Activation of Ribosomal RNA Transcription by Hepatitis C Virus Involves Upstream Binding Factor Phosphorylation via Induction of Cyclin D1

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Abstract

Hepatitis C virus (HCV) causes chronic infection in humans leading to liver cirrhosis and hepatocellular carcinoma. rRNA transcription, catalyzed by RNA polymerase I (Pol I), plays a critical role in ribosome biogenesis, and changes in Pol I transcription rate are associated with profound alterations in the growth rate of the cell. Because rRNA synthesis is intimately linked to cell growth and frequently up-regulated in many cancers, we hypothesized that HCV might have the ability to activate rRNA synthesis in infected cells. We show here that rRNA promoter–mediated transcription is significantly (10- to 12-fold) activated in human liver–derived cells following infection with type 2 JFH-1 HCV or transfection with the subgenomic type 1 HCV replicon. Further analysis revealed that HCV nonstructural protein 5A (NS5A) was responsible for activation of rRNA transcription. Both the NH2-terminal amphipathic helix and the polyproline motifs of NS5A seem to be essential for rRNA transcription activation. The NS5A–dependent activation of rRNA transcription seems to be due to hyperphosphorylation and consequent activation of upstream binding factor (UBF), a Pol I DNA binding transcription factor. We further show that hyperphosphorylation of UBF occurs as a result of up-regulation of both cyclin D1 and cyclin-dependent kinase 4 by the HCV NS5A polypeptide. These results suggest that the endoplasmic reticulum–associated NS5A is able to transduce signals into the nucleolus via UBF hyperphosphorylation leading to rRNA transcription activation. These results could, at least in part, explain a mechanism by which HCV contributes to transformation of liver cells. [Cancer Res 2009;69(5):2057–64]

Introduction

rRNA transcription, catalyzed by RNA polymerase I (Pol I), plays a critical role in ribosome biogenesis, and changes in Pol I transcription rate are associated with profound alterations in the growth rate of the cell (1). rRNA synthesis is frequently up-regulated in many cancers and transformed cells. This is likely due to the increased demand of ribosome biogenesis required for abnormally high levels of protein synthesis in cells lacking growth control. Alternatively, overexpression of rRNA could lead to excess protein synthesis and thus could be an initiating step in tumorigenesis (2). Transcription initiation by human RNA Pol I requires at least two factors in addition to Pol I: the upstream binding factor 1 (UBF1) and a species-specific factor, SL1. UBF1, a sequence-specific DNA binding protein, interacts with the template rDNA. UBF is known to activate rDNA transcription by recruiting Pol I to the rDNA promoter, stabilizing binding of SL1, and competing with nonspecific DNA binding proteins such as histone H1 (3). SL1, a protein complex containing the TATA-binding protein and three Pol I-specific TATA-binding protein–associated factors, confers promoter specificity. rRNA transcription is tightly regulated by cell cycle (5, 6). Both UBF1 and SL1 activities are regulated, at least in part, by phosphorylation during M and G1 phases. At the entry of mitosis, phosphorylation by cyclin-dependent kinase (cdk)-1/cyclin B leads to inactivation of SL1 and UBF1. As a result of SL1 phosphorylation, the ability of SL1 to interact with UBF is impaired, and Pol I transcription is repressed. In early G1 phase, rRNA transcription remains low although the activity of SL1 has been fully restored by dephosphorylation. UBF1 is activated during G1 progression by phosphorylation of Ser484 by cdk4/cyclin D1 (7) and Ser388 by cdk2/cyclin E and cdk2/cyclin A (6), resulting in full activation of rRNA transcription.

Hepatitis C virus (HCV) is an enveloped RNA virus belonging to the Flaviviridae family. HCV contains a single-stranded, plus polarity RNA genome of ~9,500 nucleotides (8). On entry into cells, the viral RNA is translated into a polyprotein, which is processed into mature viral structural and nonstructural proteins by host and viral proteases. The structural proteins include the core (C), envelope proteins E1 and E2, and p7; the nonstructural proteins include NS2, NS3, NS4A, NS4B, NS5A, and NS5B. HCV causes chronic infection in a relatively high percentage of infected patients leading to cirrhosis of liver and hepatocellular carcinoma. The effects of HCV proteins on hepatocarcinogenesis have undergone intense investigation during the recent years. These studies have implicated three viral proteins (core, NS5A, and NS3) in hepatocarcinogenesis (9). The involvement of all three proteins has been described as being in control of cell cycle through alteration of or interaction with key cellular regulator proteins such as p53, p21, and cyclins, as well as transcription factors, proto-oncogenes, and growth factors/cytokines (9).

