Mechanisms for Inhibition of Hepatitis B Virus Gene Expression and Replication by Hepatitis C Virus Core Protein

Received for publication, May 1, 2002, and in revised form, October 3, 2002
Published, JBC Papers in Press, October 24, 2002, DOI 10.1074/jbc.M204241200

Shiow-Yi Chen‡, Chih-Fei Kao‡§, Chun-Ming Chen§§, Chwen-Ming Shih‡, Ming-Jen Hsu‡, Chi-Hong Chao‡, Shao-Hung Wang***, Li-Ru You‡ ‡‡, and Yan-Hwa Wu Lee‡§§

From the ‡Institute of Biochemistry and **Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan 112, Republic of China

We have demonstrated previously that the core protein of hepatitis C virus (HCV) exhibits suppression activity on gene expression and replication of hepatitis B virus (HBV). Here we further elucidated the suppression mechanism of HCV core protein. We demonstrated that HCV core protein retained the inhibitory effect on HBV gene expression and replication when expressed as part of the full length of HCV polyprotein. Based on the substitution mutational analysis, our results suggested that mutation introduced into the bipartite nuclear localization signal of the HCV core protein resulted in the cytoplasmic localization of core protein but did not affect its suppression ability on HBV gene expression. Mutational studies also indicated that almost all dibasic residue mutations within the N-terminal 101-amino acid segment of the HCV core protein (except Arg39–Arg40) impaired the suppression activity on HBV replication but not HBV gene expression. The integrity of Arg residues at positions 101, 113, 114, and 115 was found to be essential for both suppressive effects, whereas the Arg residue at position 104 was important only in the suppression of HBV gene expression. Moreover, our results indicated that the suppression on HBV gene expression was mediated through the direct interaction of HCV core protein with the trans-activator HBx protein, whereas the suppression of HBV replication involved the complex formation between HBV polymerase (pol) and the HCV core protein, resulting in the structural incompetence for the HBV pol to bind the package signal and consequently abolished the formation of the HBV virion. Altogether, this study suggests that these two suppression effects on HBV elicited by the HCV core protein likely depend on different structural context but not on nuclear localization of the core protein, and the two effects can be decoupled as revealed by its differential targets (HBx or HBV pol) on these two processes of the HBV life cycle.

Hepatitis C virus (HCV) is a major causative agent of non-A, non-B hepatitis and is involved in the development of both chronic liver disease and hepatocellular carcinoma (1–2). The viral genome consists of a positive-stranded RNA of about 9.6 kb that encodes a large polyprotein of 3008–3037 amino acids (reviewed in Ref. 3). This polyprotein undergoes proteolytic processing by cellular signalases and viral proteases to yield at least 10 mature viral proteins classified as structural or non-structural (NS) proteins (3). The core protein, which is located at the N terminus of the polyprotein, is a component of viral capsid. It is phosphorylated (4), has both nuclear and cytoplasmic localization (reviewed in Ref. 5), and possesses several distinct functions. For example, it acts as a regulatory protein that positively or negatively modulates the cellular or viral promoters (5), although the molecular mechanism of this transcription is still not fully understood. Additionally, it interacts with a wide spectrum of cellular factors such as apolipoprotein AII (6), lymphotixin-β receptor (7–9), tumor necrosis factor-α type 1 receptor (10), heterogeneous nuclear ribonucleoprotein K (11), p53 (12), RNA helicase (13, 14), LZIP (15), 14-3-3 (16), and p21/WAF1 (17); and in most cases the core protein also affects the biological functions of its targeted proteins. Moreover, the core protein is capable of transforming primary rat embryo fibroblasts in cooperation with Ras (18) and causes hepatocellular carcinoma in certain strains of transgenic mice (19).

Hepatitis B virus (HBV), a member of the hepadnavirus family, is a DNA virus with partially double-stranded DNA genome held in a circular conformation by overlapping 5′-ends of the DNA strands (20). It is also associated with the development of liver cirrhosis and hepatocellular carcinoma (20). HBV encodes 4 overlapping reading frames that code for surface proteins (HBSAg), core protein (HBcAg), polymerase (pol), and the X protein (HBx). Among them, HBx has received much attention because it is regarded as a multifunctional protein important for the viral life cycle and viral-host interactions (reviewed in Refs. 21–24). HBx has been implicated in HBV-mediated hepatocellular carcinoma by its ability to induce liver cancer in some transgenic mice (25); it modulates a wide range of cellular functions including transcription, signal transduc-
Suppression of HCV Protein on HBV Expression and Replication

Despite containing a DNA genome, HBV replicates via reverse transcription of a linear, terminally redundant RNA progenome that is packaged into a viral capsid (48). This progenome RNA functions additionally as mRNA for the synthesis of two viral proteins, core protein and pol, which in turn interact with the package signal (termed the 5′-end of the pre-genomic RNA to initiate the RNA encapsidation process (49, 50). Encapsulation of the RNA template is under stringent control, because only the pre-genomic RNA is selectively encapsidated. In HBV, the ε package signal of the pre-genomic RNA is characterized by the presence of a stem-loop structure that is believed to serve as the docking site for the binding of the pol and is essential for both packaging and DNA priming (49–54). Synthesis of the two viral DNA strands occurs within the nucleocapsid, and it is sequential in the way that minus strand DNA synthesis occurs first by using the pol protein itself as a primer, and followed by the plus strand DNA synthesis via the concerted actions of the reverse transcriptase and RNase H activities of pol protein (54–58). Recently, it was found that the interaction of molecular chaperones (Hsp90 and Hsp60) with HBV pol is critical for the maintenance of the enzyme in a conformation competent for its functions (59–61).

The prevalence of HCV infection in patients with HBV infection has been examined in several studies (62–65). Interestingly, both clinical and animal studies have shown that HCV might exert a viral interference effect that suppresses or terminates the HBV carrier state (66–70). Still other findings suggest a reciprocal inhibition between these two viruses in patients coinfected with HBV and HCV (71–73). Along this line, our previous data indicated that the HCV core protein had the trans-suppression activity on HBV gene expression and replication (74). This trans-suppression ability of HCV core protein was positively regulated by protein kinase A and C through modulation of the phosphorylation level of its Ser\(^{70}\) and Ser\(^{116}\) residues (4). Furthermore, the suppression of HBV encapsidation by the HCV core protein was more severe when compared with that in the HBV gene expression (4, 74). However, the exact molecular mechanism for these suppression effects remains to be determined. Because HCV core protein is a multifunctional protein with several functional motifs, including basic charged residues and nuclear localization signals (74–76), and because our initial attempt to locate the suppressive domain of HCV core protein suggests the importance of the C-terminal 22-amino acid segment encompassing residues 101–122 (74), in this study we introduced mutations into the basic residues within NLSs or 22-residue suppressive domains. The properties of HCV core mutant variants were examined in order to understand whether the same domain is involved in both suppressive activities of the HCV core protein. The involvement of HBx in this suppression effect by the HCV core protein was also explored. Additionally, the in vitro commu

noprecipitation method and streptavidin-agarose affinity chromatography were adapted for the study of the mutual interaction among the HCV core protein and HBV encapsidation components, HBV pol and package signal, in an attempt to elucidate the molecular mechanism of the interference of the HBV encapsidation by the HCV core protein. Our results shown here strongly suggest that HCV core protein inhibits the HBV gene expression and viral replication through interacting with the latter two important regulators in HBV life cycle, HBx, and HBV pol proteins. Additionally, our results clearly indicate that the suppressive domain of core protein on HBV gene expression and viral replication is distinct with the former which is located mainly on the C-terminal 22 residues, whereas the latter spans almost the entire region of the HCV core protein, and several Arg residues (Arg\(^{101}\), Arg\(^{112}\), Arg\(^{114}\), and Arg\(^{115}\)) are found to be essential for both suppressive activities. Moreover, in this study we also demonstrate that this suppression of HBV gene expression and replication occurs when HCV core protein is expressed as part of the HCV polyprotein.

