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Modulation of the *trans*-Suppression Activity of Hepatitis C Virus Core Protein by Phosphorylation

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We previously demonstrated that the core protein of hepatitis C virus (HCV) can suppress gene expression and replication of hepatitis B virus (HBV) in a human hepatoma cell line (HuH-7). In this study, we have characterized the phosphorylation property of HCV core protein and examined the effect of phosphorylation on its suppressive activity of HBV. Our results indicated that both the full-length HCV core protein (22 kDa) and its processed or degraded forms (14 to 18 kDa) were phosphorylated in insect cells. As demonstrated by using the glutathione *S*-transferase fusion protein expression system and in vitro transcription and translation system, the phosphorylation of HCV core protein was carried out by protein kinase A (PKA) and protein kinase C (PKC) in vitro. In both kinase reactions, it was determined that the phosphorylated amino acid was a serine residue. The potential phosphorylated sites in core protein were identified as residues Ser-53 and Ser-116 for PKA and Ser-53 and Ser-99 for PKC. Comparison of the phosphorylation intensities of the wild type and Ser mutants suggested that Ser-99 and Ser-116 were the major phosphorylation sites for PKC and PKA, respectively. The phosphorylation of Ser-99 and Ser-116, but not Ser-53, in HCV core protein was essential for the suppressive activity of HCV core protein on HBV gene expression and replication in HuH-7 cells. Mutation of the former two serine residues to alanine or aspartate residues led to a drastic loss of the inhibitory effects of HCV core protein on HBV gene expression (both transcription and antigen production) and pregenomic RNA encapsidation, as well as the release of HBV virus particles. In contrast, the Ser-53 mutant conferred the same level of suppressive activity as the wild type did. This property is in accordance with the observation that Ser-99 and Ser-116 are the predominant phosphorylation sites in the HCV core construct. All serine mutants (including those with mutations in PKA, PKC, and both kinase recognition sites) of HCV core protein retained the ability to translocate into the nucleus. Furthermore, wild-type HCV core protein diminished its suppressive activity when cells were treated with PKA or PKC inhibitor. In conclusion, HCV core protein is a phosphoprotein and in HuH-7 cells, its *trans* suppression of HBV gene expression and replication is positively regulated by PKA and PKC. The role of phosphorylation in the control of *trans*-suppressive activity cannot be reproduced by introducing an acidic residue. In addition, our results imply that phosphorylation of serine residues by these two kinases is not a prerequisite for HCV core protein entrance into the nucleus.

Hepatitis C virus (HCV) is now recognized as the main agent of chronic hepatitis and plays a major role in the development of chronic liver disease, including hepatocellular carcinoma (11, 37). The molecular cloning of the HCV genome and development of an immunoscreening assay with recombinant HCV antigens are recent breakthroughs in research of HCV, which afflicts one million patients per year worldwide (11, 37). In spite of progress in the molecular biology of HCV, the biological characteristics of this virus remain obscure.

HCV has a positive-strand RNA genome of about 9.4 kb (10, 12, 33, 46, 55) that encodes a single large polyprotein of about 3,010 amino acids. On the basis of the hydropathy profile of the putative HCV polyprotein and partial sequence homology at the amino acid level, HCV is considered to be distantly related to flaviviruses or pestiviruses (12, 24, 33, 43). The proteins encoded in the genome of HCV are arranged in the following order: NH_2-C-E1-E2/NS1-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH, with C, E1, and E2/NS1 as virus structural proteins and NS proteins as nonstructural proteins (12, 23, 26, 55). Individual HCV proteins are processed from the precursor polyprotein by the combined actions of cellular proteinase (signalase) and virus proteinases (NS3) (4, 21, 22, 27, 28, 42, 61). The expression of the structural region of the HCV genome in recombinant systems by several laboratories has demonstrated that the amino-terminal portion of the HCV polyprotein can be processed into a basic core protein (C) of 18 to 22 kDa followed by two envelope glycoproteins of 32 to 35 kDa (E1) and 68 to 72 kDa (E2) (23, 25, 26, 50). However, the HCV structural proteins have not been characterized extensively because HCV virions have not been purified and their molecular composition has not been determined.

HCV differs from other RNA viruses in its prolonged, persistent infection, which eventually develops into hepatocellular carcinoma (7, 13, 36, 47). This could be due in part to the effect of virus gene products on the expression of host cellular genes. The most likely candidate is the HCV core protein that has been shown to *trans* suppress hepatitis B virus (HBV) genes (51). Since HCV core protein has several potential recognition sites for protein kinase A (PKA) and protein kinase C (PKC) (51), it is likely that this protein exhibits different properties pertinent to its biological functions through the phosphorylation or dephosphorylation process (29). The recent finding that the core protein of HCV is phosphorylated in insect cells (39) leads to the interesting question of whether the phosphorylation state of HCV core protein plays a role in the suppression of HBV expression. This question has been investigated in this study, and the results shown here suggest that in HuH-7 cells, the suppressive activity of HCV core protein is positively regulated by PKA and PKC.

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MATERIALS AND METHODS

Plasmids. The HCV expression construct pECE/HCVC-KF, a derivative of mammalian cell expression vector pECE (16), was described previously (51). In this construct, the expected structural protein of HCV contains the whole coding region (191 amino acids) of the core protein along with 3 amino acid residues from the envelope 1 region and 5 amino acid residues from the polylinker region of the vector pECE. Plasmid pGM3(+)/HCVS-AA was constructed by the insertion of the HCV core-envelope 1 coding region-containing *Acc*I fragment (0.8 kb) from pS7/1-216 (25, 56) into the *Acc*I site of pGEM-3Zf(+) (Promega). Plasmid pECE/HCVC-AF was constructed by subcloning the HCV core gene-containing *Kpn*I-*Fsp*I fragment (0.6 kb) from pGM3(+)/HCVS-AA into *Kpn*I-*Sma*I-digested pECE. Plasmid pSHH2.1 contains a tandem dimer of the HBV genome (subtype *ayw*) inserted at the *Eco*RI site of vector plasmid pSV08 (64).

Cell culture and transfection. A human hepatoma cell line, HuH-7 (44), was used in all DNA transfection experiments of this study. Cells (10^7 per 15-cm-diameter dish) were seeded and cultured at 37°C under 5% CO_2 for 20 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM L-glutamine. Cells were cotransfected with appropriate plasmid combinations (20 μg of DNA each) by the calcium phosphate coprecipitation method (20). The medium was changed after 20 h of incubation with DNA and was subsequently replaced and collected at 3-day intervals.

Analysis of HBV-related antigens, virus particles, and cytoplasmic core. On day 6 posttransfection, the culture medium was assayed for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen-hepatitis B core antigen (HBe/HBcAg) by using enzyme immunoassay kits (EverNew Corp., Taipei, Taiwan). Secreted HBV particles purified from 15 ml of culture medium were detected by an assay for endogenous DNA polymerase activity (31). Repaired HBV DNA purified by proteinase K digestion, phenol extraction, and ethanol precipitation was subjected to 1% agarose gel electrophoresis and then autoradiographed. The detailed procedures for preparation and detection of intracellular HBV core particle-associated nucleic acids were previously described (40, 51). Briefly, cytoplasmic core particles were immunoprecipitated with anti-core antibodies adsorbed to protein A-Sepharose 4B beads. Immunoprecipitates were treated with micrococcal nuclease and RNase-free DNase I and washed with buffers. RNA was prepared by treatment with proteinase K, phenol-chloroform extraction, and ethanol precipitation. Purified HBV core-associated RNA was then subjected to Northern (RNA) blot analysis (8), with the ^{32}P-labeled 3.5-kb HBV pregenomic RNA probe with antisense polarity prepared as described previously (51).

RNA preparation and Northern blotting. Cellular RNA was extracted by the guanidinium isothiocyanate-cesium chloride ultracentrifugation method as previously described (18). RNA samples (20 μg per lane) were electrophoresed on a 1% formaldehyde–agarose gel and then transferred to nitrocellulose paper. The HBV DNA probe was prepared from the 3.5-kb HBV fragment of pMH-9/3091 by the nick translation method (48). Prehybridization and hybridization were performed as described before (60).