Because rRNA transcription is intimately linked to cell growth and frequently up-regulated in transformed cells (10), we hypothesized that HCV might have the ability to activate rRNA synthesis in infected cells. We show here that transcription from the rRNA promoter is significantly up-regulated in cultured human liver cells following infection with type 2 JFH-1 HCV or transfection with type 1b HCV replicon. Further analysis revealed that HCV nonstructural protein 5A (NS5A) was responsible for stimulation of
rRNA transcription. The activation of rRNA transcription seems to be due to stimulation of phosphorylation of UB1, possibly as a result of up-regulation of cyclin D1/cdk4 by the NS5A polypeptide. These results could, at least in part, explain a mechanism by which HCV contributes to transformation of liver cells.

Materials and Methods

Cell cultures and transient transfection. Huh-7, Huh-7.5, and Huh-7.5.1 were maintained in RPMI/DMEM with 10% fetal bovine serum or a combination of 5% fetal bovine serum/5% newborn calf serum with or without Na-pyruvate, respectively. Penicillin-streptomycin was added in the regular medium but not during transfection using low-serum opti-MEM medium (Invitrogen, Inc.). RNA transfections were done in the presence of DIMRIE-C lipofectant (Invitrogen) according to the manufacturer's protocol. Stable cell lines containing full-length and subgenomic (type 1b) replications were made in Huh-7.5 cells by RNA transfection followed by G418 selection and single-colony purification (11, 12).

Plasmids. Plasmids phRR and pT7B were kindly provided by Dr. D.L. Johnson (University of Southern California, Los Angeles, CA; ref. 13). pT7B contains the 234-nucleotide (nt) T7B fragment (DNA fragment from the T7 bacteriophage B arm) inserted between the BamH1 and HindIII sites of pGEM vector (Promega). Human ribosomal reporter plasmid phRR contains a 1,660-bp human rDNA sequence of +150 to +1,500 followed by the 234-nt T7B DNA fragment at the 3′ end in the pBluescript SK+ (pisk) vector (Stratagene). The plasmid used for expression of synthetic NS5A fusion protein in E. coli was constructed by inserting the H7C7 HCV NSSA encoding cDNA fragment between the EcoR1 and Xho1 sites of the pET-28a vector (Novagen). The eukaryotic expression plasmid of NSSA was constructed by inserting the NSSA (H7C7) cDNA fragment into the EcoR1/Xho1 sites of pcDNA Hismax-4C mammalian expression vector (Invitrogen). The recombinant clones used for construction of mutant (deletions and point mutations) NSSA protein were constructed by PCR amplification using the appropriate primer pairs. The plasmid pTRI-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the antisense control template used to produce probes for GAPDH that serve as internal control in RNase protection assay. Infection of Huh-7.5 cells with JFH-1 HCV. The Japanese fulminating hepatitis type 2a strain JFH-1 clone (pJFH-1; provided by Dr. T. Wakita, Tokyo Metropolitan Institute, Tokyo, Japan) was used for the generation of hepatitis type 2a strain JFH-1 clone (pJFH-1; provided by Dr. T. Wakita). The titration of the supernatants containing the virus in Huh 7.5.1 cells (14). Huh-7.5.1 cells were transfected with the rDNA reporter construct phRR using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Stable cell lines containing full-length and subgenomic (type 1b) replications were made in Huh-7.5 cells by RNA transfection followed by G418 selection and single-colony purification (11, 12).

NNSA expression. The bacterial expression and purification of NS5A is described in Supplementary Fig. S1.

