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# Regulation of hepatitis C virus replication by the core protein through its interaction with viral RNA polymerase

Su-Min Kang, Jin-Kyu Choi, Seong-Jun Kim, Jung-Hee Kim, Dae-Gyun Ahn, Jong-Won Oh *

*Department of Biotechnology, Yonsei University, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-749, Republic of Korea*

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ABSTRACT

The hepatitis C virus (HCV) core protein is a structural component of the nucleocapsid and has been shown to modulate cellular signaling pathways by interaction with various cellular proteins. In the present study, we investigated the role of HCV core protein in viral RNA replication. Immunoprecipitation experiments demonstrated that the core protein binds to the amino-terminal region of RNA-dependent RNA polymerase (RdRp), which encompasses the finger and palm domains. Direct interaction between HCV RdRp and core protein led to inhibition of RdRp RNA synthesis activity of *in vitro*. Furthermore, over-expression of core protein, but not its derivatives lacking the RdRp-interacting domain, suppressed HCV replication in a hepatoma cell line harboring an HCV subgenomic replicon RNA. Collectively, our results suggest that the core protein, through binding to RdRp and inhibiting its RNA synthesis activity, is a viral regulator of HCV RNA replication.



## Introduction

Hepatitis C virus (HCV) is a major cause of chronic hepatitis and liver cirrhosis, the latter often leading to hepatocellular carcinoma [1]. HCV is an enveloped RNA virus in the *Flaviviridae* family, with a positive-sense single-stranded RNA genome of ~9.6 kb. The viral genome consists of one long open reading frame (ORF) that is flanked by untranslated regions (UTRs) at both the 5′ and 3′ ends [2,3]. The ORF encodes a single polyprotein of 3010 amino acids that is proteolytically processed by cellular and viral proteases into at least 10 polypeptides, including structural proteins and non-structural (NS) proteins [4]. The 65-kDa HCV NS5B protein carrying RNA-dependent RNA polymerase (RdRp) activity is the key player in HCV RNA replication [5]. It is a compact globular protein consisting of three sub-domains called the palm, finger, and thumb, and resembles a right hand, similar to other related enzymes [6].

HCV core protein is a viral structural protein that packages the viral RNA genome [7]. Its binding to the 5′-UTR of the HCV genome has been suggested to be important for specific encapsidation of the viral genome [8]. In addition to its roles as a structural protein, HCV core protein also displays pleiotropic functions though its interaction with cellular proteins [9,10]. Besides its roles in virus particle assembly and in pathogenesis, HCV core protein has been postulated to be involved in regulation of HCV RNA replication. It has been suggested, based on comparison of the RNA replication level of a selectable full-length HCV replicon with that of an HCV subgenomic replicon lacking HCV core/E1/E2 structural protein expression, that the decreased level of HCV RNA in cells containing the full-length replicon might be due to the presence of viral structural proteins [11]. Consistent with this hypothesis, other groups have reported that the efficiency of colony formation by the full-length replicon is about 3–4 logs lower than that of subgenomic replicons [12]. In addition, the subcellular localization of core protein is likely to overlap with that of viral RdRp, since both proteins were shown to be associated with intracellular lipid-raft structures [13,14]. Together, the results described above suggest that HCV core protein might indeed be involved in regulation of HCV RNA replication. To date, however, the precise role of core protein in HCV replication remains unknown.

In the present study, we addressed the question of how HCV core protein regulates viral RNA replication. We performed a comprehensive biochemical analysis of the interaction between HCV core protein and NS5B RdRp, and demonstrated the role of direct interaction between these proteins in modulating the function of RdRp using an *in vitro* RdRp assay and an HCV subgenomic replicon cell line.

## Materials and methods

*Cells and cell culture.* The human hepatoma cell line, Huh7, was cultured in RPMI-1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; BioWhittaker), 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin under standard culture conditions (5% $CO_2$, 37 °C). The human hepatoma stable cell lines Huh7TR-4, which expresses the

* Corresponding author. Fax: +82 2362 7265.
*E-mail address:* jwoh@yonsei.ac.kr (J.-W. Oh).