Construction of a recombinant baculovirus expression vector for the HCV core gene and expression of HCV core protein in insect cells. The BacPAK baculovirus expression system (Clontech) was employed for the expression of HCV core protein. The 0.6-kb *Xba*I-*Eco*RI fragment, which contains the HCV core gene, was excised from plasmid pECE/HCVC-AF and inserted into *Xba*I-*Eco*RI-digested pBacPAK8. The resulting construct, pBacPAK8/HCVC-AF, was digested with *Eco*RI, the 5′ protruding ends were filled in with Klenow enzyme, and the *Nhe*I linker (BioLab) (5′-CTAGCTAGCTAG-3′) was inserted into the site to introduce the amber stop codon in three different reading frames. The resulting recombinant construct, pBacPAK8/HCVC-AN, was then transfected into Sf21 cells along with BacPAK6 DNA digest (*Bsu*361 digested to remove part of the essential virus gene) by using cationic liposomes (Lipofectin reagent; Bethesda Research Laboratories). Sf21 cells were grown in Grace's medium (GIBCO Bethesda Research Laboratories) at 27°C without CO_2. Four days posttransfection, progeny viruses were recovered from culture supernatants. Recombinant viruses were selected and purified after at least two rounds of plaque purification and were amplified in Sf21 cells to yield virus stocks. The recombinant baculovirus which harbored the HCV core gene was designated BaV/HCVc191 virus. For the detection of HCV core protein expression in insect cells, Sf21 cells were infected with recombinant virus. Cells were harvested 2 days after infection and lysed in Laemmli sampling buffer (38). Aliquots of cell lysates were used for immunoblot analysis (62) with HCV patient sera or rabbit antiserum raised against glutathione *S*-transferase (GST)–HCV core fusion protein, as described previously (51) (see Fig. 1B). For ^{32}P labeling of HCV core protein, infected cells (seeded at 5×10^5 cells per 3-cm-diameter dish and infected either with wild-type baculovirus BacPAK6 or recombinant baculovirus BaV/HCVc191) were starved for phosphate on day 2 postinfection by pretreatment with phosphate-free Grace's medium containing 10% dialyzed fetal calf serum for 1 h before being labeled with $^{32}P_i$ (0.3 mCi/ml) (code no. PBS 13; Amersham) for 6 h. After being labeled, portions of cells were directly lysed by sampling buffer (39) and extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (see Fig. 1C). Alternatively, portions of cells were fractionated into soluble and insoluble fractions by extraction with EB buffer (50 mM Tris-HCl [pH 9.0], 1% Nonidet P-40, and 100 mM NaCl) and centrifugation at $15,000 \times g$ for 15 min at 4°C (39). Both fractions were resuspended in Laemmli sampling buffer and analyzed by SDS-PAGE and autoradiography (Fig. 1C).

Expression and purification of GST-HCV core fusion proteins in *Escherichia coli*. Plasmid pGEX-3KS (51), a derivative of pGEX-1 (52), was employed in the construction of a series of expression vectors for HCV core-GST fusion protein. Plasmid pGST/HCV-c122, pGST/HCV-c101, and pGST/HCV-c24 are derivatives of pGEX-3KS that directed the synthesis of different lengths of the N terminus of HCV core protein fused with the C terminus of GST (Fig. 2A). The pGST/HCV-c122 construct was expected to produce GST fusion protein which contained the 122 N-terminal amino acid residues of HCV core protein in addition to 5 amino acid residues (Arg-Pro-Cys-Ile-Met) in the junction region between GST and HCV core proteins and 10 amino acid residues from the vector at the C terminus of the fusion protein (51). Plasmid pGST/HCV-c101 was constructed by adding the *Xba*I linker (5′-CCTAGTCTAGACTAG-3′) to the Klenow-filled-in *Sac*II site. The resulting construct contains the first 101 amino acid residues of the HCV core protein followed by 4 amino acid residues (Pro-Ser-Leu-Arg) from the polylinker region. Plasmid pGST/HCV-c24 was constructed by deletion of the region from the internal *Sma*I site to the *Eco*RI site of the polylinker region. In this construct, the first 24 amino acid residues of HCV core protein were fused with GST protein and 5 additional amino acid residues (Pro-Ile-His-Arg-Val) from the polylinker region of the vector. HCV core hybrid proteins were purified by affinity chromatography with a glutathione-Sepharose 4B (Pharmacia) column (52) (Fig. 2B).

In vitro phosphorylation reaction and phosphoamino acid analysis. In vitro phosphorylation of purified GST-HCV core fusion protein was performed according to the protocols described in the technical bulletin of Promega. In brief, the labeling of HCV core fusion protein was performed by adding [γ-^{32}P]ATP (10 μCi; specific activity, >5,000 Ci/mmol) to the reaction mixture containing purified PKA (25 U of catalytic subunit; Promega) or PKC (14.4 U; Promega). The standard reaction mixtures for PKC contained 0.25 μg of protein for phosphorylation, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4), 10 mM $MgCl_2$, 1 mM unlabeled ATP, 1 mM dithiothreitol, 0.2 mg of phosphatidylserine per ml, 1.3 mM $CaCl_2$, and 10 μCi of 10-mCi/ml [γ-^{32}P]ATP. As indicated, 0.08 μM protein kinase inhibitor staurosporine (Sigma) (34) was included in the reaction. For PKA reaction, in addition to the 0.25 μg of protein for phosphorylation, buffer containing 20 mM Tris (pH 7.4), 10 mM $MgCl_2$, 1 mM ATP, and 10 μCi of [γ-^{32}P]ATP was included in the reaction mixture. After reaction at 30°C for 30 min, HCV core fusion protein was immunoprecipitated with HCV patient sera or rabbit anti-GST-HCV core fusion protein sera (51). Proteins were separated by gel electrophoresis in the presence of SDS and either transferred to an Immobilon membrane (Millipore) for phosphoamino acid analysis (32) or directly submitted to autoradiography. For phosphoamino acid analysis, phosphoproteins were hydrolyzed in 6 N HCl for 90 min at 110°C and phosphoamino acids were separated by two-dimensional high-voltage electrophoresis on cellulose plates (pH 1.9 in the first dimension and pH 3.5 in the second dimension) and identified by comigration of unlabeled phosphoamino acid standards (Sigma).

Site-directed mutagenesis of putative PKA and PKC sites in HCV core protein. The Altered Sites system (Promega) was used for in vitro mutagenesis of the HCV core gene as described by the supplier. The 0.7-kb *Hin*dIII-*Eco*RI fragment which contained the full-length HCV core gene was removed from pECE/HCVC-KF and subcloned into the *Hin*dIII and *Eco*RI sites of pSELECT-1. The resulting construct, pSELECT/HCVC, was used for site-directed mutagenesis. The oligonucleotides used for mutation were synthesized; they were identified by the position of the mutated amino acid in HCV core protein and the nature of the amino acid before and after mutation. The oligonucleotides used were Ser-53/Ala, 5′-ACCGCTCGG**C**AGTCTTCC-3′; Ser-99/Ala, 5′-CGCGGGGTG**C**CAGGAGCCATCC-3′; Ser-116/Ala, 5′-CAAATTACGCG**C**CCTACGCCG-3′; Ser-53/Asp, 5′-TTGCGACCGCTCG**TC**AGTCTTCCTAGTCG-3′; Ser-99/Asp, 5′-GAGAGCCGCGGGG**GTC**CAGGAGCCATCCTGC-3′; and Ser-116/Asp, 5′-TACCCAAATTACG**GTC**CCTACGCCGGGGGTC-3′. The boldfaced and underlined bases are mutated bases. All mutant sequences were confirmed by sequencing (49). The 0.7-kb *Hin*dIII-*Eco*RI fragments of mutant DNA in pSELECT/HCVC derivatives were then subcloned into *Hin*dIII and *Eco*RI sites of the pECE expression vector (16). Alternatively, the 0.6-kb *Acc*I-*Eco*RI fragments of mutant DNA in pSELECT/HCVC derivatives were subcloned into the *Acc*I and *Eco*RI sites of pGEM-1 (Promega) for in vitro transcription and translation analysis.