Antibodies. Monoclonal antibody (mAb) to α-actin was purchased from Oncogene Research Products. U87 (H300), cdk4 (H-22), and cdk2 (D-12) polyclonal antibodies and cyclin D1 (H-295), cyclin E (BE12), and cyclin A (C-160) mAbs were from Santa Cruz. Anti–cyclin D1 (clone Ab-4) and anti–cyclin A (clone Ab-5) mAbs and anti–cyclin D polyclonal antibody were purchased from Neomarkers, Inc., and Upstate Biotechnology. Nucleolin mAb (clone 4E2) was purchased from Research Diagnostics, Inc. Lamin B was from Sigma. Phospho-serine–specific mAb (Q5) was purchased from Qiagen, and p-UBF (Ser484) specific mAb from Santa Cruz Biotechnology. Horseradish peroxidase–labeled antinurse IgG was used for detection of primary antibody by enhanced chemiluminescence (Amersham pharmacia Biotech), and secondary goat anti-rabbit horse-radish peroxidase–labeled IgG (Upstate, Inc.) was used for detection of polyclonal primary antibodies.

RNase protection assays. RNA was extracted by TRizol (Life Technologies, Inc.) following the protocol provided by the vendor. RNase protection assays were carried out by using the RPAII kit (Ambion) as previously described (13). The isolated RNA (4 μg) was hybridized with an excess of 32P-labeled antisense transcript at 45°C overnight. The antisense transcript was generated from pT7B by using the Maxi-script kit (Ambion), pT7B was linearized with EcoR1 and used as a template to make the antisense T7B riboprobe. The DNA was transcribed by Sp6 RNA polymerase in the presence of [α-32P]CTP (specific activity, >3,000 Ci/mmol; Perkin-Elmer). The riboprobe was treated with DNase I and ethanol precipitated. The hybridized RNA was digested with 150 μL of a 1:100 dilution of highly concentrated RNaseA/RNaseT1 mixture (250 units/mL RNase A and 10,000 units/mL RNase T1) at 37°C for 30 min. The reaction was terminated by addition of 250 μL of stop buffer, and the products were precipitated and resuspended in 6 μL of loading dye and then electro- phoresed on 5% acrylamide–8 mol/L urea denaturing gels. The gels were exposed to X-ray film at −70°C, and the autoradiograms quantified by scanning with ImageJ program.

Northern and Western blot analyses. Total RNA was isolated following the TRizol reagent protocol, and Northern blot was done according to standard protocol. Radioactive T7B riboprobe (Ambion) and/or DNA probe (random primer labeling kit) was made according to the manufacturer's protocol. The blots were either scanned from the autoradiogram (ImageJ) or subjected to phosphorimager analysis (ImageQuanta) in Typhoon scanner.

For Western blots, proteins were directly transferred onto nitrocellulose membranes from SDS gels followed by immunoblotting. Antibody dilutions (200–1,000 times) were made according to the manufacturer’s suggestions. Following SDS-PAGE and Western transfer, the membranes were probed with the appropriate mAbs or polyclonal antibodies in the presence of
either 3% bovine serum albumin or 5% milk in PBS-Tween, and protein bands subsequently detected by chemiluminescence assay (Amersham Pharmacia) using horseradish peroxidase–labeled secondary antirabbit or antitumor IgG.

## Results

### rRNA synthesis is up-regulated by HCV.
To determine whether HCV is capable of activating rRNA transcription by RNA Pol I, full-length replicon–harboring cells (11) were transfected with a Pol I reporter construct containing the human Pol I promoter (13). These cells synthesize both viral structural (core, E1, E2, and p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. Transcription from the reporter plasmid was monitored by RNase protection assay (13). Accurate initiation from the Pol I promoter produces a 234-nl-long product following RNase digestion (13). The appropriate-sized RNase-protected transcript was detected at three different concentrations of the mRNA template in control Huh-7 cells (Fig. 1A, lanes 6–9). Ribosomal rRNA transcription was highly stimulated (∼12-fold) in full-length replicon–harboring cells (Fig. 1A, lanes 1–5). To examine if HCV nonstructural proteins could stimulate rRNA synthesis in the absence of structural proteins, various amounts of in vitro transcribed HCV subgenomic replicon RNA (type 1a, NS3-NS5) and the Pol I reporter plasmid were cotransfected into Huh-7 cells, and the de novo synthesized rRNA transcripts were analyzed by RNase protection assay. A titration of subgenomic replicon RNA clearly showed a dose-dependent increase in promoter-mediated transcription with as much as 10-fold increase at the highest concentration of replicon RNA compared with no replicon RNA control (Fig. 1B, lanes 1–6). While our experiments were in progress, the HCV cell culture system using the JFH-1 virus became available (14). In two separate experiments (Fig. 1C, lanes 1–4 and 5–8), infection of HuH-7.5.1 cells with the JFH-1 virus resulted in 10- to 12-fold stimulation of rRNA synthesis compared with mock-infected cells. These results suggested that HCV could activate rRNA transcription in infected cells.