EXPERIMENTAL PROCEDURES

Plasmids—HCV core protein expression construct pCEC/HCVc-KF was described previously (74). In this construct the structural protein of HCV contains the whole coding region (191 amino acids) of core protein. Plasmid pHVCc-SE, a derivative of pGEM-3Zf(+) harboring the HCV core and partial E1 region, was constructed by inserting the 1.4-kb StuI-EcoRI fragment of pCEC/HCVS-EK (74) into the HindIII (Klenow-filled) and EcoRI-digested pGEM-3Zf(+) vector. When this construct is linearized with appropriate restriction endonuclease and transcribed in vitro with SP6 RNA polymerase, the transcripts containing various sizes of HCV core gene can be produced. Plasmid pET23a/HCVc is a derivative of pET-23a harboring the 0.6-kb full-length HCV core coding region (Accl-FspI) (7). When linearized with SsaI or Clal and transcribed in vitro with T7 RNA polymerase, the resulting transcripts encode the T7-tagged 101 or 122 amino acid residue of HCV core protein (T7-C101 or T7-122) with additional 24 amino acids (including 11 amino acids of T7 tag) at the N-terminal segment of the HCV core protein. Plasmids pGST/HCVc24, pGST/HVCc101, pGST/HVCc122, and pGST/HVCc195 are derivatives of pGEX-3K that direct the synthesis of different lengths of HCV core protein fused with the C termini of GST (4, 7). Plasmids pSR\(^{116}\)/HCVc101, pSR\(^{116}\)/HCVc122, and pSR\(^{116}\)/HCVc195, the mammalian expression constructs for different lengths of HCV core protein, were described previously (14). Plasmid pSR\(^{116}\)/HCVcFL is the mammalian expression construct for the full length of HCV polypeptide. This plasmid was constructed by insertion of the 1.1-kb HindIII (filled in)/KpnI fragment consisting of the pol promoter region (HindIII-Pol fragment, 9.8 kb) and the 0.6-kb full-length HCV core coding region into the XbaI (filled in)/KpnI-digested p90/HCVFLongU (kindly provided by Apath). Plasmid pSHH2.1 contains a tandem dimer of the HCV genome inserted at the EcoRI site of the vector plasmid pSV08 (77). Plasmid pHBV-PS, a derivative of pGEM-3Zf(+) was constructed by excising the 264-bp BglII (filling-in the overhangs with Klenow enzyme)-HindIII fragment of HBV package signal sequence from plasmid pHBV3.5 (78) and then inserting it into the SmaI/HindIII-digested pGEM-3Zf(+) vector. When linearized with EcoRI and transcribed in vitro with SP6 RNA polymerase, pHBV-PS produces a 264-nucleotide transcript containing the HBV package signal, spanning HBV nucleotides 1722–1986 (HBV adw subtype, with nucleotide positions numbered from the unique EcoRI site of HBV). Plasmid pHBV97Po, a derivative of pBlueScript II KS(+)/+ harboring the 2.89-kb full-length HBV polymerase gene fragment, was provided by C. M. Chang (National Yang-Ming University, Taiwan). In this construct, the polymerase gene fragment (AluI-Sacl fragment) was derived from pMH3/3907 (79). When this construct is linearized with appropriate restriction endonuclease and transcribed in vitro with T7 RNA polymerase, transcripts coding for various length of HBV polymerase can be produced. Plasmid pHEX-X1, the mammalian expression construct for HBx under the HBx promoter control, was provided by S. J. Lo (National Yang-Ming University, Taiwan). Plasmid pGST-HBx, which can direct the expression of the full-length HBx protein fused with the C terminus of GST, was constructed by inserting the 2.60-kb PCR-generated EcoRI/HindIII fragment of the HBx gene derived from plasmid pMH3/3907, into the EcoRI/HindIII-digested pGEX-5X-1 (Amersham Biosciences). To construct plasmid pCMV-HA-HBx for generation of HA-tagged HBx, the same 462-bp EcoRI-XhoI fragment of HBx gene was
subcloned into the EcoRI/XhoI-digested pCDNA-3-HA vector (Invitrogen). Plasmid pFLAG-HBx, the mammalian expression construct for FLAG-tagged HBx, was constructed by insertion of the 462-bp PCR-generated HindIII-EcoRI fragment of the HBX gene from plasmid pMH3/3097 into the HindIII-EcoRI-digested pFLAG-CMV-2 (Eastman Kodak). Plasmid pGFP-HBVpol, which can express green fluorescent protein (GFP)-tagged HBV polymerase, was cloned by insertion of the 3-kb EcoRV-SmaI pol gene fragment from the pMH-3-BCR-EB (provided by S. J. Lo, National Yang-Ming University, Taiwan) into the EcoRI (filled in)-digested pEGFP-C3 vector (Clontech). The plasmid pFLAG-HBVpol was generated by inserting the 3-kb HindIII-SmaI DNA fragment of plasmid pGFP-HBVpol into HindIII/SmaI sites of pFLAG-CMV-2.

**HBX-EcoRI Mutagenesis of the HCV Core Protein and HBx Protein**—The “Altered Sites” system (Promega) was used for in vitro mutagenesis of the HCV core or HBX gene as described by the supplier. The construct pSELECT/HCV, a derivative of pSELECT-1 containing the full length of HCV core gene, was used for site-directed mutagenesis (4). The HCV core mutants bearing lysine and/or arginine residue substitution mutations generated in this study are listed in Fig. 1. The oligonucleotides used for mutation were synthesized and indicated by the position of the first mutated amino acid residue in the HCV core protein (see below). The mutant primers used are as follows: M9, 5'-GTCATTTTTGCTGCTGGTTAGAT-3'; M12, 5'-CAGGCTTGGTACGCTGCGTTTCCGTTGCTGGTT-3'; M17, 5'-TAACCTCTGGGCGCGCCGGCGCCGGCG-3'; M50, 5'-CCTCGGCAAGTCTGCGGCTGCGGAC-3'; M60, 5'-CGGAAATTTTGGCGCGGCGGCGGCGG-3'; M101L, 5'-CGAGACGGAGGTTAGC-3'; M105K, 5'-GCCGGGAGACGGACGGG-3'; M104L, 5'-GCCGGGAGACGGACGGG-3'; M101L, 5'-GCCGGGAGACGGACGGG-3'; M113L, 5'-GACCTACGCCGGTTTCAG-3'; M114L, 5'-CGCGCTTACGCCGGGCTC-3'; M114K, 5'-ATTACCGAGACCGGCGCGG-3'; M115A, 5'-GACCTACGCCGGTTTCAG-3'; M152K, 5'-TATACGAGACCGGCGCGG-3'; M152L, 5'-ACCGCGGGAGGCTACGCG-3'; M152G, 5'-ACCGCGGGAGGCTACGCG-3'; and M152D, 5'-ACCGCGGGAGGCTACGCG-3'. The underlined boldface bases are mutated bases. All mutant sequences were confirmed by sequencing. The 0.7-kb HindIII-EcoRI fragment of mutant DNA in pSELECT/HCV derivatives were then subcloned into HindIII-EcoRI-digested pCE plasmid and the resulting mutant construct was used for transfection.

**RNA Preparation and Northern Blotting**—Cellular RNA was extracted by using TRI reagent (Molecular Research Center) according to the instructions of the supplier (Molecular Research Center). The RNA samples were electrophoresed in a 1% formaldehyde-agarose gel and then transferred to nitrocellulose paper. Prehybridization and hybridization were performed as described previously (81). The HBV DNA probe was prepared from the 3.2-kb HBV fragment (EcoRI fragment) of pSSH2.1 by the nick translation method (82).

**In Vitro Transcription**—RNAs for in vitro translation or binding were produced by an in vitro transcription kit as suggested by the manufacturer (Promega). Recombinant phCVc-SE, which was intended for generation of full-length (C195, p22) and the truncated (C101, p11) HCV core mRNA, was linearized at FspI or SacII prior to in vitro transcription. To produce the transcripts encoding the full-length (pol) or the truncated forms (pol749 and pol587) of HBV polymerase, recombinant HBV/V70P was linearized with Smal1, Ncol, or Accl, prior to in vitro transcription with T3 RNA polymerase (see Fig. 10). To produce the vector DNA for packaging signals, EcoRI-linearized plasmid pSV08 was used. The underlined bases were subjected to in vitro transcription as described above, except that 1 μCi biotin-16-UTP (Roche Molecular Biochemicals) (final UTP concentration, 2.5 mM) was added to the transcription reaction mixture containing SP6 RNA polymerase.

**In Vitro Translation**—35S-Labeled HCV core protein and HBV polymerase or their derivatives were made in rabbit reticulocyte lysates by translation of their corresponding mRNAs, according to the manufacturer (Promega). In brief, synthesized RNAs (8–12 μg each in 4–6 μl) were incubated in a total reaction volume of 50 μl containing 35 μl of nuclease-treated rabbit reticulocyte lysate (Promega), 1 μl of RNAin (40 unit/μl), 1 μl of 1 mM amino acid mixture (minus methionine), and 3.5 μl 4°C translation mix (Promega). After 90 min incubation at 30°C, the in vitro translated HCV core protein (e.g. C101) species was also prepared in the reticulocyte lysate containing 35S-labeled amino acid as described previously (74). For in vitro synthesis of [35S]Met-labeled TT-Tagged HCV core variants (TT-C195, TT-122, and TT-C101), pET2a/HCV or appropriate restriction endonuclease (SacII and ClaI-linearized pET2a/HCV) was used as a template for the Tnt system (Promega). (35S)Met-labeled HA-tagged HBx was also produced by the TntK system (pCMV-HA-HBx) as a template. In vitro translated products were then processed for protein or RNA binding analysis.

**Expression and Purification of GST Fusion Proteins and in Vitro GST Pull Down Assay**—HCV core protein or HBx protein was expressed individually as GST fusion proteins from the expression vector pGST/HCV/c24, pGST/HCV/c101, pGST/HCV/c122, pGST/HCV/c195, or pGST/HBx. Expression and purification of the GST fusion proteins were performed as described (4). For each in vitro binding assay, 15 μl of glutathione-Sepharose 4B beads (Amersham Biociences) bound to the appropriate GST fusion protein (4 μg) was incubated with in vitro translated 35S-labeled HBV polymerase or HCV core protein with gentle rotation. The binding and washing conditions were described by Huang et al. (83). Proteins bound on the beads were eluted by sampling buffer (84), fractionated by SDS-PAGE, and detected by autoradiography.