In vitro transcription and translation of the HCV core gene and analysis of in vitro phosphorylation products. Recombinant pGEM-1 derivatives which contained the wild-type or mutated HCV core gene were used as templates for in vitro transcription by linearization with *Eco*RI (for generation of the full-length HCV core protein C191 species) (see Fig. 6A and 7A) or an appropriate restriction enzyme (e.g., *Cla*I and *Sma*I for generation of C122 and C101 species, respectively) (see Fig. 7A) and were supplied with SP6 RNA polymerase. Synthesized RNAs (15 μg each) were subjected to in vitro translation by adding rabbit reticulocyte lysates (Promega) which contained 0.25 μCi of ^{14}C-labeled amino acid mixture (final concentration, 5 μCi/ml) (code no. CFB 104; Amersham) to the reaction mixture (50 μl), incubating for 1 h at 30°C, and then chasing with 1 μl of 1 mM cold amino acid mixtures for another 1 h at 30°C. Aliquots (10 μl) of in vitro-translated products were dephosphorylated with 4 U of calf intestine phosphatase for 30 min at 37°C and immunoprecipitated with

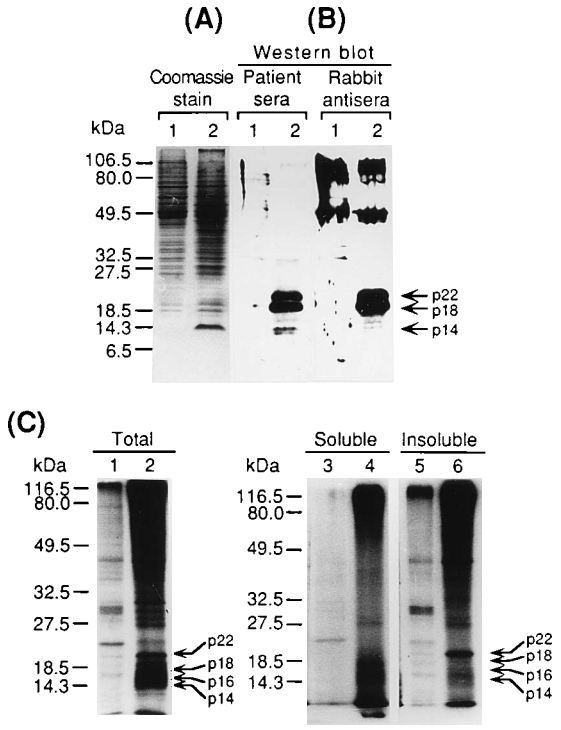

FIG. 1. Examination of in vivo phosphorylation patterns of HCV core protein expressed in insect cells. (A and B) For the detection of HCV core protein expressed in insect cells, Sf21 cells were infected with recombinant baculovirus which harbored the HCV core gene (BaV/HCVc191) and harvested on day 2 postinfection. Cell pellets from Sf21 cells infected with recombinant baculovirus (lanes 2) or without infection (lanes 1) were solubilized in sample buffer (38), analyzed by SDS-PAGE, and stained with Coomassie brillant blue (A) or immunoblotted with HCV patient sera (1:600 dilution) or rabbit anti-HCV core fusion protein sera (1:2,000 dilution) (B). The HCV core antigen was further examined by the enhanced-chemiluminescence detection method (Amersham) (51). (C) For the detection of in vivo phosphorylation patterns of HCV core protein, Sf21 cells were infected with wild-type baculovirus (BacPAK6) (lanes 1, 3, and 5) or BaV/HCVc191 (lanes 2, 4, and 6). On day 2 postinfection, cells were labeled with $^{32}P_i$ for 6 h (see Materials and Methods). Total whole-cell lysates and soluble and insoluble fractions prepared as described in Materials and Methods were solubilized in sample buffer (38) and subsequently analyzed by SDS-PAGE and autoradiography. Each lane contained the same amount of cell lysate from the same number of infected cells. The positions of p22 to p14 species are indicated by arrows.

protein A-Sepharose 4B-bound HCV patient sera. Calf intestine phosphatase-treated and protein A-bound HCV core proteins were subjected to in vitro phosphorylation reactions with PKA or PKC as described above.

Immunofluorescence. The localization of HCV antigens in transfected cells was determined by immunofluorescence microscopy (51). Cells were grown on coverslips, fixed with acetone-methanol (−20°C), probed with rabbit anti-HCV core fusion protein sera (51), and then stained with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G.

RESULTS

Full-length and truncated forms of HCV core protein are phosphorylated in insect cells. Although Lanford et al. (39) reported that C-terminal truncated forms (N-terminal 150 and 115 amino acid residues) of HCV core protein are phosphorylated in insect cells, the question of whether full-length HCV core protein is also phosphorylated remains. Here, we reexamined the phosphorylation property of HCV core protein in insect cells by infection with a recombinant baculovirus (BaV/HCVc191) that can direct the expression of full-length HCV core protein (p22) (see Materials and Methods). As shown in Fig. 1A, in addition to the expected full-length HCV core protein species, p22, three smaller molecular forms of HCV core protein, with sizes of 14 to 18 kDa (designated p14, p16, and p18, respectively), were also found in infected cell lysates examined by Coomassie blue staining. These three smaller species presumably represent degraded forms of HCV core protein since they were recognized by both rabbit anti-HCV core fusion protein sera and HCV patient sera (Fig. 1B, lanes 2) but were not detected in lysate from the mock-infection control (Fig. 1B, lanes 1). p14 was the major species of HCV core protein present in cell lysates; however, it had much lower immunoreactivity than the p22 and p18 species (Fig. 1A and B, lanes 2). When infected cells were labeled with $^{32}P_i$ for 6 h on day 2 postinfection, at least four different protein species which showed broad bands of 22 to 14 kDa were highly phosphorylated in total whole-cell lysates of recombinant virus (BaV/HCVc191)-infected cells, compared with cells infected with the wild-type baculovirus BacPAK6 (Fig. 1C, lanes 1 and 2). The fractionation of recombinant virus-infected cell lysates indicated that most of the 22-kDa species and portions of the 16- and 14-kDa species were associated with insoluble fractions (Fig. 1C, lane 6), while the majority of the 18-kDa species and portions of the 16- and 14-kDa species were found in soluble fractions (Fig. 1C, lane 4). These phosphorylated species presumably represent full-length and truncated forms of HCV core protein since their sizes are similar to those of the immunoreactive protein bands revealed on the immunoblot (Fig. 1B) and they are absent from control fractions (Fig. 1C; compare lane 3 with lane 4 and lane 5 with lane 6). Therefore, our results suggest that in insect cells, full-length HCV core protein is phosphorylated as are processed or degraded forms of p18 to p14 species. The detection of processed forms of HCV core protein in insect cells appears to be consistent with a previous report which indicated that full-length HCV core protein is degraded into an 18-kDa species when expressed in mammalian cells (50).

Phosphorylation of GST-HCV core fusion proteins by PKA and PKC. As indicated in our previous study, HCV core protein has three putative PKA recognition sites (amino acid residues 15, 53, and 116) and five PKC recognition sites (amino acid residues 11, 15, 49, 53, and 99) (51) (Fig. 2A). In order to localize the phosphorylated residues in HCV core protein, three GST-HCV core fusion protein expression vectors were constructed for the in vitro phosphorylation experiment (see Materials and Methods for a detailed explanation of plasmid construction) (Fig. 2A). These constructs can direct the synthesis of GST-HCV core fusion proteins which contain the N-terminal 24-, 101-, or 122-amino-acid fragment of HCV core protein and thus are expected to generate GST-HCV core fusion proteins of 31, 39, and 42 kDa, respectively, that can be purified by glutathione-Sepharose 4B columns (Fig. 2B). The different lengths of these HCV core fusion proteins span different PKA and PKC sites (Fig. 2A). In vitro PKC and PKA reactions indicated that the two fusion proteins GST/HCV-c101 and GST/HCV-c122, but not GST or GST/HCV-c24, could be phosphorylated by PKA and PKC (Fig. 3A and B, lanes 1 to 5). These phosphorylation signals are PKA and PKC dependent because in the absence of these two kinases (Fig. 3A and B, lanes 6 to 9) or in the presence of protein kinase inhibitor staurosporine (Fig. 3C; compare lane 3 with lane 4), the phosphorylation intensities at the expected GST-HCV core fusion protein positions (39 and 42 kDa) were absent or reduced. The lack of a phosphorylation signal in GST/HCV-c24 suggests that threonines 11 and 15 are unlikely targets for both kinases. In contrast, threonine 49 and serines 53, 99, and 116 are potential PKA and PKC recognition sites in HCV core protein.