The HCV NS5A protein up-regulates Pol I transcription.
The HCV NS5A polypeptide has been implicated in the modulation of cell cycle (16–18). To determine whether the NS5A protein could activate rRNA transcription in the absence of other viral polypeptides, the plasmids encoding the full-length NS5A and the Pol I reporter (phRR) were cotransfected into HuH-7 cells, and transcription from the reporter plasmid was examined by RNase protection assay. Transcription from the reporter DNA was stimulated ∼12-fold with 1 μg NS5A plasmid compared with the empty vector [Fig. 2A (lanes 1 and 3) and B]. Higher concentrations of NS5A plasmid were found to be inhibitory. NS5A expression as measured by immunoblotting with a polyclonal anti-NS5A antibody was found to be almost saturating at 1 μg NS5A-expressing plasmid.

Previous studies have shown that a truncated version of NS5A lacking the NH2-terminal 146 amino acids is a potent activator of RNA Pol II transcription when fused to the GAL 4 DNA binding domain in both yeast and mammalian cells (19, 20). The full-length NS5A fused to the GAL 4 DNA-binding domain does not activate transcription, suggesting that interaction with other viral or cellular proteins may be required for its transcriptional activation function. To determine whether the NH2-terminal 146 amino acids were also dispensable for rRNA transcriptional activation, the NH2-terminal 146 amino acids of NS5A were deleted, and the effect of the ΔN146 NS5A on rRNA transcription activation was examined. RNA promoter–mediated transcriptional activation by NS5A was almost totally abrogated by the deletion of the NH2-terminal 146 amino acids (Fig. 2C–E). Thus, in contrast to Pol II transcriptional activation by NS5A, which required deletion of the NH2-terminal 146 amino acids, RNA promoter–mediated Pol I transcriptional activation was dependent on the presence of the NS5A NH2-terminal 146 amino acids.

The NS5A NH2-terminal amphipathic helix and polyproline motifs are important for rRNA transcriptional activation.
The NH2-terminal 30 amino acids of NS5A (Fig. 3A) constitute an amphipathic helix, which seems to be necessary and sufficient for association of NS5A with endoplasmic reticulum membranes (21). Mutations within the amphipathic helix result in loss of membrane association and viral genome replication (15). In particular, a triple amino acid substitution mutant, with Ile8, Ile12, and Phe19 of the wt NS5A altered to code for Asp, Glu, and Asp, respectively, was also dispensable for rRNA transcriptional activation, the NH2-terminal 146 amino acids of NS5A were deleted, and the effect of the ΔN146 NS5A on rRNA transcription activation was examined. RNA promoter–mediated transcriptional activation by NS5A was almost totally abrogated by the deletion of the NH2-terminal 146 amino acids (Fig. 2C–E). Thus, in contrast to Pol II transcriptional activation by NS5A, which required deletion of the NH2-terminal 146 amino acids, RNA promoter–mediated Pol I transcriptional activation was dependent on the presence of the NS5A NH2-terminal 146 amino acids.
was completely defective in membrane (endoplasmic reticulum) association (15). To determine if NS5A membrane association was required for rRNA transcriptional activation, the three mutations (I8D, I12E, and F18D) were introduced into wt NS5A, and the effect of these mutations on rRNA transcription was determined using the Pol I reporter assay. As can be seen in Fig. 3A, all five NS5A clones containing the triple mutations (AH1–AH5) were highly defective in activating rRNA transcription compared with wt NS5A. These results suggest that interaction of NS5A with the perinuclear membranes (endoplasmic reticulum) is important for the NS5A-mediated rRNA transcriptional activation.

In addition to the amphipathic helix, the NS5A protein contains three polyproline motifs (PPM-I, PPM-II, and PPM-III). PPM-I (KLMPQLP) is located immediately following the amphipathic helix, whereas PPM-II (PLPPPR) and PPM-III (PVPPPR) are located in the COOH-terminal half of NS5A (Fig. 4A). These motifs are believed to be important for protein-protein interactions through SH3 domains of many signaling proteins (reviewed in ref. 22). To determine if the NS5A polyproline motifs are involved in activation of rRNA transcription, PPM-I, PPM-II, and PPM-III were deleted individually and the rRNA transcription activation was determined for each PPM mutant. As can be seen in Fig. 3D, all three NS5A PPM deletion mutants were defective in activating rRNA transcription compared with wt NS5A. These results suggest that interaction of NS5A with other proteins is important for rRNA transcription activation.