**In Vitro Immunoprecipitation**—In vitro translated products of HCV core protein and HBV polymerase were incubated at 4°C with either anti-HBV pol antibody (supplied by C. M. Chang) or HCV patient sera (positive for anti-HCV core protein) (74) which were pre-bound with protein A-Sepharose (20 μl packed volume) and suspended in 350 μl of NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40), and the suspension was rocked overnight. The immunoprecipitates were recovered by centrifugation and washed four times with NETN buffer, boiled in sample buffer, and analyzed by SDS-PAGE (84). The gel was dried and processed for autoradiography, and if necessary the band intensity was quantified by Phosphorimaging (Amersham Biociences).

**RNA immunoprecipitation**—HeLa or A5a cells (density of 2 × 10^6 per 10-cm dish) were cotransfected with FLAG-tagged HBx (pFLAG-HBx) or HBV polymerase (pFLAG-HBVpol) expression plasmid together with HCV core protein expression constructs (pSRa/HCV/c195, pSRa/HCV/c122, or pSRa/HCV/c101). After 24–48 h, cells were washed twice with ice-cold phosphate-buffered saline and collected by centrifugation. Western blotting with transfected cells by lysis in PBS containing 0.5% Nonidet P-40 and 1% protease inhibitor mixture (Complete, Roche Molecular Biochemicals) for 30 min on ice. The extracts were cleared by centrifugation at 10,000 × g for 20 min. Supernatants were incubated for overnight at 4°C with anti-FLAG M2 antibody-conjugated agarose beads (Sigma) in 50 μl of NETN buffer (pH 8.0), 100 mM NaCl, 50 mM Tris- HCl (pH 7.5), 20% glycerol) or binding buffer containing RNAse A (10 μg/ml). Beads were washed four times with binding buffer, and bound proteins were separated by SDS-PAGE and analyzed by Western blotting.

**Streptavidin Precipitation of Protein-RNA Complexes**—To analyze
NLSs in the HCV core protein are shown. (121), and only the relevant amino acid sequences are displayed. The HCV core protein used for the analysis was adapted from Takeuchi, or aspartate residue (121), and only the relevant amino acid sequences are displayed. The amino acid sequence of the designed for site-specific replacement of basic residues at NLS regions (75), whereas by deletion analysis the second basic amino acid residues (101, 104, 113, 114, 115, and 117), site-directed mutagenesis of individual or consecutive arginine/lysine residue or various combinations thereof were performed in order to assess their role in the suppression activity of the core protein. In the first set of 11 NLS mutants, consecutive basic residues appearing at NLS regions (residues 9/10, 12/13, 17/18, 39/40, 50/51, 61/62, and 69/70) were replaced by alanine residue (see Fig. 1A; designated as M9, M12, M17, M39, M50, M61, M69, M12/39, M17/39, M50/69, and M12/39/61, respectively). In the second set of core mutants, the six arginine residues located at residues 101–122 as described above were mutated to lysine or neutral ones (leucine in most cases or alanine). Additionally, aspartate residue was also used to replace arginine residue at 117. All together, 13 mutants in the second set of core variants were obtained (see Fig. 1B, designated as M101L, M101K, M104L, M104K, M113L, M113K, M114L, M114K, M115A, M115K, M117L, M117K, M117D, respectively).

Most Arg Residues within the 22-Residue Suppressive Domain but Not Dibasic Residues in NLS Regions of HCV Core Protein Are Important for Suppression on HBV Gene Expression—The 24 variants of the HCV core protein were analyzed for their suppressive activity on the HBV gene expression. As shown in Table I, when these substitution variants of HCV core protein expression construct (as pECE derivatives) were individually cotransfected with HBV plasmid pSHH2.1, the suppression of HBV antigen HBsAg and HBe/HBcAg production in the human hepatoma cell line HuH-7 in all the mutant constructs examined was comparable with that of the wild-type (2–3-fold suppression), except for substitution mutants M101L, M104L, M104K, M113L, M113K, M114L, M114K, M115L, and M115K. Northern blot analysis of HBV-specific transcripts (3.5 and 2.1 kb) gave similar results (Fig. 2). Therefore, these results indicate that Arg101, Arg104, Arg113, Arg114, and Arg117, but not Arg117 and dibasic residues in NLS regions, are crucial for the inhibitory effect of core protein on the HBV gene expression. Notably, mutant M101K still retained the wild-type suppressive activity, whereas this was not the case for M101L mutant, suggesting that the basic residue is required for this particular Arg residue. It should be pointed out that Arg101 is within the protein kinase C recognition motif for Ser259, and phosphorylation of this serine residue has been demonstrated to be essential for the suppressive effect of the HCV core protein (4). Thus, our present results obtained from mutants M101K and M101L are consistent with our previous study (4), as the loss or retention of the suppressive activity of the HCV core protein in these two particular mutants correlates with the functional status of the PKC site at Ser259.

HBV Viral Replication Is Affected Diversely by HCV Core Variants—When these variants of HCV core expression construct were individually cotransfected with HBV plasmid pSHH2.1, the suppression of HBV viral particle production in HuH-7, as determined from the endogenous DNA polymerase assay (see “Experimental Procedures”), was either lost completely or affected to various degrees (wild-type about 14–30-fold inhibition, mutants about 2–8-fold inhibition or 1.7–2.9-fold enhancement) in all the constructs examined, except for

![Fig. 1. Mutants of HCV core protein used in this study.](image-url)
Suppression of HCV Protein on HBV Expression and Replication

The possibility of the complex formation between HCV core protein and HBx was examined further. As shown in Fig. 6, confocal microscopy analysis using indirect immunofluorescence staining indicated that the full-length and truncated forms of HCV core protein (C195, C122, and C101) colocalized with the FLAG-tagged HBx in both nuclear and cytoplasmic compartments. Furthermore, by using the GST fusion proteins of HCV core variants harboring the full-length or N-terminal 122 or 101 amino acid residues of core protein (GST/HCVC101, GST/HCVC122, and GST/HCVC195) for pull-down analysis, results indicated that in vitro translated HA-tagged HBx could be precipitated by these three GST-core variants (Fig. 7A). A reciprocal experiment using GST/HBx for pull-down of the in vitro translated T7-tagged HCV core proteins (T7-C101, T7-C122, or T7-C195) gave a similar conclusion (Fig. 7, B and C).

Table I
Expression of HBsAg and HBeAg/BcAHg in HuH-7 cells after cotransfection with cloned HBV DNA and various HCV core mutant constructs

| Exp. no. a | HCV core plasmid | S/N ratio ± S.D. b |
|-----------|------------------|--------------------|
|           | HBsAg            | HBeAg              |
| 1         | Mock             | <1.5 ± 1.5         |
|           | Control          | 432.1 ± 11.9       |
|           | WT               | 174.4 ± 36.4       |
|           | M9               | 202.2 ± 10.0       |
|           | M12              | 255.3 ± 8.5        |
|           | M17              | 115.2 ± 13.8       |
|           | M39              | 114.8 ± 13.5       |
|           | M50              | 109.4 ± 21.0       |
|           | M61              | 190.4 ± 20.5       |
|           | M50/69           | 142.2 ± 42.7       |
|           | M12/39/61        | 110.6 ± 21.1       |
| 2         | Mock             | <1.5 ± 1.5         |
|           | Control          | 253.2 ± 35.7       |
|           | WT               | 122.2 ± 3.8        |
|           | M101L            | 520.2 ± 6.3        |
|           | M101K            | 137.7 ± 2.0        |
|           | M104L            | 122.2 ± 2.0        |
|           | M104K            | 128.0 ± 2.8        |
| 3         | Mock             | <1.5 ± 1.5         |
|           | Control          | 351.7 ± 16.3       |
|           | WT               | 122.2 ± 3.8        |
|           | M113L            | 122.2 ± 3.8        |
|           | M113K            | 122.2 ± 3.8        |
|           | M114L            | 122.2 ± 3.8        |
|           | M114K            | 122.2 ± 3.8        |
|           | M115A            | 119.0 ± 3.4        |
|           | M115K            | 123.6 ± 5.0        |
|           | M117L            | 63.0 ± 13.0        |
|           | M117K            | 58.4 ± 6.6         |
|           | M117D            | 69.0 ± 8.0         |

a HuH-7 cells are cotransfected with cloned HBV expression plasmid pSHH2.1 and HCV core construct pECE/HCV-X-FK (WT) or various HCV core mutant constructs as indicated (20 μg each, see Fig. 1 for mutant designation). Mock, without transfection; Control, cells transfected with pSHH2.1 and pECE vector.

b Data are means ± S.D. of two independent experiments. All values are given as S/N ratio (sample versus negative control).