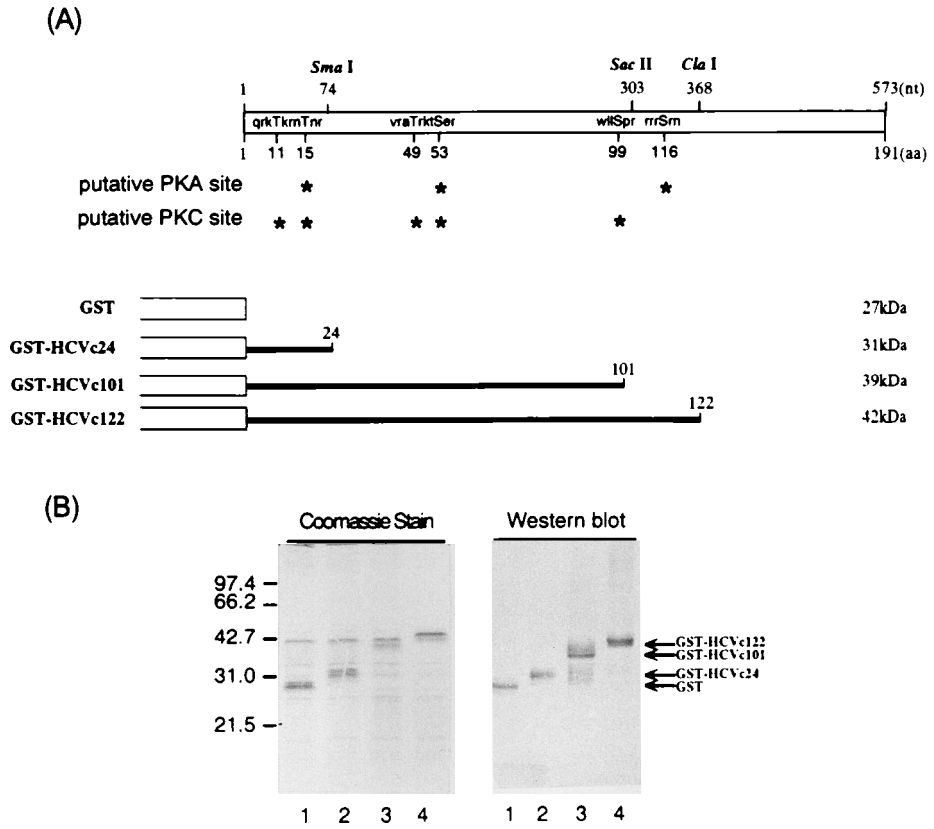

FIG. 2. Schematic representation of putative PKA and PKC sites in HCV core expression constructs and the expression of GST-HCV core fusion proteins in *E. coli*. The recognition motif is (R/K)-(R/K)-X-(S/T) and (S/T)-X-(R/K)-X for PKA and PKC, respectively (35). (A) Putative phosphorylation sites for these two kinases are indicated by asterisks. At the bottom of this panel, the molecular masses of GST and GST-HCV core fusion proteins are indicated at the right. (B) GST-HCV core fusion proteins purified by affinity chromatography were analyzed by SDS-PAGE and stained with Coomassie brillant blue or immunoblotted with rabbit anti-HCV core fusion protein sera (1:600 dilution). Goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Bio-Rad) was used as the second antibody, and the developing solution contained 0.06% 4-chloro-1-naphthol and 0.04% hydrogen peroxide. The positions of GST and GST-HCV core fusion proteins are indicated by arrows.

In the in vitro PKA phosphorylation reaction, a high-molecular-mass phosphorylated species (molecular mass, >100 kDa) was present in the GST/HCV-c122 sample, whether PKA was added or not (Fig. 3A, lanes 3 and 7). A similar phenomenon was also observed for the GST/HCV-c101 sample, though the phosphorylation intensity was much weaker (Fig. 3A, lanes 4 and 8). The exact natures of these species are not clear. Since these phenomena were observed in the PKA reaction but not in the PKC reaction, since GST and GST/HCV-c24 core fusion proteins did not exhibit this property, and since they occurred only after immunoprecipitation with HCV patient sera, not with rabbit anti-HCV core fusion protein sera (data not shown), we believe that these high-molecular-weight species may represent aggregated forms of GST/HCV-c122 or GST/HCV-c101 or are due to the association of these two GST-HCV core fusion proteins with other cellular proteins in HCV patient sera during immunoprecipitation. Phosphorylation of these high-molecular-weight species is probably mediated by protein kinase(s) contained in human sera.

Both PKA and PKC phosphorylate the serine residues of HCV core protein. To identify the phosphoamino acids in in vitro PKA- and PKC-phosphorylated GST-HCV core fusion proteins, ^{32}P-labeled GST/HCV-c122 was hydrolyzed with HCl and phosphoamino acids were separated by high-voltage two-dimensional thin-layer chromatography on cellulose plates (see Materials and Methods). As shown in Fig. 4, both PKA and PKC phosphorylated the serine residues of HCV core protein. Thus, the targets in HCV core protein for both kinases are tentatively assigned to serine 53, 99, and 116 (Fig. 2A).

Ser-99 and Ser-116 are the major PKA and PKC phosphorylated residues in HCV core protein. In order to determine which serine residue contributes to in vitro phosphorylation of HCV core protein by PKA or PKC, the mutation of a serine residue to a nonphosphorylatable alanine residue was introduced at residues 53, 99, and 116 in HCV core protein. Seven mutants were generated; in addition, three single mutants (S53A, S99A, S116A), three double mutants (S53/99A, S53/116A, and S99/116A) and one triple mutant (S53/99/116A) were obtained (see Materials and Methods for mutant construction) (Fig. 5). Full-length and truncated forms of wild-type HCV core protein and serine residue-mutated variants of HCV core protein were generated by in vitro transcription and translation reaction and subjected to in vitro PKA or PKC phosphorylation assay (see Materials and Methods). In order to quantitate the extent of phosphorylation in each HCV core construct, in vitro-translated core protein was labeled with ^{14}C-amino acids and the phosphate group was removed from proteins by phosphatase treatment before phosphorylation assays (see Materials and Methods). As shown in Fig. 6, the relative level of phosphorylation of full-length HCV core protein, p22, in the S116A mutant by PKA was reduced to 39% of that of the wild type, while in mutant S53A, 87% of the phos-

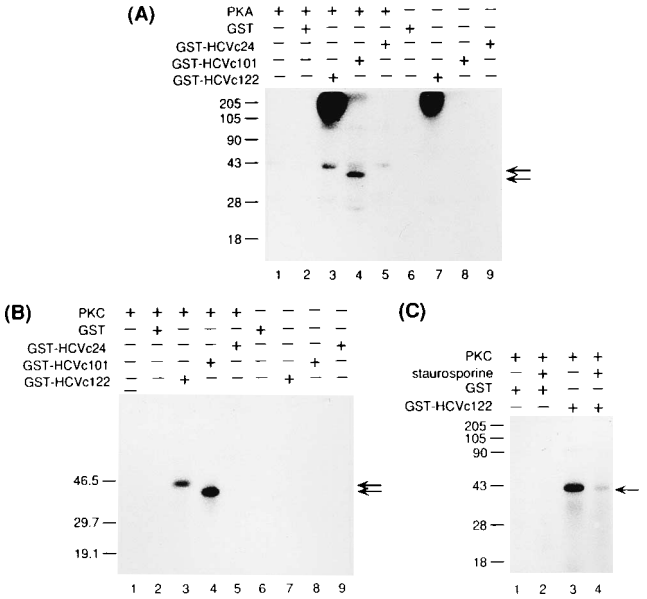

FIG. 3. In vitro PKA and PKC phosphorylation assays of GST-HCV core fusion proteins. Highly purified GST and GST-HCV core fusion proteins were subjected to in vitro PKA (A) and PKC (B and C) phosphorylation assays (see Materials and Methods). (A and B) Lanes 1 to 5, reactions carried out in the presence of PKA (A) or PKC (B); lanes 6 to 9, reactions carried out in the absence of kinase. (C) Protein kinase inhibitor staurosporine (0.08 μM) was added to the reaction as indicated. Phosphorylated GST/HCV-c101 (39 kDa) and GST/HCV-c122 (42 kDa) are indicated by arrows. Autophosphorylation of the PKA catalytic subunit contributes to some background signals of 40 to 42 kDa in lanes 1, 2, 4, and 5 in panel A.

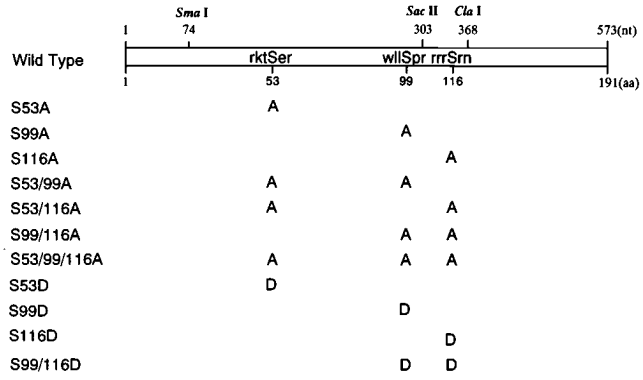

FIG. 5. Mutants of HCV core protein used in this study. All mutants carried either a single, double, or triple mutation designed for site-specific replacement of serine residues at 53, 99, or 116 with an alanine (A) or aspartate residue (D). These mutants were expressed either by transfection into HuH-7 cells or by the in vitro transcription and translation system.

phorylation level remained. However, in the S53/116A double mutant, 36% of phosphorylation was still detectable, which is about the same extent of phosphorylation in the S116A mutant. The residual phosphorylation of HCV core protein exhibited by the S53/116A double mutant might be attributable to other kinase(s) in either reticulocyte lysates or antisera. If we subtracted these phosphorylation signals from those displayed by S53A and S116A mutants, the results suggest that in the PKA reaction, 51% of phosphorylation in HCV core protein, p22, is due to Ser-116 and probably only 3% of phosphorylation is due to Ser-53. Therefore, for the PKA reaction, the major phosphorylated residue within HCV core protein is Ser-116.