UBF1 is phosphorylated by NS5A in a manner similar to serum-induced activation of UBF and Pol I transcription. Previous studies have shown that phosphorylation by G1-specific cdk/cyclin complexes at Ser484 and Ser388 of UBF activates Pol I transcription during G1 progression (6, 7). To determine whether a similar mechanism is involved in NS5A-mediated activation of Pol I transcription in vivo, phosphorylation of UBF was analyzed in Huh-7 cells following transfection with wt NS5A RNA, NS5A DNA, mutant NS5A DNA (∆N146), and either full-length or subgenomic HCV replicon RNAs. Cell-free extracts were then analyzed by Western blot with a polyclonal antibody to detect NS5A protein level and a phospho-serine mAb that detects phosphorylated forms of UBF (6, 7). The phospho-serine antibody recognizes both the basally phosphorylated (transcriptionally inactive p-UBF) and hyperphosphorylated (transcriptionally active, p-UBF*) proteins. As a positive control, parallel experiments were done with serum-starved and serum-induced Huh-7 cells. The release of liver cells from serum starvation was accompanied by not only an increase in the synthesis of the UBF protein (Fig. 4C, lanes 2 and 3) but also the generation of a new phosphorylated UBF (p-UBF*) species detectable by the phospho-serine antibody (Fig. 4C, lanes 4 and 5). As can be seen in Fig. 4A, transfection of cells with DNA or RNA encoding the NS5A as well as HCV full-length or subgenomic replicons resulted in the generation of a similar p-UBF* species observed in serum-stimulated cells (Fig. 4A, lanes 3–6). The intensity of the p-UBF* was significantly lower in cells transfected with the ∆N146 NS5A mutant (Fig. 4A, lane 7) compared with those transfected with wt NS5A. These results along with the observation that the ∆N146 NS5A mutant was defective in activating Pol I transcription (Fig. 2C) strongly suggest that NS5A-mediated activation of Pol I transcription involves activation of UBF by phosphorylation in a manner similar to that observed during serum stimulation.

The levels of cyclin D1 and cdk4 in Huh-7 cells are up-regulated by NS5A. UBF is activated during G1 progression by phosphorylation of Ser484 by cdk4/cyclin D1 and Ser388 by cdk2/cyclin E and cdk2/cyclin A, resulting in activation of rRNA transcription. To assess the cdk/cyclin levels in Huh-7 cells expressing the HCV NS5A, Western blots were done with various antibodies. The levels of both cdk4 and cyclin D1 were significantly elevated in cells transfected with a plasmid encoding NS5A but not with the ∆N146 NS5A mutant protein, as if the mutant is expressed as a truncated protein (Fig. 5A and B). The expression of cyclin D1 and cdk4 seems to be below background level in cells expressing the ∆N146 NS5A mutant protein, as if the mutant is acting in a dominant negative manner. The level of the UBF protein was not changed significantly by NS5A expression compared with the vector alone or ∆N146 NS5A controls. Cdk2, cyclin A, and cyclin E levels also did not change significantly by NS5A. These results...
suggest that NS5A could induce increased expression of cdk4/cyclin D1 in Huh-7 cells. Increased cdk4 expression occurs coincident with overexpression of cyclin D1 in many human tumors and tumorigenic mouse models. Some studies suggest that cyclin D1 overexpression leads to an increase in cdk4 translation rather than its mRNA level (23).

We also compared the levels of cdk4, cyclin D1, and UBF1 between mock- and JFH1-infected cells at a relatively high multiplicity of infection (MOI = 1). As can be seen in Fig. 5C and D, the level of cyclin D1 was considerably higher in infected compared with mock-infected cells. Up-regulation of cdk4 was also evident in HCV-infected cells; however, the degree of cdk4 activation was significantly lower in infected cells compared with those transfected with the NS5A plasmid. The cdk4/cyclin D1 complex is known to phosphorylate UBF1 at the Ser484 residue (7). We therefore examined UBF1 Ser484 phosphorylation using a mAb that specifically detects Ser484-phosphorylated UBF1 (24). As expected, the intensity of Ser484-phosphorylated UBF1 band was significantly higher (∼2.5-fold) in HCV-infected versus mock-infected cells. The level of the UBF1 protein, however, did not change significantly by infection. We did not detect any significant difference in cyclin E levels between mock-infected and HCV-infected cells. These results suggest that HCV infection of Huh-7.5.1 cells leads to activation of cdk4/cyclin D1, which in turn results in phosphorylation of UBF1 Ser484.

