Hepatitis C Virus NS5A Protein Modulates Transcription through a Novel Cellular Transcription Factor SRCAP

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Hepatitis C virus NS5A protein transcriptionally modulates cellular genes and promotes cell growth. NS5A is likely to exert its activity in concert with cellular factor(s). Using a yeast two-hybrid screen, we have demonstrated that NS5A interacts with the C-terminal end of a newly identified cellular transcription factor, SRCAP. The authenticity of this interaction was verified by a mammalian two-hybrid assay, in vitro pull-down experiment, and an in vivo communoprecipitation assay in human hepatoma (HepG2) cells. An in vitro transient transfection assay demonstrated that SRCAP can efficiently activate transcription when recruited by the Gal4 DNA-binding domain to the promoter. However, down-regulation of p21 promoter activity by NS5A was enhanced following ectopic expression of SRCAP. Together these results suggest that the interaction of NS5A and SRCAP may be one of the mechanisms by which NS5A exerts its effect on cell growth regulation contributing to hepatitis C virus-mediated pathogenesis.

Hepatitis C virus (HCV) is an important cause of morbidity and mortality worldwide, causing a spectrum of liver disease ranging from an asymptomatic carrier state to end-stage liver disease (1, 2). The HCV genome encodes a single polypeptide precursor that is cleaved by both host and viral proteases to generate structural and nonstructural proteins. The nonstructural protein 5A (NS5A) is generated as a mature protein by the action of NS3 protease in conjunction with NS4A (3, 4). NS5A is localized in the nuclear periplasmic membrane (4) and exists as phosphoproteins (p56 and p58), with the degree of phosphorylation accounting for the difference in size (5–7). Phosphorylation status of NS5A differs among HCV genotypes (8). NS5A is phosphorylated by a cellular serine/threonine kinase, and Ser321 represents a major phosphorylation site (9). However, this phosphorylation site is dispensable for interaction with NS4A and PKR. Sequence comparison of the regions surrounding the phosphorylation sites indicates an extremely high level of conservation between different strains of the HCV, but the biological significance of phosphorylation is still undefined.

Recent studies suggest that HCV NS5A protein transcriptionally modulates cellular genes, promotes cell growth (10, 11), and inhibits tumor necrosis factor-α mediated apoptotic cell death. There is also evidence that two-thirds of the NS5A protein from the C-terminal fused with Gal4 DNA-binding domain functions as a potent transcriptional activator (12, 13). Viral proteins may influence cellular genes, which in turn may be involved in the regulation of oncogenes or tumor suppressor genes. Inactivation of these genes may be a mechanism for the disruption of normal cell growth. Host factors are important components for the modulation of virus replication. Viruses also produce proteins that may interact with host factors for viral persistence by disrupting normal cell cycle. To further understand the functional role of HCV NS5A, we examined the interaction of NS5A with cellular protein(s) by yeast two-hybrid screening. Results from this study provided important information regarding the association of NS5A protein with SRCAP, a most recently identified cellular transcriptional coactivator (14).

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The entire cDNA coding region of HCV NS5A (genotype 1a, H strain) was fused in frame with the Gal4 DNA-binding domain into the pGBT9 plasmid vector (CLONTECH) at the EcoRI/SalI restriction sites (pGBT9–5A) and transformed into Saccharomyces cerevisiae yeast strain HF7c. The pGBT9–5A positive yeast colonies were grown in appropriate liquid medium lacking tryptonph and were subsequently transformed with library plasmids fused to the Gal4 activation domain, constructed in pGAD plasmid vector (CLONTECH) for screening of cellular partners. Colonies were selected on agar plates lacking histidine, tryptophan, and leucine over a 7-day period. Positive yeast transformants were picked up and replated for β-galactosidase assay by colony-lift filter procedure. A positive interaction was determined by the appearance of blue colonies. The β-galactosidase positive colonies were grown on a selective medium for plasmid isolation. Isolated plasmids were transformed into Escherichia coli KC8 strain and selected for the activation domain plasmids on M9-lev agar plates. The potential NS5A interacting cDNA inserts were retransformed into HF7c yeast strain bearing pGBT9–5A fusion gene and were grown on an appropriate selective medium for β-galactosidase assay. Positive interacting cDNA clones were analyzed by nucleotide sequencing using an automated sequencer (Applied Biosystems). Nucleotide and predicted amino acid sequences were compared with known protein sequences deposited in the GenBank by BLAST analysis.

