control from the Ministry of Health, Labor, and Welfare of Japan; by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by grants-in-aid for research for the future from the Japanese Society for the Promotion of Science; and by the Program for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR) of Japan.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 278, No. 50, Issue of December 12, pp. 50301–50308, 2003
Printed in U.S.A.

Hepatitis C Virus Non-structural Proteins in the Probable Membranous Compartment Function in Viral Genome Replication*

Received for publication, May 30, 2003, and in revised form, August 20, 2003
Published, JBC Papers in Press, September 8, 2003, DOI 10.1074/jbc.M305684200

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The molecular mechanism of hepatitis C virus(HCV) RNA replication is still unknown. Recently, a cell culture system in which the HCV subgenomic replicon is efficiently replicated and maintained for a long period in Huh-7 cells has been established. Taking advantage of this replicon system, we detected the activity to synthesize the subgenomic RNA in the digitonin-permeabilized replicon cells. To elucidate how and where this viral RNA replicates in the cells, we monitored the activity for HCV RNA synthesis in the permeabilized replicon cells under several conditions. We obtained results suggesting that HCV replication complexes functioning to synthesize the replicon RNA are protected from access of nuclease and proteinase by possible cellular lipid membranes. We also found that a large part of the replicon RNA, including newly synthesized RNA, was present in such a membranous structure but a large part of each NS protein was not. A small part of each NS protein that was resistant to the proteinase action was shown to contribute sufficiently to the synthesis of HCV subgenomic RNA in the permeabilized replicon cells. These results suggested that a major subcellular site of HCV genome replication is probably compartmentalized by lipid membranes and that only a part of each NS protein forms the active replication complex in the replicon cells.

Infection of hepatitis C virus (HCV)[1] is estimated to occur in about 3% of the worlds population. HCV infection frequently causes chronic hepatitis, which often leads to the development of liver cirrhosis and hepatocellular carcinoma after a long period (1–3). Current combination therapy with interferon-α and ribavirin, a nucleotide analogue, is effective in many patients with chronic hepatitis C (4, 5). There still are, however, a lot of patients who do not respond to these treatments. Therefore, extensive studies have been performed to develop highly effective anti-HCV drugs. Such a drug, however, has not been produced yet, possibly because of the lack of detailed information about the life cycle of this virus.

HCV is a member of the *Flaviviridae* family and contains a single-strand RNA genome of positive polarity (6). The RNA genome is ~9.6 kb in length and consists of a 5′-untranslated region of 341 nucleotides, a large open reading frame encoding a single precursor polyprotein of ~3000 amino acids, and a 3′-untranslated region of variable length (6–8). The polyprotein is processed by the host and viral proteinases to generate at least 10 functional viral proteins: core (C), envelope (E) 1, E2, p7, non-structural protein (NS) 2, NS3, NS4A, NS4B, NS5A, and NS5B (from the amino- to the carboxyl-terminal) (9–12). C, E1, and E2 are believed to form viral particles as structural proteins. p7 was recently reported to form an ion channel-like structure (13, 14). NS molecules have been considered to function in the replication of HCV subgenomic RNA (15). Using recombinant proteins produced in either bacterial or insect cells, the proteinase and helicase activities of NS3 and RNA-dependent RNA polymerase activity of NS5B have been biochemically characterized (16–20). However, it was not clear whether these recombinant proteins function in HCV genomic replication as it has been revealed that HCV genomic sequences are highly variable among all isolates and furthermore, it is unclear whether the genes for these viral enzymes were derived from infectious HCV genomes.

Recently, HCV subgenomic RNA that replicates efficiently and is maintained for a long period in the human hepatoma cell line Huh-7 was developed and called the HCV subgenomic replicon (15). Functional replicons originating from different HCV isolates have been reported (15, 21–23). The HCV subgenomic RNA was constructed by replacing the structural and part of the non-structural protein-encoding regions (C-NS2) of the HCV genome with the neomycin phosphotransferase gene (*neo*^{r}) and an internal ribosome entry site of *encephalomyocarditis* virus (21). This implies that HCV proteins encoded in this subgenomic RNA (NS3-NS5B) are functional and sufficient for this RNA replication. In this model system, it has been suggested that mutations of particular amino acids in the NS region enhanced the efficiency of the replication (24–27). It was also demonstrated that the existence of the cis-acting elements in either the 5′- or 3′-untranslated regions were required for efficient replication (27–29). Recent observations indicated that the replication of the replicon RNA could be reproduced *in vitro* using particular cellular fractions from replicon cells (30–32). It remains, however, to be elucidated how and where the HCV RNA is synthesized in the cells. So, we intended to clarify these points using digitonin-treated replicon cells of which plasma membranes were permeabilized. This permeabilized cell system is often used to monitor several cellular events,

* This work was supported by grants-in-aid for cancer research and for the second-term comprehensive 10-year strategy for cancer control from the Ministry of Health, Labor, and Welfare, through grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, grants-in-aid of research for the future from the Japanese Society for the Promotion of Science, and by the Program for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR) of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HCV, hepatitis C virus; NS, non-structural; ER, endoplasmic reticulum; DHFR, dihydrofolate reductase.

such as a nuclear protein transport as well as replication of positive-strand RNA viruses.