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tetracycline repressor, and Huh7TR-core, which expresses HCV core protein in a tetracycline-inducible manner [15], were maintained in the presence of blasticidin S (10 μg/ml) and Zeocin (100 μg/ml). Core protein expression in Huh7TR-core was induced by addition of 1 μg/ml of tetracycline for 48 h, unless otherwise specified. The Huh7 cell line R-1, which supports stable, autonomous replication of genotype 1b HCV subgenomic replicon RNA, was described previously [16].

*Plasmid construction.* The pcDNA3.1-Flag-Core plasmid expressing Flag-epitope-tagged full-length HCV core protein was described previously [15]. The pcDNA3.1-Flag-NS5B plasmid was constructed by inserting the cDNA encoding Flag-epitope-tagged full-length HCV NS5B protein into the EcoRI and XhoI sites of pcDNA3.1 (Invitrogen, Carlsbad, CA). cDNAs encoding the finger domain (amino acids 1–187), the finger–palm domain (amino acids 1–371), the palm–thumb domain (amino acids 188–591), the palm domain (amino acids 188–371), and the thumb domain (amino acids 372–591) of HCV NS5B were amplified by PCR from the pThNS5B plasmid [5] and inserted at the NheI/EcoRI site of pET-28a(+) (Novagen, Madison, WI) to obtain pET-28a(+)/NS5B(1–187), pET-28a(+)/NS5B(1–371), pET-28a(+)/NS5B(188–591), pET-28a(+)/NS5B(188–371), and pET-28a(+)/NS5B(372–591), respectively. For expression of the enhanced green fluorescence protein (EGFP)-fused HCV core protein and its deletion derivatives, pEGFP-C(1–191), pEGFP-C(1–75), pEGFP-C(1–121), pEGFP-C(1–173), pEGFP-C(76–191), and pEGFP-C(99–191) were used [10]. The cDNA encoding the full-length SARS coronavirus (SARS–CoV) capsid protein was synthesized by RT-PCR using total RNA extracted from SARS–CoV (Urbani strain)-infected Vero E6 cells, and cloned into the pTrcHisB vector (Invitrogen).

*Expression and purification of recombinant proteins.* Recombinant HCV NS5B protein with a hexahistidine tag at the N-terminus was expressed in *Escherichia coli* and purified as described previously [16]. HCV core protein was expressed in *E. coli* and purified from inclusion bodies as described previously [15]. SARS–CoV capsid protein (N) was expressed in Top10 *E. coli* cells (Invitrogen) at 25 °C overnight by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The recombinant N protein was purified by metal affinity chromatography using Ni-nitrilotriacetic acid (NTA)-agarose (Qiagen, Hilden, Germany), heparin–Sepharose (Amersham Biosciences, Piscataway, NJ), and SP-Sepharose columns (Amersham Biosciences) sequentially.

*Western blot analysis and immunoprecipitation. In vitro* immunoprecipitation of HCV core and NS5B proteins was performed using 1 μg of each recombinant protein. The two proteins in buffer A (50 mM Tris–HCl, pH 8.0; 1 mM dithiothreitol [DTT]; 50 mM NaCl; 5 mM $MgCl_2$; 10% glycerol) were incubated with an anti-HCV core antibody (Virogen, Watertown, MA) for 1 h on a rotator at 4 °C. For co-immunoprecipitation experiments, Huh7TR-4 or Huh7TR-core cells were transfected with pcDNA3.1-Flag-NS5B using FuGENE6 (Roche, Switzerland) and treated with 1 μg/ml of tetracycline for induction of core protein expression. At 48 h post-transfection, cells were harvested and washed twice with cold PBS, resuspended in lysis buffer A (100 mM Tris–HCl, pH 8.0; 150 mM NaCl; 1% Triton X-100; 10 mM NaF; 1 mM $Na_3VO_4$; 17.5 mM β-glycerophosphate) supplemented with an EDTA-free protease inhibitor cocktail (Roche) and incubated on ice for 30 min. After centrifugation, cell lysates was incubated with an anti-Flag antibody (Sigma–Aldrich, Saint-Louis, MO) for immunoprecipitation of the Flag-tagged NS5B protein. For domain mapping of the NS5B-interacting region of HCV core protein, the subgenomic replicon cell line (R-1) was transfected with pEGFP, pEGFP-C(1–191), pEGFP-C(1–75), pEGFP-C(1–121), pEGFP-C(1–173), pEGFP-C(76–191), or pEGFP-C(99–191) using FuGENE6 (Roche) and harvested 48 h post-transfection. Cell lysates were incubated with an anti-GFP monoclonal antibody (BD Biosciences, Palo Alto, CA). Immunocomplexes were recovered by adsorption to Protein G-Sepharose or Protein A-Sepharose (Qiagen). Cell lysates and immunoprecipitates were subjected to SDS–PAGE and electroblotted onto nitrocellulose membrane (Amersham Biosciences). Western blot analysis was performed as described previously [15] using an anti-core antibody (Virogen), a Penta-His antibody (Qiagen), an anti-Flag antibody (Sigma–Aldrich), an anti-NS5B monoclonal antibody (provided by S.B. Hwang at Hallym University, Korea), an anti-GFP antibody (Santa Cruz Biotechnology), or an HCV patient serum (provided by K.H. Han at Yonsei University College of Medicine). Proteins associated with antibodies were visualized using appropriate peroxidase-conjugated secondary antibodies and a chemiluminescent substrate (ECL, GE Healthcare Life Sciences, Piscataway, NJ).