Mammalian Two-hybrid Analysis—A mammalian expression plasmid encoding VP16, a hybrid polypeptide containing the transactivation domain of herpesvirus VP16 (15), was fused to NS5A (VP16–5A). Gal-SRCAP expression plasmid DNA (14) was used in this study. HepG2 and NIH3T3 cells were cotransfected with 1 μg of Gal4 responsive reporter gene (G5E1b-CAT), 2 μg of VP16–5A, and Gal-SRCAP or Gal-ΔSRCAP effector plasmids. CAT assay was performed as described.

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1 The abbreviations used are: HCV, hepatitis C virus; CAT, chloramphenicol acetyl transferase; PAGE, polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; CDK, cyclin-dependent kinase.

2 A. K. Ghosh and R. B. Ray, unpublished observation.
earlier (10). In all the transfection experiments, β-galactosidase gene was included to normalize the transfection efficiency.

In Vitro Pull-down Experiment—The NS5A genomic region was cloned in frame with histidine-tag (His-NS5A) into proEXHTA plasmid vector (Life Technologies, Inc.), and expressed in E. coli BL21 cells. Bacterial extracts were immobilized on nickel-nitrilotriacetic acid beads and incubated with in vitro translated [35S]methionine-labeled SRCAP. Beads were washed, and proteins were analyzed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography (16). His-MBP-1 (an unrelated protein) was used similarly as a negative control.

Coimmunoprecipitation—HepG2 cells grown in 35-mm plates were transfected with 1 µg of the CMV-NS5A or pcDNA3 control plasmid using LipofectAMINE (Life Technologies, Inc.). Cell lysates were prepared after 48 h of transfection in 0.3 ml of low stringency lysis buffer (150 mM NaCl, 10 mM Hapes, pH 7.6, 0.1% Nonidet P-40, 5 mM EDTA) containing protease inhibitors (2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM phenylmethylsulfonylfluoride). Each of the cell lysates were incubated with mononclonal antibody to SRCAP and immobilized on staphylococcus protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech). Immunoprecipitates were separated by SDS-PAGE, followed by Western blot analysis using NS5A or SRCAP specific antibody.

Immunofluorescence Study—HepG2 cells were grown on glass coverslips and fixed with 4% paraformaldehyde in 10% fetal bovine serum. Cells grown overnight were transfected with CMV-NS5A using LipofectAMINE for immunofluorescence and colocalization study using a similar method described earlier (17). Cells were washed after 48 h of transfection and fixed with 3.7% formaldehyde in phosphate-buffered saline for 30 min. After fixing, cells were washed twice with phosphate-buffered saline and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 5 min. Cells were incubated with a murine monoclonal antibody to SRCAP, anti-NS5A rabbit polyclonal antibody or both the antibodies for 1 h at room temperature. Cells were washed and incubated with fluorochrome-conjugated secondary antibodies for 30 min at room temperature. Finally, washed cells were mounted for confocal microscopy using Bio-Rad 1024 confocal microscope. Fluorescence images were superimposed digitally to allow fine comparison. Colocalization of green (fluorescein isothiocyanate) and red (tetramethylrhodamine B isothiocyanate) signals in a single pixel produces yellow color, whereas separated signals remain green or red.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)—Cyttoplasmic RNA was isolated from NIH3T3 cells transfected with various plasmid DNAs, stable NIH3T3neo and NIH3T3NS5A cells (10) using the TRIzol (Life Technologies, Inc.) kit. RNA (2 µg) was used for RT-PCR as described previously (18, 19). RT-PCR reaction was performed using specific primers for p21 cDNA (forward primer: 5'-T-GTCGTCAGAACCATACTG-3'; reverse primer: 5'-AGGGCTTCCTC-TTCCGAGA-3'), luciferase cDNA (forward primer: 5'-TTCGCAGCTA- CCGTATGTT-3'; reverse primer: 5'-CCCTGGAGATGAGGATTT-3'), GAPDH cDNA (forward primer: 5'-GAACATTCCACATCATGG-3'; reverse primer: 5'-CATGGTGGGATGAGGATTT-3'). Reaction was carried out at 48 °C for 45 min in reverse transcription, followed by PCR at 94°C for denaturing, 55°C for annealing, and 72°C for extension. For quantitative evaluation, we initially performed the RT-PCR reaction over a range of cycles (20, 25, 30, 35, and 40) and 25–30 cycles were observed to be within the logarithmic phase of amplification. GAPDH was used in the quantitative RT-PCR analysis as an internal control.