The Nuclear Localization of HCV Core Protein Is Not Required for Its Suppression Activity—Because mutation in the NLS regions affected the suppressive effect of the core proteins on HBV replication to various degrees but retained a comparable wild-type level of suppression on HBV gene expression (Figs. 2 and 3 and Table I), it is pertinent to know whether these mutational effects resulted from the influence on the subcellular localization of HCV core protein. As shown in Fig. 4, all NLS core mutants displayed nuclear localization except for the M50/69 mutant, in which cytoplasmic localization of core protein was observed. This strongly implies that the bipartite NLS located at residues 50–70, but not other NLSs, serves as a functional NLS in the full-length HCV core protein. Because M50/69 mutant retained its suppression activity on HBV gene expression and also conferred considerable degrees of suppression (about 5-fold) on HBV particle release (Figs. 2 and 3, Table I), it seems that nuclear localization of core protein is not required for its suppression activity on HBV gene expression and replication.

HBx Mediates the Suppression of HBV Gene Expression but Not Replication by the HCV Core Protein—Next, we elucidated the suppression mechanism of core protein on HBV gene expression. Because HBx is the trans-activator for HBV transcription (29), it is likely that the suppression of HBV gene expression by the HCV core protein is mediated through HBx. To assess this possibility, we analyzed the effect of HCV core protein on the HBV gene expression from HBx null plasmid pHBV(X-). As shown in Fig. 5A (lanes 1–5) and Table II, in contrast to the case of pSHH2.1 harboring the wild-type HBV genome, HCV core protein did not exhibit any inhibition on the production of HBsAg, HBeAg, or HBV transcript derived from this HBx null plasmid pHBV(X-). Moreover, when HBx expression construct (pHEX-X1) was cotransfected with this pHBV(X-) plasmid, the suppression of HCV core protein on HBV gene expression was recovered (Fig. 5A, lanes 6 and 7; Table II). However, the suppression of HBV particle production was retained in this pHBV(X-) and HCV core plasmid cotransfected cells (Fig. 5B), suggesting that the mechanism for suppression of HBV gene expression and replication by the HCV core protein is decoupled.

It is a matter of functional missense site of PKA at Ser216, still led to a loss of the inhibitory activity of the core protein. A considerable enhancement (about 2.9-fold) of HBV particle production was also noted in NLS mutant M50 and to a less extent (1.7–1.8-fold) in mutants M9, M17, and M61 (Fig. 3), although the molecular mechanism for this effect is still unclear.

Based on these results, it appears that introducing substitution mutations into the basic residues of NLS regions or the 22-residue suppressive domains impart various effects to the suppressive activity of the core protein on HBV viral replication.

The possibility of the complex formation between HCV core protein and HBx was examined further. As shown in Fig. 6, confocal microscopy analysis using indirect immunofluorescence staining indicated that the full-length and truncated forms of HCV core protein (C195, C122, and C101) colocalized with the FLAG-tagged HBx in both nuclear and cytoplasmic compartments. Furthermore, by using the GST fusion proteins of HCV core variants harboring the full-length or N-terminal 122 or 101 amino acid residues of core protein (GST/HCVC101, GST/HCVC122, and GST/HCVC195) for pull-down analysis, results indicated that in vitro translated HA-tagged HBx could be precipitated by these three GST-core variants (Fig. 7A). A reciprocal experiment using GST/HBx for pull-down of the in vitro translated T7-tagged HCV core proteins (T7-C101, T7-C122, or T7-C195) gave a similar conclusion (Fig. 7, B and C).
Fig. 2. Northern blot analysis of HBV transcription in HuH-7 cells cotransfected with HBV plasmid and various HCV core mutants. HuH-7 cells were cotransfected (20 μg each) with HBV plasmid pSHH2.1 and HCV core expression construct pECE/HCVC-KF (WT) and its various mutant derivatives that carried the mutations as indicated. Total cellular RNAs were prepared from cells at day 6 post-transfection and probed with 32P-labeled HBV DNA as described under "Experimental Procedures." The same blot was rehybridized with 32P-labeled 18S or glyceraldehyde-3-phosphate dehydrogenase gene fragment (G3PDH). The positions of 3.5- and 2.1-kb HBV-specific transcripts are indicated by arrows. Mock, without transfection; control, cells transfected with pSHH2.1 and vector pECE. The designations for HCV core mutants are shown in Fig. 1.

Moreover, in vitro coimmunoprecipitation experiment using anti-FLAG antibody for immunoprecipitation also confirmed that FLAG-HBx formed a complex with these three forms of HCV core protein in transfected HeLa cells, albeit the two truncated forms of core protein exhibited only weak interaction with FLAG-HBx compared with that of the full-length core protein (Fig. 7D).

All together, our results indicate that HCV core protein associates with HBx, and this interaction regulates the suppression of HBV gene expression by the core protein of HCV. Additionally, because the suppression-impaired mutant C101 still retained its ability to interact with HBx, this implies that the binding of HBx is necessary but not sufficient for the suppression effect of HCV core protein on HBV gene expression.

HCV Core Protein Forms a Complex with HBV Polymerase—The suppression of HBV encapsidation process by the HCV core protein may be because of the direct interaction of this protein with the HBV pol. To test this possibility, a coimmunoprecipitation experiment was performed. Fig. 8A shows the in vitro translated, 32P-labeled full-length of HCV core protein (C195, p22) and HBV pol protein (92 kDa) used in this study. In addition to the p22 species, minor protein species with a size of 18 (p18) or 44 kDa (p44) was detected in the translation products of HCV core protein, which presumably represent the degradation (p18) or dimer form (p44) of p22 as noted previously (4). When in vitro translated HCV core protein and HBV pol protein were incubated together and immunoprecipitated with HCV patient sera (positive for anti-HCV core protein, see Fig. 8B), HBV pol was coprecipitated with HCV core protein (Fig. 8B, lane 2). This coimmunoprecipitation of pol protein depends on the presence of HCV core protein (Fig. 8B, compare lanes 1 and 2). In vitro binding analysis using purified GST/HCV core fusion proteins harboring various core domains for pull-down affinity assay of in vitro translated HBV pol protein suggested that N-terminal 101- or 122-amino acid fragment of core protein but not its 24-amino acid fragment could interact with HBV pol protein (Fig. 8C). An in vivo coimmunoprecipitation experiment using anti-FLAG antibody for immunoprecipitation revealed that both the full-length and the truncated HCV core variants (C195, C122, and C101) could form a complex with the FLAG-tagged HBV pol protein in transfected HeLa cells (Fig. 8D). Notably, this complex formation between HCV core and HBV pol proteins was not mediated by RNA, because RNase treatment of coimmunoprecipitates did not disrupt their interaction (Fig. 8D). Furthermore, indirect immunofluorescence microscopy analysis revealed that all three forms of HCV core protein colocalized with the GFP-tagged HBV pol in the cytoplasmic compartment when these expression plasmids were cotransfected into HeLa cells (Fig. 9). Taken together, these results suggest that both the full-length and the HBV core variant of HCV core protein can associate with the HBV pol in vitro and in vivo. Detection of the complex formation between the full-length HCV core protein and the HBV core-pol fusion protein was also noted previously (87).

Mapping the Interaction Domains of HCV Core Protein and HBV Polymerase—To delineate the interaction domain of HCV
core protein on HBV pol protein, a similar coimmunoprecipitation experiment was performed on the C-terminal truncated forms of HBV pol generated from in vitro translation of the 3’/H11032-end truncated transcripts of HBV pol gene (see “Experimental Procedures”). The truncated HBV pol used in this study harbored the N-terminal 749 and 567 amino acid residues, respectively (Fig. 10, A and B). These two truncated HBV pol were designated as pol749 or pol567 with sizes around 76 or 60 kDa, respectively (Fig. 10B). We found that the full-length HCV core protein could associate with the pol749 but not with the smaller truncated form pol567 (Fig. 10C, compare lane 4 with lane 6). Interestingly, the truncated pol749 had much stronger binding affinity to HCV core protein as compared with that of the full-length HBV pol (Fig. 10C, compare lane 2 with lane 4). These results demonstrate that HCV core protein binds to the C-terminal central region of HBV pol through amino acids 567–749, which resides in the junction region between reverse transcriptase and RNase H domains of pol protein (88). This interacting region has been shown to be crucial for RNA encapsidation and reverse transcription activities of HBV pol protein (53, 88, 89).

**HCV Core Protein Inhibits Complex Formation between HBV Polymerase and ε Sequence**—The HCV core protein has RNA binding ability (90). To determine whether the strong suppression of HBV encapsidation process by the HCV core protein may be the result of the binding of HCV core protein to HBV package signal (ε), we studied the binding affinity of the HCV core protein to the ε sequence using the streptavidin-biotin-mediated binding assay (53; also see “Experimental Procedures”). When in vitro translated HCV core protein was incubated with the biotin-labeled HBV ε sequence, the presumed RNA-core protein complex could be bound to the streptavidin-agarose (Fig. 11A). The binding of the HCV core protein to the ε sequence was found to be enhanced in a concentration-de-
ependent manner (Fig. 11A, lanes 3–6). Moreover, this binding of HCV core protein on the streptavidin-agarose was HBV ε sequence-specific because in the absence of biotinylated ε RNA core protein was not retained by the streptavidin-agarose (Fig. 11A, lane 1), and the binding signal of HCV core protein could be ablated by an excess of unlabeled ε sequence but not by the unrelated *Escherichia coli* cct RNA (354 nucleotides) of ColE7 operon (91) (Fig. 11B, lanes 2–4). Notably, both the full-length (p22) and truncated forms (p18) of HCV core protein could bind the ε sequence (Fig. 11A, lanes 5 and 6). Because p18 was present in a lesser amount in the in vitro translated products (Fig. 8A), the display of similar intensity of both forms of the HCV core protein in the precipitate implies that the affinity of p18 species to the ε sequence is much stronger as compared with that of the full-length p22 species.