*Confocal microscopy.* The Huh7TR-core cell line was cultured in eight-well chamber slides (Nunc, Rochester, NY) to ~50% confluence and transfected with pcDNA3.1-Flag-NS5B. At 48 h post-transfection, cells were fixed and treated as described previously [15]. Cells were then incubated with a monoclonal anti-core protein antibody (Virogen) and anti-Flag antibody (Sigma–Aldrich). Primary antibodies were detected by fluorescein isothiocyanate- and Texas Red-coupled secondary antibodies. Nuclei were visualized by staining with 1 μM 4′,6′-diamidino-2-phenylindole (DAPI) in PBS for 10 min. Confocal images were obtained using a Bio-Rad Radiance 2000 laser scanning confocal microscope.

*Enzyme assays. In vitro* RNA polymerase activity assays were performed using the full-length $(His)_6$-tagged NS5B protein as described previously [16]. For the (−)3′-UTR template, which represents the complementary sequence of the 5′-UTR, 200 ng of RNA was used. RdRp reactions with the poly(A) template (1 μg) were conducted in the presence of 10 pmol of oligonucleotide $(U)_{20}$ as a primer. Heat-denatured RdRp products were resolved on an 8 M urea, 5% polyacrylamide gel. The gels were dried after fixing and then exposed to X-ray film for autoradiography. RdRp product levels were determined by densitometric analysis of autoradiographs using Scion Image from the National Institutes of Health or by measuring the cpm using a Perkin Elmer Topcounter.

*TaqMan real-time quantitative reverse transcription-PCR.* Total RNA was extracted from R-1 cells using TRIZOL LS reagent (Invitrogen) and purified according to the manufacturer's instructions. HCV RNA levels were quantified by real-time quantitative RT-PCR using a primer pair and TaqMan probe targeting a region within the HCV 5′-UTR as described previously [16]. Cellular glyceraldehyde-3-phosphate dehydrogenase mRNA from the same extracts was used as an internal control.

## Results

### *Direct interaction between HCV core protein and NS5B RdRp*

To investigate the interaction between HCV core protein and NS5B RdRp, and the effect of this protein interaction on viral RNA synthesis *in vitro*, both full-length core and NS5B proteins were expressed and purified from *E. coli* as fusion proteins with an N-terminal hexahistidine. Using highly purified full-length recombinant core and NS5B proteins, as assessed by Coomassie staining (Fig. 1A), we performed immunoprecipitation experiments to demonstrate the direct interaction between these proteins. The core protein was immunoprecipitated with an anti-core protein antibody. As seen in Fig. 1B, immunoprecipitation of core protein co-immunoprecipitated NS5B (lane 2). We further confirmed the interaction in human hepatoma cells by co-immunoprecipitation experiments. Huh7TR-core cells expressing core protein were transfected with pcDNA3.1-Flag-NS5B. Flag-tagged NS5B in detergent-solubilized cell lysates were immunoprecipitated with an anti-Flag antibody, and the resulting immunocomplexes were examined for the presence of core protein by