Discussion

Transcription of rRNA genes by RNA Pol I ultimately determines the number of ribosomes and, consequently, the potential for cell growth and proliferation in response to changes in the cellular environment including neoplasia. We show here that HCV, which causes hepatocellular carcinoma in chronically infected patients, isable to deregulate rRNA transcription in cultured liver cells. Infection of Huh-7.5.1 cells with type 2 JFH-1 HCV leads to rRNA transcriptional activation (Fig. 1C). This observation is supported by results obtained from Huh-7 cells that stably express and replicate full-length replicon RNA and those that are transiently

Figure 3. NS5A NH2-terminal amphipathic helix and polyproline motifs are essential for rRNA promoter–mediated transcriptional activation. A, schematic representation of amphipathic helix (AH) and PPM domains of NS5A are shown. Triple substitution mutations (I8D, I12E, and F18D) as shown were engineered into the NH2-terminal NS5A amphipathic helix domain as reported previously (15). B, five different triple substitution mutant clones (named AH1–AH5) were examined for the ability to activate rRNA promoter–mediated transcription in transient transfection assay by transfecting 1 μg of wt or mutant NS5A plasmid in 60-mm plates for 48 h. The rRNA promoter–mediated transcription from the reporter plasmid pH2R was examined by Northern blot analysis with 50 μg total RNA using the radiolabeled T7III probe as described in Materials and Methods. Endogenous 18S and 28S rRNA were visualized by ethidium bromide staining and served as internal loading control. C, polyproline motif deletion constructs (PPM-I–PPM-III) were used along with the pH2R reporter plasmid to determine the role of individual PPMs in rRNA promoter–mediated transcriptional activation under conditions described in B. D, Western blot analysis was done to detect expression of wt (lanes 1 and 10), AH1 to AH5 mutants (lanes 2–6), and PPM-I to PPM-III mutants (lanes 7–9) using a polyclonal anti-NS5A. Vinculin mAb (Sigma) was used to detect vinculin as internal control. Lane 11, negative control without (W/O) NS5A expression.
transfected with RNA encoding only the viral nonstructural proteins (subgenomic replicon; Fig. 1A and B). Expression of the HCV NS5A polypeptide in the absence of other viral proteins in Huh-7 cells results in significant stimulation of rRNA transcription (Fig. 2A and B). Our results are consistent with a number of previously published observations that showed activation of rRNA gene transcription by oncogenic proteins and growth factors that induce cell growth and proliferation (25–27). The tumor suppressor proteins p53 (28), pRB (5, 29), PTEN (30), and p14ARF (24), on the other hand, have been shown to decrease rRNA gene transcription.

Although the full-length NS5A polypeptide does not enter the nucleus, the NH2-terminal deleted forms containing the nuclear localization signal were found to localize to the nucleus (31, 32), suggesting that an altered form of NSSA could act as a potential transcriptional activator in the nucleus. Indeed, previous studies have shown that a truncated version of NS5A lacking the NH2-terminal 146 amino acids is a potent activator of RNA Pol II transcription when fused to the GAL 4 DNA binding domain in yeast and mammalian cells (31, 32). Our analysis, however, showed that the NH2-terminal 146 amino acids of NS5A that contain the NH2-terminal 30-amino-acid amphipathic helix were required for activation of rRNA transcription (Fig. 3). The amphipathic helix has been shown to be required for its interaction with endoplasmic reticulum membrane. Deletion of the entire amphipathic helix or three point mutations within the amphipathic helix, previously shown to interfere with NSSA membrane binding (15), almost completely abrogated rRNA transcriptional activation by NS5A.