Because HCV core protein has binding affinity for both HBV pol and ε sequence, it may compete for the complex formation between HBV pol and ε sequence, resulting in the suppression

| Exp. no. | Plasmid | HBsAg | HBeAg |
|----------|---------|-------|-------|
| 1+       | Mock    | <1.5  | <1.5  |
|          | pSHH2.1 + pECE | 395.7 ± 6.8   | 117.1 ± 1.9 |
|          | pSHH2.1 + pECE/HCVC-KF | 119.9 ± 19.8 | 48.8 ± 1.5 |
|          | pHBV(X)+ pECE | 338.2 ± 1.6   | 114.9 ± 1.0 |
|          | pHBV(X)+ pECE/HCVC-KF | 415.1 ± 3.7   | 134.8 ± 0.4 |
| 2+       | Mock    | <1.5  | <1.5  |
|          | pHBV(X)+ pECE + pHEX-X1 | 503.8 ± 7.6   | 157.8 ± 1.9 |
|          | pHBV(X)+ pECE/HCVC-KF + pHEX-X1 | 170.5 ± 7.6  | 62.3 ± 1.8 |

* Data are means ± S.D. of two independent experiments. All values are given as S/N ratio (sample versus negative control).

* HuH-7 cells are transfected with pSHH2.1 or its mutant derivative pHBV(X) together with vector pECE or HCV core construct pECE/HCVC-KF (20 µg each). Mock, without transfection.

* HuH-7 cells are transfected with pHBV(X) and HBx expression construct pHEX-X1 together with vector pECE or HCV core construct pECE/HCVC-KF (14 µg each).
of the virus encapsidation process. To assess this possibility, we studied the competition of HCV core protein with HBV pol for binding to Ε sequence. As shown in Fig. 11C, in vitro translated HBV pol bound to Ε sequence with sequence specificity because the binding signal of HBV pol in the precipitates of streptavidin-agarose could be blocked by an excess of the unlabeled Ε sequence but not by the unrelated E. coli cei RNA (Fig. 11C, lanes 2–4). A pilot analysis for the determination of the saturation amount of Ε sequence that could bind to a fixed amount of HBV pol was conducted prior to the competition experiment. As shown in Fig. 11D, a saturation level was achieved when 60 pmol of biotin-labeled Ε sequence was present in the binding reaction mixture containing 0.2 pmol of in vitro translated HBV pol (Fig. 11D, lane 6). It is noted that this similar amount of biotinylated Ε sequence could bind about 1.5 pmol of in vitro translated, full-length of HCV core protein (see Fig. 11A, lane 5).

To examine whether the presence of increasing amounts of HCV core protein might impair the binding of HBV pol to its package signal, various amounts of HCV core protein (0.4–2.0 pmol) were incubated with a fixed amount of HBV pol (0.2 pmol) and biotin-labeled RNA (60 pmol). The remaining HBV pol bound to the biotin-labeled RNA was then detected by the streptavidin-agarose binding assay (Fig. 11E). As predicted, addition of the full-length HCV core protein to the reaction mixture containing HBV pol and Ε RNA reduced the pol bound to Ε in a concentration-dependent manner (Fig. 11E, lanes 2–4). It was found that at the highest concentration examined (molar ratio of HCV core/HBV pol about 10-folds), HCV core protein reduced the pol signal more than 50% (Fig. 11E, lane 4). Only the truncated form of HCV core protein, p18, was coprecipitated by the streptavidin-agarose beads (Fig. 11E, lanes 3 and 4). The full-length HCV core protein (p22) was present as a complex with HBV pol in the supernatant recovered from the binding assay because it could be coimmunoprecipitated by the antiserum against HBV pol (Fig. 11E, lanes 1 and 2). All together, our results indicate that the full-length HCV core protein interferes with the ability of HBV pol to bind to Ε RNA.

HCV Core Variant Binds to HBV Polymerase and Ε Sequence but Cannot Disrupt the pol-ε Complex Formation—Earlier works (74) indicate that the N-terminal 101-amino acid segment (C101) of the HCV core protein do not have the suppression ability in the HBV encapsidation process. To examine whether this loss of the suppressive effect in the shorter construct of HCV core protein correlates with the loss of binding ability to HBV pol protein or Ε sequence, a similar coimmunoprecipitation or RNA binding assay was performed on this truncated C101 species. To circumvent the low level of [35S]methionine labeling in C101 species (only one Met at the first initiation codon), 3H-labeled C101 or the 35S-labeled T7-C101...
core fusion protein (see “Experimental Procedures”) was used in these experiments. As shown in Fig. 12, A and B, the data suggest that this truncated form of the HCV core protein (C101 or T7-C101) retained its ability to bind with HBV pol and ε sequence. We next examined the ability of this suppression-defective core protein to compete with HBV pol for binding of ε sequence. Fig. 12C indicated that the presence of increasing amounts of the C101 species (molar ratio 7–35-fold) did not disrupt the complex formation of HBV pol–ε because the pol signal in the precipitates of streptavidin-agarose remained unchanged. This loss of the competition activity of the HCV core mutant protein did not result from its N-terminal fusion with T7 tag, because similar fusion did not affect the competition ability of the full-length of the HCV core protein (data not shown). Surprisingly, C101 species (T7-C101) was also found in the precipitates of streptavidin-agarose (lanes 3–5), and immunoprecipitates (IP) were analyzed by SDS-PAGE followed by immunoblotting (WB) with human anti-HCV core sera (top panel) or monoclonal anti-FLAG antibody (middle panel) for the detection of HCV core protein or FLAG-pol. The immunoprecipitation experiment was performed in the presence of RNase A (10 μg/ml). Also shown at the bottom of the panel is the immunoblotting of transfected cell extracts with anti-HCV core sera without immunoprecipitation.

HCV Core Protein Retains Its Inhibitory Effect on HBV Expression and Replication—Our study indicated that expression of HBV antigens and production of HBV particles were suppressed by HCV core protein, and this suppression is mediated through the interaction with HBV two regulatory proteins, HBx and HBV pol proteins. Questions arise regarding whether these interactions and effects on HBV also occur in the context of full-length HCV polyprotein. To investigate this, we examined the suppression ability of HCV polyprotein on HBV by cotransfection of HCV polyprotein expression construct pSRCa/HCV-FL (see “Experimental Procedures” for plasmid construction) with HBV plasmid pSNIH2.1. As shown in Fig. 13A, both HBV antigens, HBe/HBcAg and HBsAg, were reduced 3-fold by the HCV polyprotein. A strong inhibitory effect (about 20-fold) on HBV particle release was also observed in pSRCa/HCV-FL and pSNIH2.1 cotransfected cells (Fig. 13B). Thus, the same level of suppressive effect was observed by the HCV polyprotein expression construct pSRCa/HCV-FL and by the HCV core expression construct pECE/HVC-C-KF (see Table I and Figs. 2 and 3). Western blot analysis indicated that the expression level of HCV core protein generated from proteolytic processing of the polyprotein in pSRCa/HCV-FL transfected cells was comparable with that of core protein expressed by HCV core expression construct pECE/HVC-C-KF (Fig. 13C). These findings imply that the inhibitory effect is due predominantly to the core protein, and the contribution of the other HCV viral proteins, if any, is probably minor. Thus, the core protein expressed as part of
the full-length polyprotein also has the suppressive effects on HBV gene expression and replication.

Next, we examined whether in the presence of other HCV viral proteins the core protein can associate with HBx or HBV pol protein. In this experiment we used the HuH7-derived cell lines harboring subgenomic HCV RNA replicon (NS3 to NS5B) (Ava.5 cells) (see “Experimental Procedures”) for cotransfection. The expression of NS3 protein in Ava.5 cells was detected by immunoblot, which presumably was generated from proteolytic processing of HCV polyprotein (Fig. 14A). When FLAG-tagged HBx or HBV pol protein expression construct together with HCV core protein expression construct (pSRa/C195) were cotransfected into Ava.5 cells, in vivo coimmunoprecipitation experiments using anti-FLAG antibody for immunoprecipitation revealed that HCV core protein was coprecipitated with the FLAG-tagged HBx or HBV pol protein in Ava.5 cells (Fig. 14, B and C, lanes 4 and 5). RNase A treatment of the coimmunoprecipitates did not eliminate their interaction (compare lanes 4 and 5), suggesting that the complex formation between HCV core protein and HBx or HBV pol is not mediated by RNA. Therefore, our observations of the interaction as well as the effects on HBV elicited by HCV core protein as presented above likely reflect the context of full-length HCV polyprotein.