Cell biological and biochemical analyses have demonstrated that all HCV NS proteins are directly or indirectly associated with inner cellular membranes and colocalize on the rough endoplasmic reticulum (ER) membranes (33–36). So, we expected the active HCV replication complexes to be retained on the inner cellular membranes in permeabilized replicon cells. In this paper, we report that the functional replication complexes are retained in permeabilized replicon cells and its activity is easily detected by using this system. We also obtained data suggesting that a part of each NS protein in the cells, which is probably located in a membranous compartment, forms the active replication complex and contributes to the synthesis of HCV subgenomic RNA.

EXPERIMENTAL PROCEDURES

Cell Cultures—The human hepatoma cell line Huh-7 was grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml nonessential amino acids (Invitrogen), and 100 μg/ml penicillin and streptomycin sulfate (Invitrogen). MH-14 cells were cultured in the same medium with 500 μg/ml G418 (GENETICIN, Invitrogen).

Sequencing Analysis—The nucleotide sequence of the HCV subgenomic replicon RNA in MH-14 cells was determined by reverse transcription-PCR-based DNA sequencing as described previously (21).

Plasmid Construction—The plasmid pGEM-NN was constructed by inserting the cDNA fragment of the subgenomic replicon from pNNRZ2 (21) into the TA site of pGEM-T-Easy vector (Promega, Madison, WI). The cDNA fragment was obtained by PCR using the oligonucleotides 5′-GCCAGCCCCCGATTGGGGGCGACAC-3′ and 5′-ACATGATCTGCAGAGAGGCCAG-3′ and the plasmid pNNRZ2 as primers and a template, respectively.

In Vitro Transcription—pGEM-NN was linearized with PvuI or SpeI for plus or minus strand subgenomic HCV RNA synthesis, respectively, and used as a template for *in vitro* RNA synthesis with MEGA script Sp6 or T7 kit (Ambion, Austin, TX), respectively.

Preparation of RNA and Northern Blot Analysis—RNA was extracted from cells and a reaction mixture with Sepasol RNA I and II super reagent (Nacalai Tesque, Kyoto, Japan), respectively, according to the manufacturer's protocol. Northern blot analysis was performed as described previously (37). For the preparation of the ^{32}P-labeled probe, the EcoRI fragment of pNNRZ2 was labeled with a Ready-to-Go DNA labeling beads (-dCTP) kit (Amersham Biosciences) in the presence of 50 μCi of [α-^{32}P]dCTP (Amersham Biosciences).

Western Blotting—The preparation of the cell lysate, SDS-PAGE, and immunoblotting were performed as previously described (10). The antibodies used in the immunoblotting were those against HCV NS3, NS4A (α-NS4A), NS4B (NS4B-52), NS5A (α-NS5A), NS5B (NS5B-14) (21), dihydrofolate reductase (DHFR) (34), BiP/Grp78 (StressGen, Victoria, BC, Canada), and Calnexin-NT (StressGen). Anti-NS3 antibody was a gift from Dr. M. Kohara (Tokyo Metropolitan Institute of Medical Science).

Indirect Immunofluorescence—Indirect immunofluorescence analysis was essentially performed as described previously (38), with minor modifications. 1.0 × 10^5 cells were seeded on poly-L-lysine (Sigma)-coated coverslips. Three days post-seeding, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) for 30 min at room temperature. The cells were treated with pre-chilled 100% methanol for 10 min at −20 °C after the fixation. In the case of permeabilized cells, permeabilization with digitonin followed by washing with buffer B (see below) was performed before or after fixation with 4% paraformaldehyde and then the cells were permeabilized completely with chilled 100% methanol after fixation. The antibodies against NS5A (α-NS5A), NS5B (NS5B-12), and protein-disulfide isomerase (StressGen) were used as primary antibodies. NS5B-12 was a gift from Dr. M. Kohara (Tokyo Metropolitan Institute of Medical Science). The fluorescent secondary antibodies were Alexa 568 goat anti-mouse IgG (H+L) and Alexa 488 goat anti-rabbit IgG (H+L) conjugates (Molecular Probes, Eugene, OR). The nucleus was visualized by staining with 4′,6-diamidino-2-phenylindole. All imaging experiments were performed on a Leica SP2 confocal microscope (Leica Microsystems, Germany).