Luciferase Assay—HepG2 cells were transfected with 4 µg of a reporter plasmid (WLP-luc, p21 promoter linked with luciferase gene), 2 µg of CMV-NS5A (suboptimal dose), and 1 µg of CMV-SRCAP using LipofectAMINE. 48 h after transfection, luciferase activity was measured as described previously (10). Briefly, cells were treated with lysis buffer (Promega), and luciferase activity in the lysates was assayed by integrating the total light emission over 10 s using a luminometer (Optocomp II, MGM Instruments). The luciferase activity was normalized based on protein concentration.

RESULTS AND DISCUSSION

We have previously demonstrated that the NS5A protein of HCV transcriptionally modulates cellular genes and promotes murine fibroblast cell growth into a tumorigenic phenotype (10). Because the predicted amino acid sequence of NS5A does not possess a known DNA binding motif, it appears that NS5A transcriptionally regulates these cellular genes either by direct interaction with general transcription factor(s) or through a cofactor. To explore the potential targets of NS5A protein, a yeast two-hybrid screen was performed. Yeast strain H7FC was transformed with pGBT9–5A and colonies were selected on dropout agar medium lacking tryptophan. A few randomly picked colonies were grown, and extracted proteins were subjected to SDS-PAGE followed by Western blot analysis using a monoclonal antibody to the Gal4 DNA-binding domain. Results indicated pGBT9–5A fusion protein expression in all the yeast transformants (data not shown). The expression was also confirmed with a monoclonal antibody to NS5A. For the yeast two-hybrid screening, H7FC yeast cells expressing NS5A were transformed with library plasmid DNAs and selected the candidate colonies on the basis of their ability to grow in the appropriate selection medium and turning on the LacZ gene. Yeast transformants, positive for the activation of two reporter genes, were identified from $2 \times 10^9$ independent transformants. We initially identified 30 β-galactosidase positive clones grown in histidine-deficient selective medium. Plasmid DNA was isolated from 17 clones, amplified in bacteria, and retransformed into H7FC yeast cells expressing pGBT9–5A gene for confirmation of the positive interaction. Six clones indicated positive growth on selective medium and in β-galactosidase assay following retransformation. On further testing of these clones for interaction with a battery of heterologous baits in yeast, three clones were found to specifically interact with pGBT9–5A and not with other heterologous protein baits. All three clones were sequenced and analyzed by the BLAST program. Sequence analysis revealed that these isolates represent an independent overlapping cDNA with homology to a recently identified coactivator SRCAP (14).

The mammalian version of the conventional two-hybrid assay was used to ascertain whether the candidate NS5A-interacting protein associates with NS5A in mammalian cells. For this purpose, we constructed mammalian expression plasmid vectors that encode a VP16–5A and Gal-SRCAP fusion proteins. The mammalian two-hybrid assay was performed by transfecting HepG2 or NIH3T3 cells with a Gal4-responsive reporter gene (GSE1b-CAT) and pairwise combinations of the appropriate expression vectors. Reporter gene activity was determined by measuring CAT activity in cell lysates from each transfected culture. A significant increase in CAT activity was observed following coexpression of VP16–5A and Gal-SRCAP hybrids (Fig. 1). However, CAT activity was not enhanced by coexpression of the VP16–5A and Gal4 vector. CAT activity was detected in Gal-SRCAP and VP-16 vector transfected cells, although the level of CAT expression was much lower as compared with hybrid. We obtained overlapping cDNAs of SRCAP interacting with NS5A from yeast two-hybrid assay. Initial mapping data suggests that NS5A associates with the C-terminal 62 amino acids of SRCAP. To confirm that the C-terminal region of SRCAP indeed interacts with NS5A, we used a C-terminal deletion mutant of SRCAP (Gal-SRCAP) in mammalian two-hybrid assay. Results demonstrated that the C-terminal deletion mutant can no longer interact with NS5A under a similar condition (Fig. 1).