**DISCUSSION**

Our laboratory previously demonstrated that the HCV core protein has the trans-suppression activity on HBV gene expression and replication, and phosphorylation of Ser99 and Ser116 residues in HCV core protein is essential for this suppressive effect (4, 74). In this study, we demonstrated that this suppression of HBV gene expression and replication also occurs when HCV core protein was expressed in the context of the intact HCV polyprotein (Fig. 13). We also extended our previous work to map the trans-suppressive domain of HCV core protein responsible for the suppressive activity on HBV gene expression and replication. According to our earlier work (74), the N-terminal 122-amino acid fragment, but not the 101-amino acid fragment, of the HCV core protein retained the same suppressive effect as the full-length core protein. Thus, it is likely that the region between amino acids 101 and 122 of the HCV core polypeptide is responsible for the suppressive activity of the HCV core protein. In this study, we mutated six arginine residues (Arg101, Arg104, Arg113, Arg114, Arg115, and Arg117) within this 22-amino acid segment of the HCV core polypeptide, and we studied the mutational effects on the core protein’s suppressive effect (see Fig. 1B). Our results (Figs. 2 and 3 and Table I) indicated that mutation of Arg117 did not interfere with the
trans-suppressive activity of the HCV core protein, and the role of Arg^{101} on the trans-suppressive activity of the core protein was involved in phosphorylation of Ser^{109} by protein kinase C, which is essential for the suppressive activity of the HCV core protein. Arg^{104} mutant still blocked HBV encapsidation but did not confer any effect on HBV gene expression (Figs. 2 and 3 and Table 1). However, single substitution mutation at Arg^{113}, Arg^{114}, or Arg^{115} led to the loss of both suppressive effects (Figs. 2 and 3 and Table 1). Thus, these results support the notion that the amino acid segment between 101 and 122 residues is an important domain for the trans-suppression activity of HCV core protein on HBV transcription and viral encapsidation. Furthermore, our results strongly suggest that both suppressive activities are at least in part mediated through different amino acid residues of the HCV core protein (e.g. Arg^{105}), albeit some arginine residues are crucial for both suppressive effects (e.g. Arg^{101}, Arg^{113}, Arg^{114}, and Arg^{115}).

The finding that several arginine residues within the 22-residue suppressive domain are critical for the inhibitory activity of the HCV core protein raises the question concerning whether other basic residues located outside this segment play any role in the suppressive activity of the HCV core protein. Because the N-terminal 101 residues of HCV core polypeptide contain three independent NLSs and one bipartite NLS, in this work we also individually or jointly mutated dibasic residues located within these NLSs regions including residues at 9/10, 12/13, 17/18, 39/40, 50/51, 61/62, or 69/70 of the HCV core protein (see Fig. 1A). We showed that all these NLS mutants retained the trans-suppressive activity on HBV gene expression (Fig. 2 and Table 1). However, most of their suppressive activity on HBV virion replication was lost to a different degree (Fig. 3), indicating that the trans-suppressive domains of HCV core protein involved in inhibiting HBV gene expression and virion replication are rather different. Whereas the suppressive domain of HCV core protein on HBV gene expression may be located solely on amino acid residues 101–122, the important residues for suppression of HBV encapsidation probably span the entire region (122 amino acid residues) of the HCV core protein. In addition, consistent with our earlier work, this study also demonstrated that the core protein exhibited much stronger suppression activity on the HBV replication (15–30-fold inhibition) as compared with its inhibitory effect on the HBV gene expression (2–4-fold). Moreover, it was noted that in some mutants the multiple mutations of several dibasic residues adversely led to a partial recovery of the suppression ability of the HCV core protein on HBV replication but not on HBV gene expression. Specifically, multiple mutations introduced into dibasic residues at position 50/51 and 69/70 in M50/69 core mutant partially restored the suppression ability of core protein (5-fold suppression) on HBV replication as compared with that of mutation at 50/51 (M50; 2.9-fold enhancement) or 69/70 (M69; 2-fold inhibition) (Fig. 3). Similarly, M17/39 core mutant still retained certain strength of suppression ability (8-fold suppression), whereas M17 core mutant barely had the suppression ability (1.7-fold enhancement) (Fig. 3). Taken together, our results imply that the ability to suppress HBV replication is more sensitive to mutations within the NLS region of core protein and that the structural context of the core protein rather than the amino acid residue itself is more important for its suppression on HBV replication.

In this study, we also examined the functional NLS of HCV core protein by mutational analysis (see Fig. 1A). Based on
immunofluorescence study (see Fig. 4), we found that the functional NLS governing the nuclear entry of the HCV core protein actually resembles the bipartite configuration (85) consisting of two clusters of basic residues separated by a 17-amino acid spacer and is located within residues 50–70 (KRTSER-SQPGRQRQPFPK) (93). The characteristics of core protein NLS differ from the prototypic NLSs consisting of short stretches of basic amino acids as found in NLS1, NLS2, or NLS3 (75, 76) (Fig. 1A). This discrepancy can be due to the different methods used for the identification of NLS. As noted, mapping the NLS by deletion analysis, as reported by other groups, presumably unmasks cryptic NLS. This bipartite NLS of HCV core protein has a property similar to a number of viral proteins, such as HDAg of HDV (92), Tof protein of HTLV-1 (93), Bel 1 protein of human foamy virus (94), and tegument pp65 (UL83) of HCMV (95). Moreover, it should be noted that M50/69 core mutant lost the nuclear transport activity but to some extent retained its suppression activity on HBV, suggesting the dispensability of nuclear targeting for the inhibitory ability of HCV core protein.

One of the major findings in this work is the observed inability of HCV core protein to inhibit HBV gene expression in HBx null mutant background (see Fig. 5A; Table I). This result, together with the detection of the complex formation between HCV core protein and HBx (see Figs. 6, 7, and 14B), provides an important clue for the HCV core protein-mediated inhibitory effect on HBV gene expression. Given that HBx can transactivate HBV enhancer and promoters (28–30), it becomes appealing that the HCV core-mediated inhibition of HBV transcription acts through directly interacting with this key trans-

**Fig. 11.** Competition binding analysis of the HCV core protein to HBV encapsidation components, HBV polymerase, and e RNA.

A, analysis of the e RNA-binding affinity of the full-length HCV core protein. The binding affinity of the HCV core protein to the biotin-labeled e RNA was examined by the streptavidin-biotin-mediated binding assay (see “Experimental Procedures”). In this RNA-protein binding reaction, the biotin-labeled e RNA (Bio-e) (60 pmol) was incubated with increasing amounts of in vitro translated HCV core protein, ranging from 0.4 to 3.0 pmol (lanes 3–6), and the HCV core protein-e RNA complexes were precipitated with streptavidin-agarose beads. The bound labeled proteins were released by boiling in sample buffer and detected by SDS-PAGE and autoradiography. Lane 1, binding assay in the absence of Bio-e; lane 2, binding assay in the absence of core protein. The positions of the full-length (p22) and the truncated form (p18) of the HCV core protein are indicated. B, e RNA-binding specificity of the full-length HCV core protein. In this RNA-protein binding reaction, the Bio-e (30 pmol) was incubated with in vitro translated HCV core protein (1.0 pmol) in the presence or absence of 6-fold excess competitor RNA as indicated and the HCV core protein-e RNA complexes were precipitated with streptavidin-agarose beads and detected by autoradiography. C, e RNA-binding specificity of the HBV polymerase (pol). In this RNA-protein-binding reaction, the experimental conditions were similar to that described in B except that the Bio-e (30 pmol) was incubated with in vitro translated HBV pol (0.2 pmol) in the presence or absence of 6-fold excess competitor RNA as indicated. D, binding of HBV polymerase to biotinylated e RNA. The in vitro translated HBV pol (0.2 pmol) was incubated with increasing amounts of biotinylated e RNA (Bio-e), ranging from 7.5 to 120 pmol (lanes 3–7) or without Bio-e (lane 2) in a total of 350 μl of reaction buffer (see “Experimental Procedures”), and the RNA-protein complexes were precipitated with streptavidin-agarose beads and displayed by SDS-12% PAGE. E, competition binding assays. Adding the increasing amounts of HCV core protein (0.4–2 pmol) to the HBV pol (0.2 pmol) and biotinylated e RNA (60 pmol) binding reactions (final volume 350 μl), the truncated form (p18) of HCV core protein was detected in the pol-RNA complexes, which in turn decreased the complex formation of HBV pol-e RNA (compare lanes 3 and 4 with lanes 1 and 2) as detected by streptavidin-agarose affinity chromatography (see “Experimental Procedures”). F, the supernatant recovered from the binding reaction mixtures of lanes 2 and 4 in E was further precipitated with antiserum against HBV pol (see “Experimental Procedures”), and the precipitates were analyzed by SDS-PAGE and autoradiography. Lanes 1 and 2, immunoprecipitates obtained from supernatants of binding reactions in lanes 2 or 4 of E, respectively; lanes 3 and 4, immunoprecipitates of HCV core protein or HBV pol by antiserum against HCV core protein or HBV pol, respectively. The positions of the HCV core protein (p22 and p18) and HBV pol (pol) are indicated.
Except that the truncated version of 14C-labeled HCV core protein (C101) obtained by the legend to Fig. 8 (A) was used in this study. All binding assays were identical to the experimental conditions described in the legend to Fig. 8B except that the truncated version of 14C-labeled HCV core protein (C101) obtained by in vitro translation (see “Experimental Procedures”) was used in this study. B, analysis of the ε RNA-binding affinity of the truncated version of HCV core protein. All experimental conditions were identical to those of Fig. 11A legend except that the binding affinity of the HCV core protein to the biotin-labeled ε RNA was examined on the truncated version of 35S-labeled T7-C101 (1.4–7.0 pmol) (lanes 3–5). Lane 1, binding assay in the absence of Bio-ε; lane 2 binding assay in the absence of T7-C101. C, competition binding assay. The experimental conditions were identical to those of Fig. 11E legend, except that the in vitro translated T7-C101 (1.4–7.0 pmol) was used for competition analysis. Lane 1, binding assay in the absence of both Bio-ε and T7-C101 (lane 1) or T7-C101 (lane 2). The positions of the truncated form of the HCV core protein (C101 or T7-C101) and HBV pol (pol) are indicated.