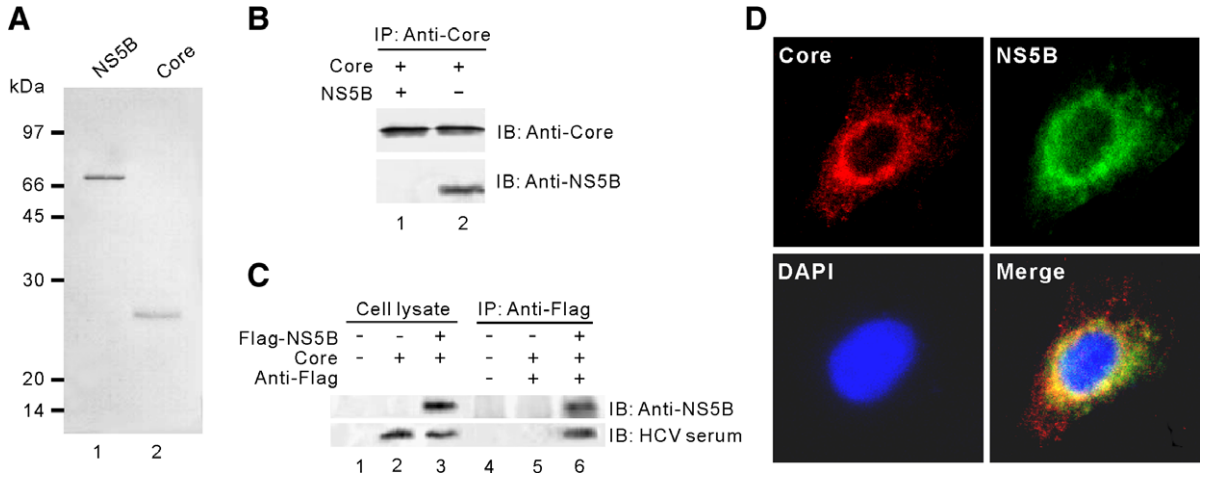


**Fig. 1.** Interaction of HCV core protein with NS5B RdRp. (A) Purified recombinant NS5B and core proteins. Each of the recombinant proteins (400 ng) was loaded on a 12% SDS–PAGE gel and visualized by Coomassie staining. (B) Recombinant NS5B and core proteins were used to demonstrate NS5B-core protein interaction by immunoprecipitation (IP) of protein complexes with a monoclonal anti-HCV core protein antibody. The immunoprecipitated complexes were analyzed by immunoblotting (IB) for core protein and NS5B protein using an anti-core monoclonal antibody and an anti-NS5B monoclonal antibody. (C) Cell lysates of Huh7TR-core transfected with pcDNA3.1-Flag-NS5B, and Huh7TR-4, the parental cell line of Huh7TR-core, were used to immunoprecipitate protein complexes using an anti-Flag antibody or an irrelevant antibody (–). Cell lysates and immunoprecipitated complexes were analyzed for Flag-tagged NS5B and core protein using a monoclonal anti-NS5B antibody and an HCV patient serum, respectively. (D) Partial co-localization of HCV core protein with NS5B protein. Huh7TR-core cells were transiently transfected with a plasmid expressing the Flag-tagged HCV NS5B. At 48 h post-transfection, cells were examined by indirect double immunofluorescence staining of the Flag-tagged NS5B protein (green) and core protein (red) using a mixture of polyclonal anti-Flag antibody and monoclonal anti-core protein antibody. Nuclei were visualized by DAPI staining. The merged image is shown in the bottom right panel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

immunoblot analysis using an HCV patient serum. The results shown in Fig. 1C reveal the interaction between core protein and NS5B in cellular context (lane 6).

Given that core protein interacted with NS5B *in vitro* and in cellular context, we examined whether core protein and NS5B colocalize in cells by confocal immunofluorescence microscopy.