On the other hand, in the PKC phosphorylation reaction, the C-terminal-truncated p14 (C122) and p11 (C101) species, but not the full-length p22 (C191) of the wild-type HCV core construct, were phosphorylated (Fig. 7B). Portions of the p22 and p14 species of HCV core protein were degraded into a p11 molecular species (Fig. 7A). However, these degraded forms of p11 did not exhibit any significant phosphorylation signal, unlike the p11 species generated by in vitro translation (Fig. 7B).

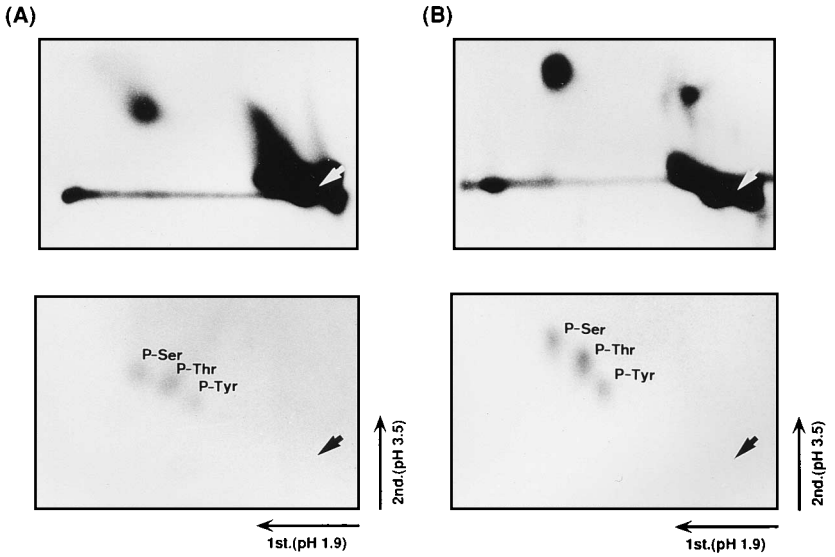

FIG. 4. Phosphoamino acid analysis of in vitro PKA- or PKC-phosphorylated GST/HCV-c122 protein. In vitro PKA- (A) and PKC (B)-phosphorylated GST/HCV-c122 proteins were separated by SDS-PAGE and electrotransferred to an Immobilon membrane (Millipore). Sliced membrane which contained phosphorylated GST/HCV-c122 protein was acid hydrolyzed and processed for two-dimensional high-voltage thin-layer electrophoresis on a cellulose plate (see Materials and Methods). Origins are indicated by arrows. Autoradiography of acid-hydrolyzed ^{32}P-labeled phosphoamino acid (upper boxes); ninhydrin staining of phosphoamino acid standards (P-Ser, P-Thr, and P-Tyr) (lower boxes).

A comparison of the relative phosphorylation intensities of wild-type p14 and serine mutants indicates that serine 53 is not the major phosphorylation site for PKC. While around 81% of phosphorylation remained in the S53A mutant, in mutant S99A, phosphorylation was reduced to 60% of that of the wild type (Fig. 7D). The result of subtracting the phosphorylation intensity of S53A or S99A from that exhibited by the S53/99A double mutant (40%) suggests that Ser-99 has about twofold the phosphorylation of Ser-53. Therefore, neither PKA nor PKC produced a very high phosphorylation reaction at Ser-53. Instead, Ser-99 and Ser-116 of HCV core protein are the major phosphorylation residues for PKC and PKA, respectively. Additionally, since S53/116A and S53/99A still show significant levels of phosphorylation (36 to 40%), it is obvious that the core protein of HCV is also phosphorylated on additional residues in vitro.

Intriguingly, in addition to the p22 and p14 species of HCV core protein that produce phosphorylation signals in both kinase reactions, there are some strong phosphorylation signals that appear for several other protein species (Fig. 6 and 7). For example, in the PKA phosphorylation reaction, at least six different protein species (p18, p28, p32, p44, p55, and p200) were phosphorylated (Fig. 6B). p44 might represent the dimer form of p22 since the ^{14}C signal that reflected in vitro-translated HCV core protein appeared at the corresponding position (Fig. 6A). However, the rest of these species may result from coprecipitation of HCV core protein and cellular proteins present in reticulocyte lysates or antisera during the processes of in vitro translation and immunoprecipitation. Since their phosphorylation intensities directly correlated with the phosphorylation intensities of HCV core protein p22 variants (Fig. 6B), the phosphorylation of HCV core protein may regulate its association with other proteins and hence their phosphorylation. Alternatively, the phosphorylation of HCV core protein may affect the extent of phosphorylation of other proteins associated with it. A similar situation was also observed in the PKC phosphorylation reaction in which six different protein species, p20, p25, p30, p46, p80, and p100, were phosphorylated, and their phosphorylation intensities were parallel to that of p14 in a particular HCV core construct (Fig. 7D). The observed interactions between core protein and these coimmunoprecipitated phosphorylated proteins are rather specific; in control samples without HCV core protein (Fig. 6B and 7D, lanes C), most of them were absent, except for p28, p55, and p200 species, when there was a slight background in the control sample (Fig. 6B, lane C). However, it is rather intriguing to

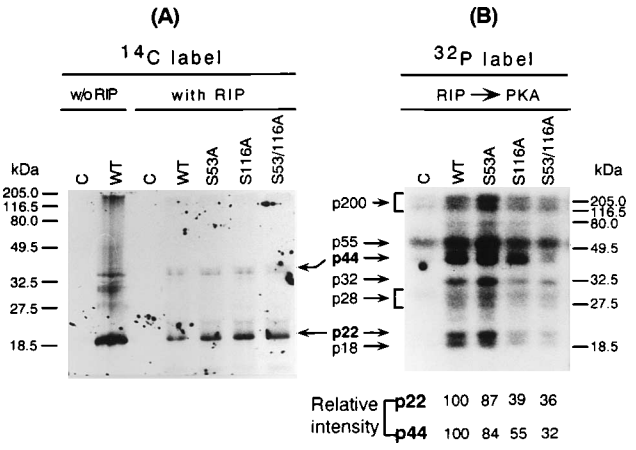

FIG. 6. In vitro PKA phosphorylation assay of in vitro-translated HCV core protein variants. Full-length (p22) HCV core protein variants were generated by in vitro transcription and translation reaction as described in Materials and Methods. One-fifth (10 μl) of each in vitro-translated HCV core protein variant (^{14}C labeled) was immunoprecipitated with HCV patient sera (RIP) and analyzed by SDS-PAGE and autoradiography (A). Another one-fifth (10 μl) of each in vitro-translated HCV core protein variant was subjected to in vitro PKA phosphorylation assay, and recovered samples were analyzed by SDS-PAGE and autoradiography (^{32}P labeled) (B) (see Materials and Methods). The relative phosphorylation signals of HCV core protein p22 and p44 species (indicated below each lane in panel B) were quantitated with a soft laser densitometer (Molecular Dynamics) by normalization with each ^{14}C signal of p22 or p44. The positions of p22, p44, and other protein species that produced phosphorylation signals are indicated by arrows. The results with in vitro-translated HCV core protein before immunoprecipitation (w/o RIP) are also shown. Lanes C, in vitro-translated products from the *Eco*RI-linearized pGEM-1 vector. Lanes WT, S53A, S116A, and S53/116A, in vitro-translated products from *Eco*RI-linearized pGEM-1 derivatives which carried the wild-type or a mutated HCV core gene. The designations of these mutants are shown in Fig. 5.