An in vitro binding assay was used to verify the physical interaction between the viral protein NS5A and SRCAP. Histidine-tagged NS5A (His-NS5A) was expressed in bacteria, immobilized onto nickel-nitrilotriacetic acid beads and incubated with [35S]methionine-labeled SRCAP generated by in vitro translation. The proteins binding onto beads was then subjected to SDS-PAGE, followed by autoradiography. Results of the in vitro binding assay exhibited a specific band of SRCAP retained by the His-tagged NS5A nickel-nitrilotriacetic acid beads (Fig. 2A). However, an unrelated cellular protein (His-MBP-1) when used as a negative control under similar experi-
in vivo, endogenous SRCAP physically associates with SRCAP.

The detection of endogenous SRCAP (ascertained from the migration of standard protein molecular weight luminescence (ECL). The molecular weight of the NS5A band was also via Biotech) was used for detection of the peroxidase signal by chemi-conjugate (anti-rabbit IgG/horseradish peroxidase, Amersham Pharmacia Biotech).

Immunoprecipitates were separated by SDS-PAGE (7.5%) and 2\% cell lysates were immunoprecipitated with a monoclonal antibody to NS5A. A secondary antibody conjugate (anti-rabbit IgG/ 

To investigate the ability of NS5A for association with endogenous SRCAP in vivo, a coimmunoprecipitation experiment was performed with lysates of NS5A-transfected HepG2 cells. SRCAP-specific antibody was used to precipitate the protein complex, followed by Western blot analysis with a specific antibody to detect the NS5A protein (Fig. 2B). Interestingly, NS5A was coprecipitated with endogenous SRCAP as evident from the specificity of the antibody and the size of the NS5A protein in the immunoblot. Vector-transfected control HepG2 cell lysates when analyzed similarly did not exhibit a NS5A specific band. The blot was stripped and reprobed with a specific antibody to SRCAP, and an endogenous SRCAP band was detected in both the lanes. A similar experiment was performed using an unrelated monoclonal antibody of the same isotype as a negative control. This negative control antibody did not exhibit a detectable reactivity with either SRCAP or NS5A (data not shown). Results suggested that endogenous SRCAP forms a complex with HCV NS5A. Thus, specific association of SRCAP and NS5A in HepG2 cells was demonstrated using the mammalian two-hybrid system, in vitro pull-down assay, and the coimmunoprecipitation analysis.

We further examined whether NS5A protein can colocalize with the endogenous SRCAP. We initially investigated the localization of endogenous SRCAP by indirect immunofluorescence in HepG2 cells using a monoclonal antibody to SRCAP. Immunofluorescent staining of the SRCAP protein showed a predominant perinuclear localization with occasional nuclear staining. Similarly, cells transfected with CMV-NS5A plasmid DNA when stained with polyclonal antisera, exhibited perinuclear staining as shown earlier (4). To compare the subcellular localization of SRCAP with NS5A, HepG2 cells were transfected with CMV-NS5A, and immunofluorescent staining was performed with antibodies to NS5A and SRCAP. Confocal microscopy showed a significant colocalization of the endogenous SRCAP with NS5A (Fig. 3). There was no detectable staining when normal control sera were used.