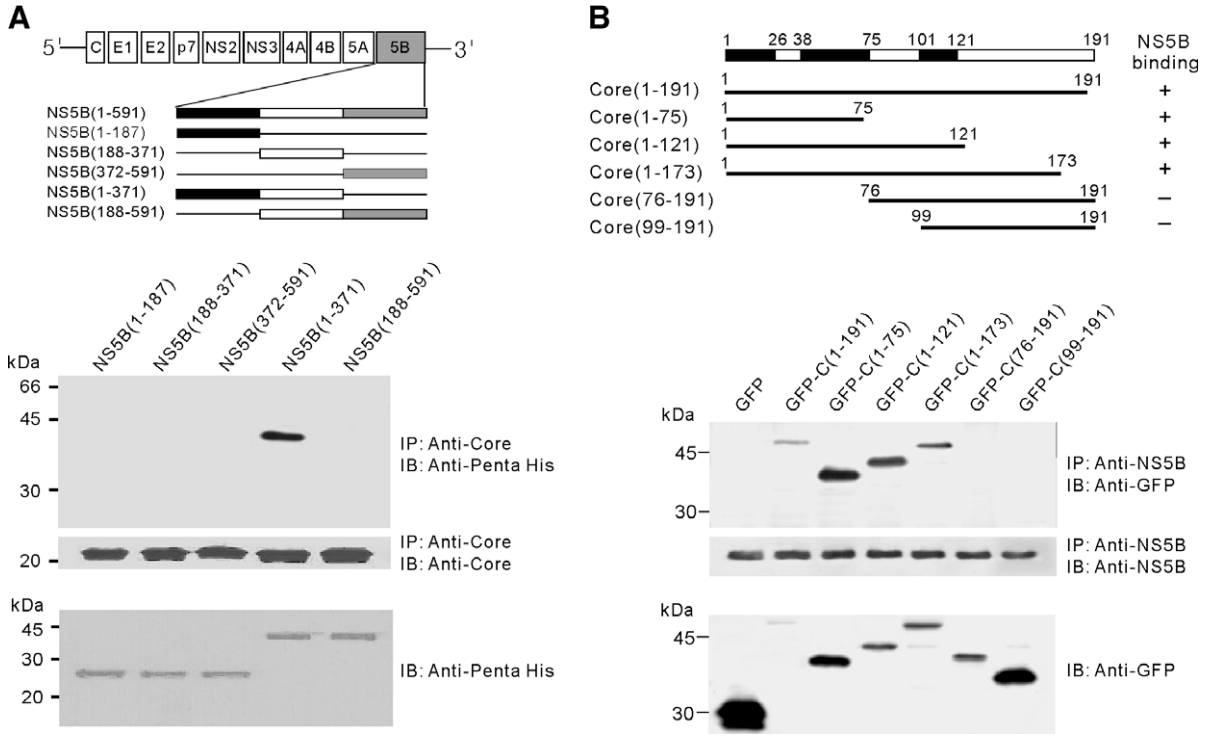


**Fig. 2.** Mapping of the core protein domain interacting with NS5B protein. (A) Schematic representation of the full-length HCV NS5B and its derivatives fused to $(His)_6$ (upper panel). Amino acid numbers of each domain are indicated in parentheses. Recombinant core protein was immunoprecipitated with a monoclonal anti-core protein antibody, and immunoprecipitates were analyzed by immunoblotting using a monoclonal Penta-His or anti-core protein antibody (middle panel). Equal amounts of input NS5B subdomain protein added to the immunoprecipitation experiments were subjected to SDS–PAGE followed by immunoblotting using a monoclonal Penta-His antibody (bottom panel). (B) Schematic representation of the full-length HCV core protein and five deletion mutants fused to GFP at the N-terminus (upper panel). Cell lysates from HCV subgenomic replicon R-1 cells transfected with the plasmids indicated above the blot were used to immunoprecipitate protein complexes using an anti-NS5B monoclonal antibody. Immunoprecipitated NS5B and core proteins as well as the input core proteins were subjected to immunoblotting with the indicated antibodies.

Huh7TR-core cells were transfected with pcDNA3.1-Flag-NS5B. At 48 h post-transfection, the cells were fixed, permeabilized, and dual-labeled with monoclonal anti-core protein and rabbit anti-Flag antibodies to determine subcellular localization of core and NS5B proteins. As shown in Fig. 1D, anti-core protein staining of transfected cells produced a punctate staining pattern predominantly in perinuclear regions. As shown in the merged image, co-localization of the two proteins was observed in a predominantly perinuclear pattern as well as in several speckled regions within the cytoplasm.