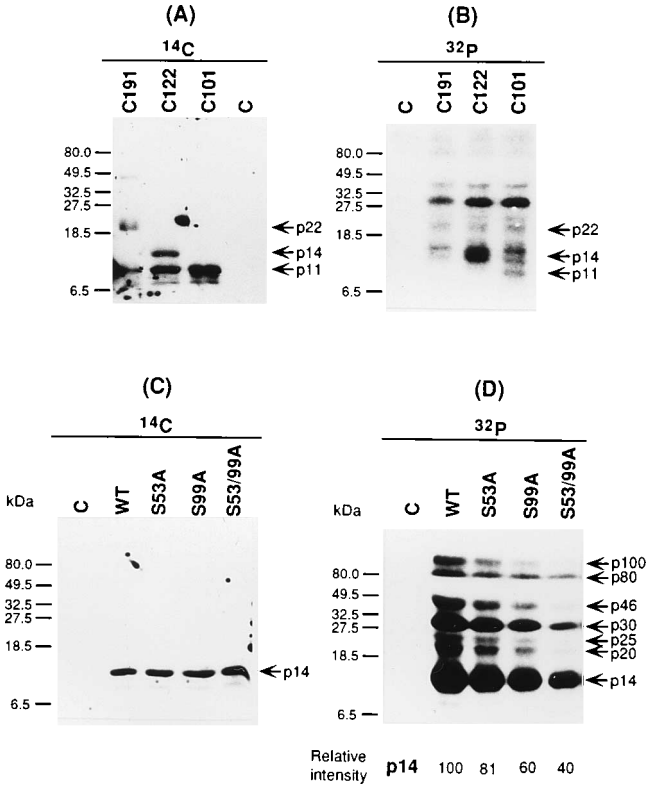

FIG. 7. In vitro PKC phosphorylation assay of in vitro-translated HCV core protein variants. (A and B) Full-length (p22) and truncated forms (p14 and p11) of wild-type HCV core protein were generated by in vitro transcription and translation reaction as described in Materials and Methods. One-fifth (10 μl) of each in vitro-translated HCV core protein variant (^{14}C labeled) was immunoprecipitated with HCV patient sera and analyzed by SDS-PAGE and autoradiography (A). Another one-fifth (10 μl) of each in vitro-translated HCV core protein variant was subjected to in vitro PKC phosphorylation assay, and recovered samples were analyzed by SDS-PAGE and autoradiography (^{32}P labeled) (B) (see Materials and Methods). For production of p22, p14, and p11 molecular species of HCV core protein, the template pGEM-1 derivative which carried the HCV core gene was linearized with *Eco*RI (C191), *Cla*I (C122), or *Sma*I (C101) before in vitro transcription. (C and D) All experimental conditions were the same as those described for panels A and B, except that the truncated forms (p14) of wild-type or mutant HCV core protein generated by in vitro transcription and translation reaction were used as the samples for in vitro PKC phosphorylation assay (see Materials and Methods). The relative phosphorylation signals of p14 variants (shown below each lane in panel D) were quantitated with a soft laser densitometer (Molecular Dynamics) by normalization with each ^{14}C signal of p14. The positions of p14 and other protein species that produced phosphorylation signals are indicated by arrows. The designations above lanes are described in the legend to Fig. 6, except that the in vitro transcription reaction was performed on the *Cla*I-linearized template.

TABLE 1. Expression of HBsAg and HBe/HBcAg in HuH-7 cells after cotransfection with cloned HBV DNA and various HCV core mutant constructs which harbored Ser-to-Ala or -Asp substitution mutation(s)[a]

Expt no.	HCV plasmid[b]	S/N ratio ± SD	
		HBsAg	HBe/HBcAg
1	Mock	<1.5	<1.5
	Control	305.1 ± 4.2	45.7 ± 2.3
	Wild type	155.1 ± 20.7	22.5 ± 2.9
	S53A	165.2 ± 5.5	22.0 ± 2.6
	S99A	294.0 ± 36.1	39.6 ± 0.1
	S116A	300.3 ± 19.2	41.1 ± 0.5
	S53/99A	331.3 ± 37.7	41.1 ± 1.1
	S53/116A	297.4 ± 3.8	35.6 ± 0.6
	S99/116A	299.8 ± 21.6	41.6 ± 1.1
	S53/99/116A	300.8 ± 20.4	45.3 ± 4.0
2	Mock	<1.5	<1.5
	Control	590.0 ± 3.0	97.0 ± 0.5
	Wild type	363.7 ± 7.4	51.8 ± 0.2
	S53D	342.0 ± 6.0	41.0 ± 0.6
	S99D	595.1 ± 3.4	91.0 ± 0.8
	S116D	582.6 ± 7.8	94.2 ± 0.1
	S99/116D	581.7 ± 6.8	89.2 ± 0.1

[a] Data are means ± standard deviations of two independent experiments and are given as S/N ratios (sample versus negative control). Cells were cultured in 15-cm-diameter petri dishes.

[b] Mock, without transfection; control, cells transfected with pSHH2.1 and vector pECE; wild type, S53A, S99A, S116A, S53/99A, S53/116A, S99/116A, S53/99/116A, S53D, S99D, S116D, and S99/116D, cells transfected with pSHH2.1 and pECE/HCVC-KF or its derivatives which carried the mutation(s) indicated. The designations for HCV core mutants are those from Fig. 5.

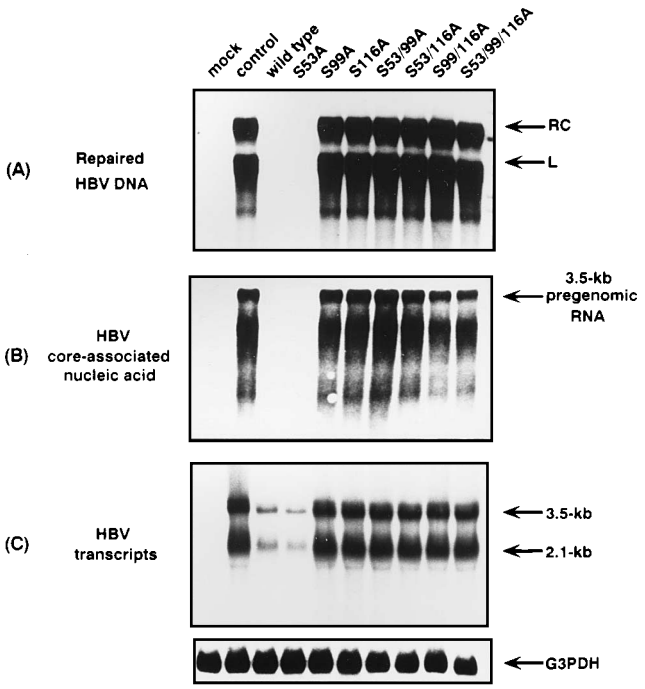

FIG. 8. Effects of HCV core protein variants with Ser-to-Ala substitution mutations on HBV expression. HuH-7 cells were cotransfected with HBV plasmid pSHH2.1 and various HCV core constructs. On day 6 posttransfection, released HBV particles, cytoplasmic HBV core-associated nucleic acid, and total cellular RNAs were prepared for HBV endogenous polymerase activity assay (A) or Northern blotting with the in vitro-transcribed ^{32}P-labeled antisense polarity of HBV pregenomic RNA riboprobe (B) or nick-translated ^{32}P-labeled HBV DNA probe (C), respectively (see Materials and Methods). The nitrocellulose membrane of panel C was rehybridized with the ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase gene fragment (G3PDH) (63). The positions of relaxed-circular (RC) and linear (L) forms of HBV DNA, HBV 3.5-kb pregenomic RNA, and 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; wild type, S53A, S99A, S116A, S53/99A, S53/116A, S99/116A, and S53/99/116A, cells transfected with pSHH2.1 and pECE/HCVC-KF or its derivatives which carried the mutation(s) indicated. The designations for HCV core mutants are those from Fig. 5.

find a wide spectrum of cellular proteins associated with full-length and truncated forms of the core protein. Nevertheless, this unusual property may partially account for the technical difficulty involved in the immunoprecipitation of HCV core protein from cell lysates, a problem encountered by us and others (50).

Phosphorylation regulates the suppressive activity of HCV core protein. According to our previous study, HCV core protein can exert a suppressive effect on HBV gene expression (antigen production and transcript production) and virus RNA encapsidation (51). It is pertinent to know whether this suppressive activity of HCV core protein is affected by its phosphorylation status. When seven variants of an HCV core protein expression construct (as pECE derivatives) which harbored single or multiple serine-to-alanine substitution mutations at phosphorylation sites Ser-53, Ser-99, and/or Ser-116 were individually cotransfected with HBV plasmid pSHH2.1, the suppression of HBsAg production and HBe/HBcAg production in human hepatoma cell line HuH-7 was lost in all the constructs examined, except for the one with the mutation at Ser-53 (Table 1, experiment 1). Northern blot analysis of HBV-specific transcripts (3.5 and 2.1 kb) gave similar results (Fig. 8C), as did analysis of secreted HBV particles by endogenous-DNA polymerase repair assay (Fig. 8A) and quantitation of cytoplasmic core-associated HBV RNA (Fig. 8B). Therefore, these results indicate that phosphorylation at Ser-99 and Ser-116 is crucial for the suppressive activity of HCV core protein. Both PKA and PKC are presumably involved in regulation of the suppressive activity of HCV core protein. This conclusion is further supported by the results from the inhibitor experiment. As shown in Fig. 9, when HuH-7 cells were treated with a PKA or PKC inhibitor, HA1004 or H7 (35), respectively, the inhibitory activity of wild-type HCV core protein, as judged by assays of secreted HBsAg (Fig. 9A) and virus particles (Fig. 9B), was completely lost, compared with that of cells without an inhibitor. Taken together, our results indicate that the suppressive activity of HCV core protein in HuH-7 cells is positively regulated by PKA and PKC through the phosphorylation of Ser-99 and Ser-116. In addition, the observation of strong impairment of the encapsidation of the HBV pregenome (at least 20-fold repression) and to a lesser extent on transcription and antigen production (about 2- to 4-fold) by HCV core protein is consistent with our previous study (51).