HCV may benefit by regulation of cellular gene(s) leading to the disruption of normal cell growth. Viral genes can override cellular control mechanisms, which in untransformed cells regulate cell cycle progression in response to various antiproliferative signals. HCV often causes persistent infection and is a silent disease. In HCV persistently infected cells, the continued presence of viral gene products is likely to be detrimental for host cells. We have previously shown that HCV NS5A protein transcriptionally down-regulates p21 activity (10). Cell cycle progression is driven by the sequential activation of cyclin-dependent kinases (CDKs), which are subject to regulation by positive (cyclins) and negative (CDK-inhibitory proteins) effectors (20). One such effector is the universal CDK inhibitor p21/waf1 (21, 22). p21 may participate in apoptosis, and increased p21 expression correlates with enhanced cell death under certain conditions (23, 24). p21 protein binds and inhibits the activity of CDKs by preventing the phosphorylation of critical CDK substrates and by blocking cell cycle progression (21, 25). To further examine the effects of NS5A and SRCAP on a natural promoter, HepG2 or NIH3T3 cells were transfected with a reporter construct (p21 promoter linked with luciferase gene), CMV-NS5A and CMV-SRCAP as the effector plasmids. Results from the luciferase assay suggested that inhibition of p21 promoter activity by NS5A protein was higher in presence of SRCAP (Fig. 4). However, CMV-SRCAP alone at the same concentration did not show a significant effect on p21 promoter activity. A similar result was observed in NIH3T3 cells. To further examine the effect of NS5A on p21 promoter at the transcriptional level, a quantitative RT-PCR was performed using total RNA from stably or transiently transfected cells. This assay was designed to separately analyze the effect of NS5A and SRCAP directly on the p21 mRNA level or on the

![Image](54x512 to 291x729)

**FIG. 1. Interaction of NS5A with SRCAP in mammalian two-hybrid system.** HepG2 cells were transfected with 1 \( \mu \)g of G5E1b-CAT reporter gene, 2 \( \mu \)g of VP16-5A, and 2 \( \mu \)g of Gal-SRCAP or Gal-\( \Delta \)SRCAP. CAT assay was performed 48 h posttransfection. The amount of DNA was kept constant in each transfection by adding the empty vector DNA. SRCAP-NS5A hybrid shows high level of CAT activity as compared with Gal-SRCAP or NS5A alone.

![Image](100x257 to 247x438)

**Panel A:** in vitro translated [\( ^{35} \)S]methionine-labeled SRCAP was subjected to pull-down analysis with His-NS5A or His-MBP-1 (negative control) fusion protein. Ten percent of the input protein (in vitro translated SRCAP) was loaded in the gel for comparison. The image was edited from the same x-ray film. **Panel B:** in vitro coimmunoprecipitation of endogenous SRCAP with HCV NS5A. HepG2 cells were transfected with NS5A expression plasmid or empty vector plasmid. Cells were lysed after 48 h. Vector-transfected (\( \text{lane 1} \)) and NS5A-transfected (\( \text{lane 2} \)) cell lysates were immunoprecipitated with a monoclonal antibody to SRCAP. Immunoprecipitates were separated by SDS-PAGE (7.5%) and immunoblotted with a rabbit antibody to NS5A. A secondary antibody conjugate (anti-rabbit IgG/ 

**FIG. 2. NS5A protein physically associates with SRCAP.** Panel A, in vitro translated [\( ^{35} \)S]methionine-labeled SRCAP was subjected to pull-down analysis with His-NS5A or His-MBP-1 (negative control) fusion protein. Ten percent of the input protein (in vitro translated SRCAP) was loaded in the gel for comparison. The image was edited from the same x-ray film. **Panel B:** in vitro coimmunoprecipitation of endogenous SRCAP with HCV NS5A. HepG2 cells were transfected with NS5A expression plasmid or empty vector plasmid. Cells were lysed after 48 h. Vector-transfected (\( \text{lane 1} \)) and NS5A-transfected (\( \text{lane 2} \)) cell lysates were immunoprecipitated with a monoclonal antibody to SRCAP. Immunoprecipitates were separated by SDS-PAGE (7.5%) and immunoblotted with a rabbit antibody to NS5A. A secondary antibody conjugate (anti-rabbit IgG/ 

![Image](54x512 to 291x729)