*Mapping of the domains required for the interaction between NS5B and core proteins*

To determine the domain of NS5B interacting with core protein, we performed co-immunoprecipitation experiments using a series of truncation mutants of $(His)_6$-tagged-NS5B (Fig. 2A, upper panel) mixed with an equal amount of purified core protein. Core protein interacted with NS5B(1–371) but not with other truncation mutants of NS5B, including NS5B(1–187), NS5B(188–371), NS5B(372–591), and BS5B(188–591), suggesting that the finger–palm domain of NS5B (amino acids 1–371) is critical for core protein binding.

To map the NS5B interacting domain, we transiently expressed various GFP-tagged core protein truncations in HCV subgenomic replicon cells (R-1) and examined NS5B-immunoprecipitates for the presence of core protein by immunoblot analysis. As shown in Fig. 2B, GFP-C(1–191), GFP-C(1–75), GFP-C(1–121), and GFP-C(1–173) co-immunoprecipitated with NS5B, but neither GFP-C(76–191) nor GFP-C(99–191), which lack the N-terminal 75 amino acids, bound to NS5B, indicating that amino acids 1–75 of the core protein constitute the minimal region required for NS5B binding.

*Core protein binding to NS5B interferes with RdRp activity in vitro and suppresses HCV replication in HCV subgenomic replicon cells*

To test the effect of core protein on HCV RNA synthesis *in vitro*, we performed RdRp assays using HCV 3′ (−)UTR, which does not to bind to core protein [17], with increasing amounts of core protein or without core protein. As shown in Fig. 3A, dose-dependent inhibition of RNA synthesis was observed, with effective inhibition at a concentration as low as 0.5-fold molar excess of core protein over NS5B. Additionally, with a homopolymeric RNA/oligo$(U)_{20}$ substrate [5], a similar inhibitory effect of core protein was observed (Fig. 3B); however, SARS–coronavirus core protein did not inhibit HCV RNA synthesis (lane 7). The inhibitory effect of the core protein on HCV replication was also confirmed by over-expressing the core protein in Huh7-derived R-1 cells, which support autonomous replication of a genotype 1b HCV subgenomic replicon RNA. Core protein expression resulted in a concentration-dependent inhibition of HCV RNA replication, with ~80% inhibition when the cells were transfected with 6 μg of the core protein-expressing vector (Fig. 4A). More importantly, GFP-C(1–191) was more effective than GFP-C(99–191), in suppressing HCV RNA replication in the subgenomic replicon cell line R-1 (Fig. 4B): GFP-C(99–191) does not interact with NS5B as shown in Fig. 2B and lacks the known minimal RNA binding domain (amino acids 1–75) [18]. Collectively, these results suggest that core protein affects RNA synthesis by means of its binding to the RdRp.

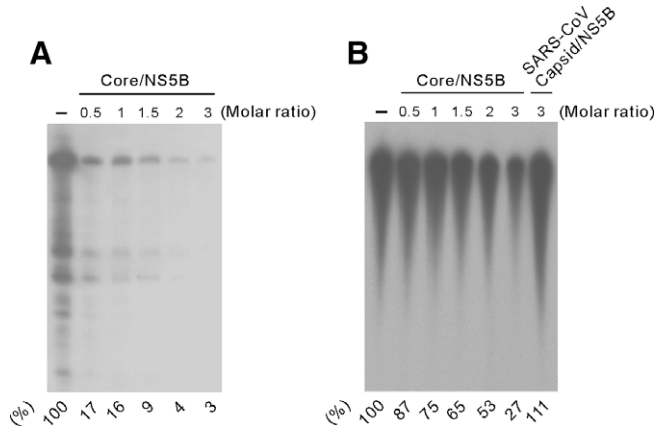


**Fig. 3.** Inhibition of NS5B RdRp activity by core protein *in vitro*. Purified NS5B was used for an RdRp activity assay using poly(A)/oligo$(U)_{20}$ (A) or (−)3′-UTR (B) as a template, in the presence of the indicated amounts of purified HCV core protein or SARS–CoV capsid protein. The molar ratios of the added capsid proteins to NS5B protein are indicated above the autoradiogram. Labeled RdRp products were resolved in an 8 M urea–5% polyacrylamide gel and subjected to autoradiography. The product levels expressed as percent of control (first lane) are shown at the bottom of each autoradiogram.