Fig. 12. HCV core protein variant could bind to HBV polymerase and ε RNA but could not eliminate the pol-ε complex formation. A, interaction of HCV core protein truncated variant with HBV pol. All binding assays were identical to the experimental conditions described in the legend to Fig. 8B except that the truncated version of 14C-labeled HCV core protein (C101) obtained by in vitro translation (see “Experimental Procedures”) was used in this study. B, analysis of the ε RNA-binding affinity of the truncated version of HCV core protein. All experimental conditions were identical to those of Fig. 11A legend except that the binding affinity of the HCV core protein to the biotin-labeled ε RNA was examined on the truncated version of 35S-labeled T7-C101 (1.4–7.0 pmol) (lanes 3–5). Lane 1, binding assay in the absence of Bio-ε; lane 2 binding assay in the absence of T7-C101. C, competition binding assay. The experimental conditions were identical to those of Fig. 11E legend, except that the in vitro translated T7-C101 (1.4–7.0 pmol) was used for competition analysis. Lane 1, binding assay in the absence of both Bio-ε and T7-C101 (lane 1) or T7-C101 (lane 2). The positions of the truncated form of the HCV core protein (C101 or T7-C101) and HBV pol (pol) are indicated.

Fig. 13. Effect of intact HCV polyprotein on expression of HBV antigens and HBV endogenous DNA polymerase activity in HuH-7 cells. HuH-7 cells were cotransfected with HBV plasmid pSHH2.1 (2.5 μg) and HCV polyprotein expression construct pSRa/HCV-FL (17 μg) or pSRa vector (5 μg). The total amount of plasmid DNA used for transfection was 19.5 μg each by adding pGEM-3Zf(-) plasmid DNA, if applicable. The medium fraction on day 6 post-transfection was detected for HBsAg and HBeAg/HBcAg (A) and HBV endogenous DNA polymerase activity (B) (see “Experimental Procedures”). S/N ratio, sample versus negative control. The positions of relaxed circular (RC) and linear (L) forms of HBV DNA are indicated by arrows. C, analysis of HCV core protein expression in pSRa/HCV-FL or pECE/HCV-KF transected cells. HuH-7 cells (6-well) were cotransfected with pSHH2.1 (0.5 μg) and HCV core protein expression construct pECE/HCV-PL or pECE/HCV-KF (3.6 kb, 1.0 μg) (lane 2) or HCV polyprotein expression construct pSRa/HCV-FL (17 μg, 3.75 μg) (lane 3). The total amount of plasmid DNA used for transfection was 4.25 μg each by adding pGEM-3Zf(-) plasmid DNA, if applicable. The detection of HCV core protein expression in transfected cells was examined by immunoblot using anti-HCV core sera.

Activator of HBV. Confocal microscopy analysis of HCV core protein and FLAG-tagged HBx coexpressed cells showed the colocalization of HBx and HCV core protein in both nuclear and cytosolic compartments (see Fig. 6). However, in line with our results indicating that the nuclear transport of core protein is not a prerequisite for inhibition of HBV gene expression and the findings that HBx regulates transcription at either subcellular compartment (24, 27, 96), likely the suppression of HBV gene expression by HCV core protein may take place via forming a complex with the cytoplasmic HBx. However, the involvement of nuclear HBs-HBV core complex in this suppression effect is not formally excluded. Furthermore, when considering that both HBx and HCV core proteins are the promiscuous regulators affecting a plethora of cellular activities or interacting with a long list of cellular proteins involved in transcription, cell growth, and apoptotic cell death (reviewed in Refs. 5, 23, and 24), presumably the presence of these two viral proteins or their complex formation during dual infection of both hepatitis viruses may aggravate or counteract their individual activity or cellular functions. Apparently, a more comprehensive survey and comparison of their activities or targets may shed some light on this issue. Along this line, it is noted that both viral proteins have the same cellular targets, such as p53 and 14-3-3 (12, 16, 97–103). However, in contrast to HCV core protein (12), HBx down-regulates apoptosis and transcriptional activation mediated by this tumor suppressor (97–101). In the case of 14-3-3, both viral proteins stimulate Raf-1 kinase or Ras/Raf-1/mitogen-activated protein kinase pathway through direct targeting to this scaffold protein or its associated complex including MEKK1, SEK1, and stress-activated protein kinase (16, 102). Additionally, a recent study (103) has demonstrated that among more than 10 viral proteins of HCV and HBV including HBx, the core protein of HCV is the most potent signal transducer on several intracellular signals, especially NF-κB, AP-1, and serum response element (SRE)-associated pathways. Because activation of these signaling pathways by
HBx or HCV core proteins plays an important role in liver injury, cirrhosis, and hepatocellular carcinoma (104–107), this may partially account for the clinical observation of increasing severity of liver disease in patients with dual infection of these two viruses (69, 108–110).

Assembly of the replication-competent HBV nucleocapsid involves the complex association of at least three different components, including pre-genomic RNA, core protein, and HBV polymerase (49, 50, 111, 112). In this study, we have shown that the full-length HBV core protein can complex with HBV polymerase to prevent the binding of the pol protein to its package signal (Figs. 8, 10, and 11). Because binding of the HBV pol to /H9280 is a prerequisite for HBV pre-genomic RNA packaging (49, 111), we anticipated that the full length of HCV core protein would inhibit encapsidation of the pre-genomic RNA into a nucleocapsid, thus confirming the observation made with in vivo transfection experiments (4, 74). The possibility that the suppression of HBV encapsidation by the HBV core protein is in part due to complex formation with another encapsidation component-HBV core protein was also explored. However, our preliminary results provide no support for this hypothesis, because similar coimmunoprecipitation experiments on the HCV and HBV core proteins failed to detect their association (data not shown). Another alternative hypothesis suggested from the present study (see Fig. 11) could imply that the binding of processed forms of HCV core protein (e.g., p18) to /H9280 sequence may preclude the binding of the HBV pol to the /H9280 sequence, which in turn also affects the encapsidation process. In viewing that the amount (60 pmol) of HBV /H9280 sequence used in this study was in large excess to that of HBV pol (0.2 pmol) or HCV core protein (about 0.4 to 2 pmol), this possibility seems less likely. Thus, based on our results shown here, it appears that the major cause for suppression of HBV encapsidation by the HCV core protein is due to inhibition of HBV pol binding to the /H9280 sequence by the formation of inactive HBV pol-HCV core protein complex. The essence of this model is that of all three essential encapsidation components, only the HBV pol presents as trace amounts, thus the selection of HBV pol protein instead of HBV core protein or /H9280 sequence as a target for suppression of HBV encapsidation by the HCV core protein appears as the most promising mechanism.

In the present study, the in vitro analysis of the mutual

---

**Fig. 14.** HCV core protein can associate with HBx or HBV pol in the presence of HCV nonstructural proteins (NS3 to NS5B). A, analysis of NS3 expression in Ava.5 cells. The cell extracts prepared from HuH-7 or Ava.5 cells were analyzed by SDS-PAGE followed by immunoblotting with rabbit anti-HCV NS3 sera for the detection of NS3. The amounts of protein loaded are as follows: 25 µg for lanes 1 and 4; 50 µg for lanes 2 and 5; and 75 µg for lanes 3 and 6. B and C, in vivo coimmunoprecipitation of HBx or HBV pol and HCV core protein. Ava.5 cells were cotransfected with FLAG-tagged HBx (B) or FLAG-tagged HBV pol expression construct (C) together with the HCV core expression construct pSRa/HCVc195 (see “Experimental Procedures”) (10 µg each). The cells extracts prepared from the transfected cells were immunoprecipitates (IP) by anti-FLAG (M2) antibody-conjugated agarose resins (lanes 1–5), and immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting (WB) with human anti-HCV core sera for the detection of HCV core protein. All experimental conditions are similar to the legend of Fig. 7D and Fig. 8D except that the coimmunoprecipitation experiments of lanes 1–4 of B and C were performed in the presence of RNase A (10 µg/ml).
interaction between the C-terminal truncated form of the HCV core protein (C101) and the HBV encapsidation components strongly suggests that, unlike the full-length core protein, this mutant form of the core protein cannot compete with HBV pol for binding to the packaging signal, although it can form a complex with HBV pol protein or with the ε sequence. Loss of the competition ability of this core variant appears to be consistent with the loss of the in vivo suppressive effect of this variant on HBV encapsidation (74). Additionally, results obtained from the suppression-defective mutant (C101) also imply that the amino acid segment beyond amino acid residue 101 may be important for the interference effect on the HBV pol function.