**FIG. 4.** HCV NS5A Interacts with SRCAP. Panel A, in vitro translated [\( ^{35} \)S]methionine-labeled SRCAP was subjected to pull-down analysis with His-NS5A or His-MBP-1 (negative control) fusion protein. Ten percent of the input protein (in vitro translated SRCAP) was loaded in the gel for comparison. The image was edited from the same x-ray film. **Panel B:** in vitro coimmunoprecipitation of endogenous SRCAP with HCV NS5A. HepG2 cells were transfected with NS5A expression plasmid or empty vector plasmid. Cells were lysed after 48 h. Vector-transfected (\( \text{lane 1} \)) and NS5A-transfected (\( \text{lane 2} \)) cell lysates were immunoprecipitated with a monoclonal antibody to SRCAP. Immunoprecipitates were separated by SDS-PAGE (7.5%) and immunoblotted with a rabbit antibody to NS5A. A secondary antibody conjugate (anti-rabbit IgG/ 

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transcription of a tagged luciferase reporter gene. Results suggested down-regulation of p21 promoter activity at the transcriptional level by NS5A alone or together with SRCAP (Fig. 5).

We report that the SRCAP molecule physically associates with HCV NS5A protein. SRCAP was isolated in three independently derived cDNA clones in yeast two-hybrid screening. The interaction of HCV NS5A protein with SRCAP was confirmed by mammalian two-hybrid assay, pull-down experiments, and coimmunoprecipitation studies. HCV NS5A colocalizes with SRCAP at the perinuclear membrane of HepG2 cells. Preliminary mapping analysis suggests that the binding of NS5A occurs with SRCAP through the C terminus (62 amino acids), which contains highly charged residues. HCV NS5A also possesses several acidic domains (12, 13). Whether SRCAP-binding domain of NS5A resides within one of these acidic domains remains to be elucidated. NS5A is suggested as a potent transcriptional activator. However, it exerts a negative regulatory activity on the p21 promoter in favor of promoting cell growth (10). SRCAP alone increases the E1b promoter activity when brought closer by Gal4 DNA-binding domain to the promoter sequences (14). Although SRCAP is defined as a coactivator, it behaves like a corepressor when associated with NS5A to exert a negative effect on the p21 promoter. We propose NS5A may recruit endogenous SRCAP to repress the p21 promoter. Transcription factors that activate in one circumstance and repress in another have been documented, and the molecular basis for these transitions are quite diverse (for review, see Refs. 26 and 27). NS5A has recently been demonstrated binding with a different cellular factor Grb2 at the SH3 domain, which perturbs the mitogenic signaling pathways (28). Interaction between HCV NS5A and SNARE-like protein (hVAP33) has recently been reported (29). Our observations add a novel cellular protein SRCAP to the list of NS5A-interacting cellular factors. To our knowledge, this is the first report of dissecting the transregulatory activity of NS5A and implicates its relation with a cellular factor. We propose that the recruitment of SRCAP by NS5A is one of the mechanisms of its transcription-modulatory activity. This may also explain the growth-promoting activity of HCV NS5A.

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**Fig. 4.** Transcriptional regulation of p21 promoter by NS5A and SRCAP. Effector plasmid (CMV-SRCAP and/or CMV-NS5A) was cotransfected with 4 μg of WWP-luc as the reporter plasmid in HepG2 cells. The total amount of plasmid DNA (7 μg) was kept constant by the addition of empty vector in each transfection. Cell extracts were prepared 48 h posttransfection and luciferase activity was determined. In each set of experiments, triplicate transfections were performed, and relative luciferase activity is presented. p21 promoter activity was suppressed when NS5A and SRCAP was coexpressed.

**Fig. 5.** Panel A, quantitative RT-PCR analysis for endogenous p21 or GAPDH mRNA from NIH3T3neo (lane 1) and NIH3T3NS5A (lane 2) cells. Panel B, detection of luciferase mRNA by RT-PCR from transiently transfected p21-luciferase (lane 1), p21-luciferase with NS5A (lane 2), and p21-luciferase with NS5A and SRCAP (lane 3). GAPDH was used as an internal control.
HCV NS5A Interacts with SRCAP

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