![Figure 4](image-url). HCV replicon and NSSA expression induce phosphorylation of UBF in Huh-7.5 cells. A, phospho-UBF Western blot. Forty micrograms of total protein from nuclear extracts derived from Huh-7 cells transfected with empty vector (lane 2), 1 mg wt NSSA DNA (lane 3), 5 mg NSSA RNA (lane 4), 7.5 mg genomic replicon RNA (lane 5), and 1 mg N146 NSSA DNA (lane 7) were used for Western blot analysis with phospho-serine mAb to detect hyperphosphorylated (p-UBF*) and basally phosphorylated UBF1 (p-UBF). In lane 6, 40 mg of nuclear extract from Huh-7 cells stably transfected with HCV subgenomic replicon were used. Lane 1, migration of molecular weight marker proteins. B, immunoblot of the same lanes as shown in A except that polyclonal anti-UBF1 was used to detect total UBF1 protein. C, Huh-7.5 cells were serum starved (SS), followed by subsequent serum induction (SI). The levels of hyperphosphorylated (p-UBF*) and basally phosphorylated (p-UBF) UBF1 proteins were determined by Western blot analysis using phospho-serine mAb (lanes 4 and 5) and polyclonal anti-UBF1 (lanes 2 and 3). Lane 1, molecular weight marker proteins.

![Figure 5](image-url). NS5A expression and JFH-1 virus infection up-regulate cyclin D1 and cdk4 expression. A, Huh-7.5 cells were transfected with plasmids (1 mg each) encoding vector alone (Vec), wt NS5A, and ΔN146 NS5A. Cells were harvested after 48 h and nuclear extracts were made for Western blot analysis with antibodies as detailed in Materials and Methods. Fifty micrograms of protein were used for the Western blot analyses. Nucleolin was used as an internal control. The expressions of wt NS5A and ΔN146 NS5A are shown in Supplementary Fig. S2B. B, quantification of the data in A averaged from two separate experiments. C, nuclear extracts (50 mg) derived from mock-infected and JFH1-infected (MOI = 1) Huh-7.5.1 cells were used for Western blot analyses with various antibodies as in A except that a phospho-Ser484-specific UBF1 antibody, which specifically recognizes the phospho-Ser484 of UBF1, was used (24). Anti-lamin A was used to detect lamin A in nuclear extract as an internal loading control. D, quantification of the data in C averaged from two separate experiments.

(Figs. 2C–E and 3B). These results suggest that unlike the ability of NS5A to activate Pol II transcription, activation of Pol I transcription requires NS5A-membrane interaction, and that Pol I transcriptional activation may not necessarily require NS5A nuclear entry.

The NS5A NH2-terminal amino acids 26 to 32 constitute a class I polyproline motif (KLMPQLP). Two additional closely spaced class II polyproline motifs (amino acids 342–347 PLPPP and amino acids 350–355 PVPPPR) are located near the COOH terminus of NS5A. The class II polyproline motifs are able to bind to the SH3 domains of a number of cellular signaling proteins (22). Our results suggest that all three PPMs play important roles in rRNA promoter–dependent transcriptional activation because deletion of each motif results in almost complete lack of rRNA transcriptional activation (Fig. 3D). Taken together, our mutagenesis studies suggest that both NS5A membrane binding and its interaction with other cellular proteins possibly involved in signal transduction are necessary for activation of rRNA transcription.