Effects of the replacement of Ser-53, Ser-99, and Ser-116 phosphorylation sites with Asp on the *trans*-suppression activity of HCV core protein. Phosphorylation of a protein causes an increase in negative charge. Therefore, it is possible that the effects of phosphorylation on protein function could be reproduced by the addition of a negative charge at the phosphorylation site. To examine this hypothesis, we constructed HCV core protein expression vectors in which phosphorylation sites Ser-53, Ser-99, and Ser-116 were replaced with aspartate residues (Fig. 5). The *trans*-suppression activities of these mutant HCV core proteins on HBV gene expression (HBsAg- and

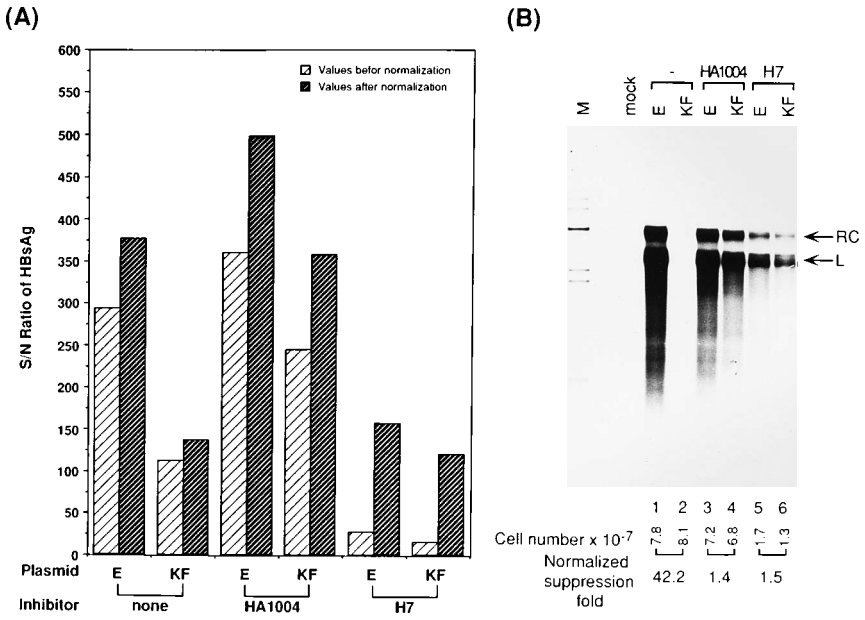

FIG. 9. Effects of HBV expression in kinase inhibitor-treated HuH-7 cells cotransfected with cloned HBV and HCV DNAs. HuH-7 cells were cotransfected with pSHH2.1 and pECE (E) or pECE/HCVC-KF (KF). On day 2 posttransfection, cells were treated with 0.1 mM HA1004 or H7 inhibitor (Sigma). Media collected on days 3 to 6 posttransfection were assayed for HBsAg with an EverNew enzyme immunoassay kit (A) or for HBV endogenous DNA polymerase activity (B) (see Materials and Methods). Cell numbers were determined with a hemacytometer, and the S/N (sample versus negative control) ratio of HBsAg (A) and the fold suppression (B) obtained after proportional normalization at 10^8 cells are indicated. M, *Hin*dIII fragments of lambda DNA. The positions of relaxed-circular (RC) and linear (L) forms of HBV DNA are indicated by arrows.

HBV-specific transcript production) and virus particle release were similar to those of mutants generated by the replacement of Ser with Ala at each corresponding site (Table 1, experiment 2) (Fig. 10). The same extent of abrogation of the *trans*-suppression activity in the S99A and S99D mutants or the S116A and S116D mutants strongly suggests that an acidic residue cannot mimic the effect of phosphorylation and further implies that the integrities of Ser-99 and Ser-116, but not that of Ser-53, are important for the *trans*-suppression activity of HCV core protein.

Mutations of PKA and PKC recognition sites Ser-53, Ser-99, and Ser-116 of HCV core protein did not block the entrance of core protein into the nucleus. We previously indicated that HCV core protein can target the nuclei of hepatoma cells on day 6 posttransfection, while it remains mainly in the cytoplasm on day 3 when visualized by indirect immunofluorescence (51). The subcellular localization of HCV core protein mutants was investigated to elucidate whether phosphorylation affects nuclear transport. As shown in Fig. 11, when the Ser-53, Ser-99, and/or Ser-116 sites of HCV core protein were replaced by alanine or aspartate residues, all mutant forms, including the three single mutants (S53D, S99D, and S116D), two double mutants (S53/99A and S53/116A), and one triple mutant (S53/99/116A), of HCV core protein still retained the ability of the wild type to translocate into the nucleus on day 6. Thus, the loss of suppression activity in these mutant forms of HCV core protein, if any, is not accounted for by decreased expression or defective nuclear localization. Although this finding strongly suggests that the phosphorylation of these serine residues by PKA and PKC is not required for nuclear translocation of HCV core protein, whether phosphorylation at other sites may play a role in regulation of nuclear transport remains an open question.

DISCUSSION

Phosphorylation is one of the major mechanisms by which the activities of protein factors can be regulated (29). Phosphorylation can regulate the activities of transcription factors at multiple levels, including nuclear transport, dimerization,

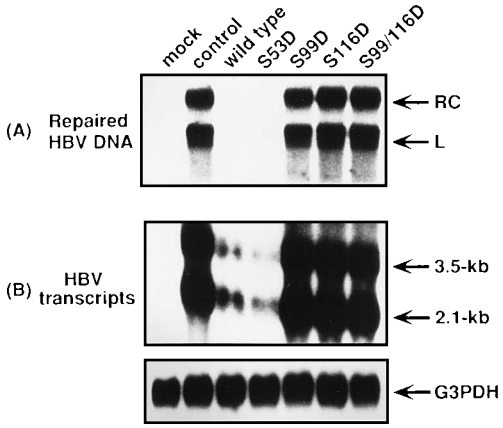

FIG. 10. Effects of HCV core protein variants with Ser-to-Asp substitution mutations on HBV expression. HuH-7 cells were cotransfected with HBV plasmid pSHH2.1 and various HCV core constructs. On day 6 posttransfection, released HBV particles and total cellular RNAs were prepared for HBV endogenous polymerase activity assay (A) and Northern blotting with the nick-translated ^{32}P-labeled HBV DNA probe (B) (see Materials and Methods). The nitrocellulose membrane of panel B was rehybridized with the ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase gene fragment (G3PDH) (63). The positions of relaxed-circular (RC) and linear (L) forms of HBV DNA and 3.5- and 2.1-kb HBV-specific transcripts are indicated by arrows. The designations mock, control, and wild type are identical to those described in the legend to Fig. 8. The designations for HCV core mutants are those from Fig. 5.