Cell Permeabilization and Synthesis of HCV Subgenomic RNA—Cells of about 80% confluency in 12- or 6-well plates were precultured in complete Dulbecco's modified Eagle's medium containing 5 μg/ml actinomycin D (Nacalai Tesque) for 2 h, then washed with cold buffer B: 20 mM HEPES-KOH (pH 7.7 at 27 °C), 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, and 2 mM dithiothreitol. The cells were permeabilized by incubation in buffer B containing 50 μg/ml digitonin for 5 min at 27 °C and the reaction was stopped by washing twice with cold buffer B. The permeabilized cells were, then, incubated for 4 h at 27 °C in the labeling reaction mixture: 2 mM manganese(II) chloride, 1 mg/ml acetylated bovine serum albumin (Nacalai Tesque), 5 mM phosphocreatine (Sigma), 20 units/ml creatine phosphokinase (Sigma), 50 μg/ml actinomycin D, 500 μM ATP, CTP, and GTP (Roche Diagnostics), and 10 μCi of [α-^{32}P]UTP (Amersham Biosciences) in buffer B (pH 7.7), for 4 h at 27 °C unless otherwise specified. The reaction was terminated by the addition of Sepasol RNA I or II super reagent.

For the treatments with micrococcal nuclease and/or Nonidet P-40, the permeabilized cells were pre-washed twice with buffer D: 20 mM HEPES-KOH (pH 7.7 at 27 °C), 110 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, and 1 mM $CaCl_2$. Then the permeabilized cells were incubated in buffer D containing 0.1 unit/ml micrococcal nuclease (United States Biochemical Corp.), with or without 0.5% Nonidet P-40 for 15 min at 37 °C or 0.5% Nonidet P-40 for 15 min on ice.

Slot Blot Hybridization—RNA products synthesized in the permeabilized cells in the presence of 200 μCi of [α-^{32}P]UTP were fractionated by denaturing agarose gel. The 8-kb RNA bands were eluted from the gel using RNade (BioOne, Carlsbad, CA). To increase the hybridization signal, the ^{32}P-labeled 8-kb RNA eluted from the gel was subjected to alkaline hydrolysis to generate fragments of ~250 nucleotides in length and used in hybridization. Newly synthesized replicon RNA in intact replicon cells was metabolically labeled by adding 1200 μCi of [^{32}P]orthophosphate to the culture medium (see below) and handled in the same manner. For detection of plus and minus strand replicon RNA, minus and plus strand replicon RNA were prepared as riboprobes by *in vitro* transcription, respectively, as described above. Then 2 μg of each riboprobe was applied to a nylon membrane filter (Hybond-N, Ambion). Slot blot hybridization was performed as described previously (39), except that ULTRAhyb (Ambion) was used for hybridization buffer.

Metabolic Labeling—For metabolic labeling of the replicon RNA, after a preculture in phosphate-free Dulbecco's modified Eagle's medium (Invitrogen) containing 2% dialyzed fetal bovine serum, 200 μg/ml G418, and 5 μg/ml actinomycin D for 2 h, 4 × 10^5 cells were cultured for 12 h in the same medium with 100 μCi of [^{32}P]orthophosphate (Amersham Biosciences), as described previously (21).

Proteinase K Treatment—After permeabilization of the replicon cells, the cells were scraped into 400 μl of cold buffer B and transferred to a siliconized tube. The cells were treated with various concentrations of proteinase K at 37 °C for 5 min. The reaction was terminated by the addition of 1 mM phenylmethylsulfonyl fluoride, and followed by trichloroacetic acid precipitation. The trichloroacetic acid precipitates were solubilized in 1× sample buffer containing 1 mM phenylmethylsulfonyl fluoride and used in Western blotting analysis.

RESULTS

Selection of Replicon Cell Clones in Which Replicon RNA Efficiently Replicated—We reported the establishment of a cell clone, NN 50-1, in which HCV subgenomic replicon RNA originating from the HCV genome isolated from the cultured human T cell line MT-2C infected with HCV *in vitro* efficiently replicated (21). Furthermore, we obtained a cell clone, MH-14, in which the HCV subgenomic RNA level is nearly 5-fold higher than that in NN 50-1 (data not shown). Nucleotide sequence analysis revealed that the replicon RNA in MH-14 cells bore two point mutations in the NS4B and NS5A encoding regions. One was a thymine to cytosine transition at nucleotide position 5985 (the number corresponds to the nucleotide number of the HCV genotype 1b genome) in the NS4B-encoding region without an amino acid substitution. Another was a cytosine to adenine transition at nucleotide 6953 in the NS5A-encoding region, resulting in the substitution of arginine for serine at amino acid position 2204. The substitution at amino acid 2204 has already been reported as one of the adaptive mutations enhancing the efficiency of viral replication (25). Then we used MH-14 cells, refereed as the replicon cells, in the following