What is the mechanism of RNA Pol I transcription activation by NS5A? Our results clearly show that expression of NS5A alone in Huh-7 cells is sufficient to generate an additional form of UBF (p-UBF*), which is recognized by a mAb to phospho-serine,
suggesting that NS5A expression leads to activation of kinase(s) that phosphorylate UBF. This phosphorylation of UBF seems to be analogous to UBF hyperphosphorylation following serum treatment of serum-starved cells, an event known to activate rRNA transcription. Activation of RNA pol I transcription during G1 progression has been shown to be mediated by UBF phosphorylation of Ser484 by cdk4/cyclin D1 (7) and Ser388 by cdk2/cyclin E and cdk2/cyclin A (6). Indeed, the levels of both cyclin D1 and cdk4 are significantly elevated by expression of NS5A in HuH-7 cells or infection of HuH-7.5.1 cells by HCV (Fig. 5). Our observation that cyclin D1 is activated by HCV NS5A is consistent with a previous report that showed activation of cyclin D1 promoter in HCV replicon–bearing cells (33). These results and the fact that UBF1 Ser484 phosphorylation is stimulated by HCV infection (Fig. 5C) are consistent with the idea that NS5A is able to activate cdk4/cyclin D1, which results in phosphorylation of UBF at Ser484 leading to activation of Pol I transcription (Fig. 6). Up-regulation of cyclin D1, a β-catenin–regulated gene, could result from activation of phosphoinositide 3-kinase and subsequent stabilization of β-catenin following expression of NS5A (34). NS5A-mediated activation of the cdk4/cyclin D1 complex and consequent hyperphosphorylation of UBF possibly require NS5A membrane binding because the ΔN146 mutant fails to activate cyclin D1/ckd4 and promote UBF phosphorylation (Figs. 4 and 5). In fact, the levels of both cyclin D1 and cdk4 seem to be lower in cells expressing ΔN146 NS5A than in vector-expressing cells (Fig. 5A), suggesting that the NH2-terminal deletion mutant is acting in a dominant negative manner. This is also supported by the observation that interaction among cyclin D1, cdk4, and UBF1 is drastically reduced in HuH-7.5 cells transfected with the ΔN146 NS5A mutant compared with those that express wt NS5A (Supplementary Fig. S2). Additionally, for unknown reasons, the level of cyclin E seems to be significantly elevated in ΔN146 NS5A–expressing cells compared with those expressing the wt NS5A protein. The involvement of NS5A has previously been described as being in control of cell cycle through interaction with key cellular regulator proteins such as p53 and p21 (16–18, 35, 36) and cdk/cyclins (15, 16, 37), as well as transcription factors (38–40), proto-oncogenes (39), and growth factors/cytokines (38, 39, 41–43).

Although results presented here strongly suggest that NS5A-mediated activation of cyclin D1/ckd4 is likely responsible for rRNA transcriptional activation, there are other pathways that could also lead to rRNA transcription activation in HCV-infected cells. For example, it has been reported that core, NS3, and E2 proteins of HCV can activate the mitogen-activated protein kinase/extracellular signal–regulated kinase (ERK) kinase/ERK pathways (44). ERK has been shown to act directly on UBF and transcription intermediary factor 1A (45), leading to stimulation of rRNA synthesis. In fact, a previous report has suggested that expression of the HCV core protein activates transcription by all three RNA polymerases (46). However, whether Pol I transcription activation by the core occurs through cell cycle regulatory kinases was not investigated. It is possible that this functional redundancy (by core and NS5A) exists to ascertain total deregulation of Pol I transcription in HCV-infected cells.

Another potential cellular target that directly modulates rRNA transcription is the retinoblastoma protein (Rb). Rb inhibits Pol I transcription by binding UBF and preventing it from recruiting SL1 (5, 47, 48). Increased production of cyclin D1 in HCV-infected cells could lead to assembly of cyclin D1 into a functional kinase (cdk4 and cdk6) complex, which phosphorylates and inactivates the retinoblastoma protein. This could lead to release of UBF and consequent activation of Pol I transcription. Alternatively, NS5B-dependent ubiquitination of pRb and its subsequent degradation via the proteasome (49) could also lead to UBF release and activation of Pol I transcription. Many other transcription factors, such as Pol II factor E2F, Jun, Ap-2, and Pol III factor TFIIIB, are also activated following inactivation of Rb by cyclin D/ckd4 and cyclin D/ckd6 complexes. Thus, transcription of genes by all three classes of RNA polymerases could potentially be activated by elevated levels of cyclin D1.

Amplification or overexpression of cyclin D1 plays pivotal roles in the development of human cancers such as parathyroid adenoma, breast cancer, colon cancer, lymphoma, melanoma, and prostate cancer (50). Of the three D-types of cyclins, it is cyclin D1 overexpression that is predominantly associated with human tumorigenesis and cellular metastases. Recent evidence suggests that in addition to its original description as a cdk-dependent regulator of the cell cycle, cyclin D1 also performs cell cycle–or cdk-independent functions (50). Cyclin D1 associates with and regulates the activity of transcription factors, coactivators, and corepressors that control histone acetylation and chromatin remodeling proteins. Thus, activation of cyclin D1 in HCV-infected cells could lead to a number of important changes, including ribosome biogenesis, which ultimately lead to initiation and/or maintenance of hepatocellular carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Activation of Ribosomal RNA Transcription by Hepatitis C Virus Involves Upstream Binding Factor Phosphorylation via Induction of Cyclin D1

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