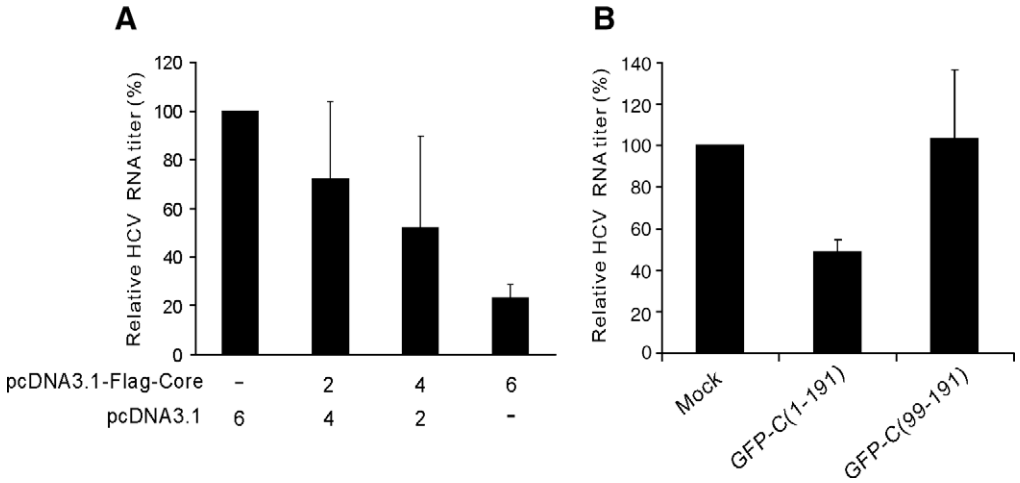


**Fig. 4.** Suppression of HCV RNA replication by core protein. (A) R-1 cells were transfected with increasing amounts of pcDNA3.1-Flag-Core. pcDNA3.1 was used to normalize the total amount of DNA used per plate. At 48 h post-transfection, cells were harvested for analysis of HCV genome copy number by quantitative real-time RT-PCR. Histograms represent the HCV RNA copy number relative to that in cells transfected with an empty expression vector, whose value was taken as 100%. Data represent the means ± SE. of triplicate samples. (B) R-1 cells were transfected with either an empty vector or a vector expressing GFP-C(1–191) or GFP-C(99–191). HCV genome copy number was estimated as in (B).

## Discussion

In the present study, we have uncovered an interaction between HCV core protein and NS5B RdRp. Our results suggest that the inhibitory effect of core protein can be attributed to its direct binding to RdRp. Interestingly, the N-terminal interacting domain contains the Glu-18 residue, which is one of two amino acids identified to be important for the dimerization and catalytic activity of RdRp [19,20]. Thus, the functional consequence of the direct interaction between HCV core protein and NS5B may be inhibition of RdRp activity by blocking NS5B dimerization or by inducing a conformational change.

Core protein and HCV infection are known to induce activation of PKR, an interferon-induced kinase responsible for the phosphorylation of the translation initiation factor eIF2α [21,22]. We also observed that, in cells expressing core protein, both HCV internal ribosome entry site (IRES)- and cap-dependent translation are inhibited by activation of PKR (data not shown). These results underscore the contribution of core protein in the negative regulation of viral genome translation as well as in the regulation of the polymerase activity. When core protein accumulates in infected cells, both viral replication and IRES-mediated translation might be blocked to switch the HCV life cycle from viral replication to packaging of the genome and release of virions. The modulatory function of core protein in HCV replication thus may play a significant role at the late stage of infection. Alternatively, our results may suggest a novel mechanism by which HCV core protein controls the viral replication level through its interactions with the NS5B, likely for the establishment of persistent infection. In other RNA viruses, coat protein was shown to enhance or inhibit RNA replication, likely depending on expression level of coat protein [23]. Similarly, the coat protein of alfalfa mosaic virus is required for initial viral RNA infection and translation [24], but a higher concentration seems to inhibit viral replication [25]. Our results are thus consistent with the possibility that HCV core protein plays more than one role, depending on the stage of the HCV life cycle and the interaction of core protein with various viral and/or cellular proteins.

In summary, we show that HCV core protein binds to NS5B RdRp, a key viral protein required for replication of the HCV genome. The functional consequence of this direct interaction was inhibition of the activity of NS5B, which in turn might contribute to negative regulation of HCV replication.

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