Along this line, our results as shown here indicate that mutation of Arg<sup>113</sup>, Arg<sup>114</sup>, or Arg<sup>115</sup> residue of HCV core protein severely impairs its suppression ability on HBV encapsidation, and the fact that phosphorylation of Ser<sup>69</sup> and Ser<sup>116</sup> also modulates the suppression ability of HCV core protein on HBV encapsidation (4) all support this explanation. Intriguingly, in this study we also found a stimulating effect of HBV replication (1.7–2.9-fold) in several core mutants like M50, M9, M17, and M61 (see Fig. 3). The exact molecular mechanism for this effect is not clear. In view of the fact that the conformation of HBV pol or HBV pol-ε complex dictates the efficiency of the HBV pregenome RNA encapsidation process (113–116), presumably the presence of these HCV core variants directly or indirectly induces the formation of a more competent, productive conformation of HBV pol or HBV pol-ε complex for the encapsidation process. If this notion is correct, one may predict that the effect of HCV core protein on HBV replication may depend on the structural context of the HCV core protein. This may well explain the variation or even the lack of the interference effect between these two viruses in some clinical cases (73, 117) or in the transgenic mice model (118). As noted, HCV core protein used for our experiment is genotype 1b, and in general the different genotype of HCV may impart amino acid substitution on HCV core protein, even though this protein is highly conserved.

Mapping studies reveal that the junction region between reverse transcriptase and RNase H domains of the HBV pol is important for binding to HCV core protein (see Fig. 10). These two regions are the most critical for HBV pre-genomic RNA encapsidation and reverse transcriptional reaction (53, 88, 89, 119). Several studies (53, 88, 89, 119) have suggested that mutation at these two regions severely impairs the HBV virion formation including the pre-genomic RNA packaging. Considering the importance of these two regions in HBV pol function, it is perhaps not surprising that the inhibition of the binding of pol protein to ε is mediated through the interaction of the HCV core protein with the reverse transcriptase/RNase H domains of the pol protein. A relevant question arises whether this complex formation may also inhibit the reverse transcriptase and RNase H activities of the pol protein.

Within the last few years, considerable progress has been made in understanding the roles of the HBV pol and cellular factors in HBV nucleocapsid assembly. It is interesting to note that translation and packaging of HBV pol are two intimately coupled events in hepadnaviruses (50, 120), and a more recent study involves a role of Hsp90 and its partner p23 or Hsp60 as participants in the interaction of the HBV pol with ε (59–61). In view of these results, it is particularly interesting that the suppression of HBV encapsidation by the HCV core protein may take place during the translation of HBV pol before its interaction with other cellular factors for nucleocapsid assembly. The question of whether the suppression of HBV encapsidation by HCV core protein has to be mediated via Hsp90, Hsp60, or other cellular factors remains to be clarified.

Our observations presented here confirm and extend previous studies on the suppression of HBV gene expression and encapsidation by the HCV core protein and establish the in vivo mechanistic model for the inhibition mechanism of the HCV core protein on these two processes of HBV. This study further suggests that binding with HBV trans-activator HBx or encapsidation components per se is not a guarantee of the suppression activity. Additionally, the functional domains for suppressive activity of the HCV core protein are distinct in such a way that it is more stringent on the core protein structure for inhibition on HBV replication as compared with that for inhibition on HBV gene expression. Moreover, HCV core protein bears the trans-suppression ability on gene expression of the HBV and several cellular and viral promoters (reviewed in Ref. 5), but so far it exhibits much stronger suppression activity on the HBV encapsidation process. Further understanding of the molecular mechanisms involved in both suppression activities of the HCV core protein will be helpful for designing this protein as an antiviral drug specifically against HBV replication.

Acknowledgments—We thank C. M. Rice and Apath for generously providing the HCV plasmid p90/HCVFLlong and Ava.5 cells. We also thank S. J. Lo, C. M. Chang, and C. K. Chak for providing plasmids or antibodies. We are grateful to M. T. Hsu for critical reading and comments on this manuscript.

REFERENCES
1. Choo, Q.-L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) Science 244, 359–362
2. Saito, I., Miyamura, T., Okabayashi, H., Katayama, S., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Choo, Q.-L., Houghton, M., and Kuo, G. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6547–6549
3. Reed, K. E., and Rice, C. M. (2000) Curr. Top. Microbiol. Immunol. 242, 55–84
4. Shih, C.-M., Chen, C.-M., Chen, S.-Y., and Lee, Y.-H. W. (1995) J. Virol. 69, 1160–1171
5. Lai, M. M. C., and Ware, C. F. (2000) Curr. Top. Microbiol. Immunol. 242, 117–134
6. Barba, G., Harper, F., Tahara, T., Kohara, M., Golunetz, S., Matsuura, Y., Eder, G., Schaff, Z. S., Chapman, M. J., Miyamura, T., and Brechot, C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1205–1209
7. Chen, C.-M., You, L.-R., Hwang, L.-H., and Lee, Y.-H. W. (1997) J. Virol. 71, 9417–9426
8. Masumoto, M., Hsieh, T.-Y., Zhu, N., Vanarsdale, T., Hwang, S. B., Jung, K.-S., Gorbaleyna, A. E., Lo, S.-Y., Ou, J.-H., Ware, C. F., and Lai, M. M. C. (1997) J. Virol. 71, 1301–1309
9. You, L.-R., Chen, C. M., and Lee, Y. H. W. (1999) J. Virol. 73, 1672–1681
10. Zhu, N., Khoshan, A., Schneider, R., Masumoto, M., Dennert, G., Ware, C. F., and Lai, M. M. C. (1998) J. Virol. 72, 3691–3697
11. Hsieh, T.-Y., Masumoto, M., Chou, H. C., Schneider, R., Hwang, S. B., Lee, A. S., and Lai, M. M. C. (1998) J. Biol. Chem. 273, 17561–17569
12. Lu, W., Lo, S. Y., Chen, M., Wu, K., Fung, Y. K., and Ou, J. H. J. (1999) Virology 264, 134–141
13. Mamiya, N., and Worman, H. J. (1999) J. Biol. Chem. 274, 15751–15756
14. You, L., Chen, C. M., Yeh, T. S., Tsai, Y. T., Mai, R. T., Lin, C. H., and Lee, Y. H. W. (1999) J. Virol. 73, 2841–2853
15. Jin, D. Y., Wang, H. L., Zhou, Y., Chun, A. C., Kühler, K. V., Hou, D. Y., Kung, H., and Jeang, K. T. (2000) EMBO J. 19, 729–740
16. Aoki, H., Hayashi, J., Moriyama, M., Arakawa, Y., and Hino, O. (2000) J. Virol. 74, 1736–1741
17. Wang, F., Yoshida, I., Takamatsu, M., Ishido, S., Fujita, T., Oka, K., and Hotta, H. (2000) Biochem. Biophys. Res. Commun. 273, 479–484
18. Ray, R. B., Laggning, L. M., Meyer, K., Steele, R., and Ray, R. (1996) J. Virol. 70, 4438–4445
19. Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, M., Matsuura, Y., Kimura, S., Miyamura, T., and Koike, K. (1998) Nat. Med. 4, 1065–1067
20. Ganem, D., and Varmus, H. E. (1987) Annu. Rev. Biochem. 56, 651–693
21. Koike, K. (1995) Intervirology 38, 134–142
22. Cernichi, J. A. (1996) Trends Microbiol. 4, 270–274
23. Murakami, S. (1999) Intervirology 42, 81–99
24. Murakami, S. (2001) J. Gastroenterol. 36, 651–660
25. Kim, C. M., Koike, K., Saito, I., Miyamura, T., and Jay, G. (1991) Nature 351, 317–320
26. Diao, J., Garces, R., and Richardson, C. D. (2001) Cytokine Growth Factor Rev. 12, 189–205
27. Ganem, D. (2001) Science 294, 2299–2300
28. Faktor, O., and Shaul, Y. (1999) Oncogene 19, 861–872
29. Nakatake, H., Chisaka, O., Yamamoto, S., Matsuura, K., and Koshi, R.
Mechanisms for Inhibition of Hepatitis B Virus Gene Expression and Replication by Hepatitis C Virus Core Protein
Chi-Hong Chao, Shao-Hung Wang, Li-Ru You and Yan-Hwa Wu Lee

J. Biol. Chem. 2003, 278:591-607.
doi: 10.1074/jbc.M204241200 originally published online October 24, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204241200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 120 references, 59 of which can be accessed free at
http://www.jbc.org/content/278/1/591.full.html#ref-list-1