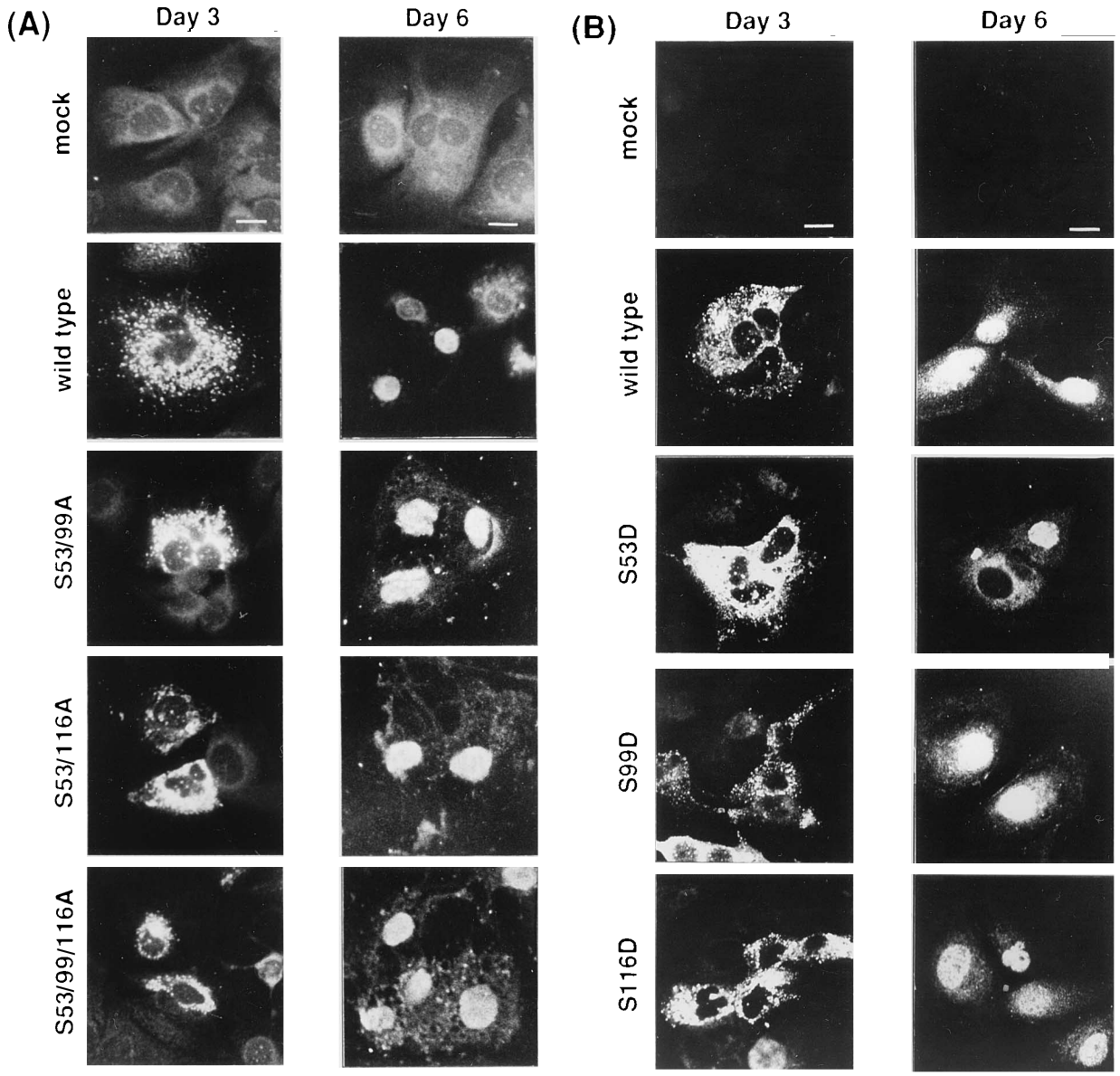

FIG. 11. Indirect immunofluorescence analysis of HCV core protein variant expression in transfected HuH-7 cells. HuH-7 cells were transfected with HCV constructs which harbored Ser-to-Ala (A) or Ser-to-Asp (B) substitution mutations and at days 3 and 6 posttransfection, cells were processed for the detection of HCV core proteins by indirect immunofluorescence staining with rabbit anti-HCV core fusion protein sera (1:600 dilution) (see Materials and Methods). Wild type, S53/116A, S53/99A, S53/99/116A, S53D, S99D, and S116D, cells transfected with plasmid pECE/HCVC-KF or its derivatives which carried the designated mutations. The designations for HCV core mutants are those from Fig. 5. All photographs were taken at the same magnification. Bar, 10 μm.

DNA binding, and transcriptional activation (29). In this study, we have demonstrated that HCV core protein is phosphorylated by PKA and PKC and that phosphorylation of Ser-99 and Ser-116 by these two kinases regulates the suppressive activity of HCV core protein on HBV gene expression and replication in HuH-7 cells. Additionally, this work excludes the phosphorylation of these two serine residues from a role in regulation of the core protein's nuclear transport. However, the precise role of phosphorylation in regulation of the suppressive actions of HCV core protein is not yet clear. Two basic mechanisms may account for modulation of the *trans*-suppression activity by phosphorylation. In the first, phosphorylation increases the affinity of the *trans*-suppressive domain for a component of transcriptional machinery (e.g., promoter, enhancer, or transcriptional factor) or encapsidation (e.g., pregenomic RNA, core, or polymerase of HBV) (5, 6) by generation of negatively charged acid residues. The second mechanism is that phosphorylation acts by affecting the conformation of the suppressive domain, which increases the affinity of HCV core protein for its target(s) (30). Our preliminary results indicate that the replacement of Ser-99 and Ser-116 with either aspartate or alanine abrogates the suppressive activity of HCV core protein equivalently, strongly suggesting that the mechanism by which phosphorylation modulates the activity of HCV core protein involves more than merely the introduction of negatively charged phosphate groups at Ser-99 and Ser-116 per se. Phosphorylation of these two serine residues possibly catalyzes phosphorylation at more distant sites in HCV core protein and

consequently may well elicit other, more significant alterations, such as conformational changes. A similar allosteric model for phosphorylation-induced modulation of transcriptional activity has been proposed for the transcriptional factor Oct-2 (57), and cyclic AMP (cAMP) response element-binding protein (19, 41). Further understanding of the biochemical mechanism by which phosphorylation affects the inhibitory activity of HCV core protein will require the identification of the physiological target with which it interacts.

Our finding that PKC fails to phosphorylate the full-length core protein (p22) unless the protein has undergone truncation (Fig. 7) suggests that native, unphosphorylated HCV core protein is presumably folded in a structural conformation that precludes phosphorylation of potential recognition sites by PKC. In contrast, at least the Ser-53 and Ser-116 sites are accessible to PKA (Fig. 6). Therefore, the tertiary structure of the intact protein is a critical determinant for substrate recognition by kinases. Specifically, this may explain our observation that Thr-11, Thr-15, and Thr-49 are cryptic sites for both kinases in vitro. However, this finding does not preclude a role for phosphorylation of HCV core protein at these sites. Indeed, they may be unmasked in some physiological conditions and thus become targets for kinases.

The significance of the requirement for phosphorylation by two kinases is presently unknown. In principle, phosphorylation at multiple sites by different protein kinases can result in regulation at several distinct levels. Its occurrence in HCV core protein may be important for a proper order in folding core protein. In light of our findings that intact HCV core protein (p22) is not a substrate for PKC and that phosphorylation at both Ser-99 and Ser-116, catalyzed by PKA and PKC, is essential for *trans* suppression, one can think of a possible scenario in which the two kinases may function in a sequential manner. It is possible that phosphorylation of Ser-116 precedes that of Ser-99 and that the placement of phosphate groups at these two sites provides the requisite conformation of core protein for interaction with its target(s). Consistent with this notion are several well-documented examples, glycogen synthase (17), the modulator subunit of protein phosphatase 1 (2, 15), cAMP response element modulator (14), and phosphoprotein of vesicular stomatitis virus (54).

HCV core protein is a multifunctional protein with several functional motifs, including basic charged residues, putative nuclear localization signals and DNA binding motif, and several PKA and PKC recognition sites (51). Our initial attempt to locate the suppressive domain of HCV core protein suggests the importance of the C-terminal 21-amino-acid segment which encompasses residues 101 to 122. Within this region are two Arg-rich motifs, the SPRG sequence which constitutes the putative DNA binding motif (53) and the recognition site for PKA. From this study, it is clear that our earlier report of the loss of suppressive activity by the N-terminal 101-amino-acid segment of HCV core protein may be partially accounted for by the lack of phosphorylated Ser-116 and the loss of phosphorylation at Ser-99 because of truncation of the recognition site for PKC (Fig. 2A). This 21-residue suppressive segment may also be involved in the interaction of HCV core protein with other protein components, such as the basal transcriptional factors. Support for this notion comes from our recent finding that a chimera which contains the GAL4 DNA binding domain (residues 1 to 147) and amino acid residues 101 to 191 of HCV core protein confers *trans*-repression activity on a reporter gene with GAL4-binding sites (9). In such a chimera, the replacement of Ser-116 within HCV core protein with an alanine residue does not alter its suppressive activity (9), suggesting that overall conformation of the suppressive domain rather than acidity alone is vital to *trans* suppression. The remaining issue of whether the six arginine residues (residues 101, 104, 113 to 115, and 117) (51) within this segment play a role is currently being investigated in our laboratory.

Virus capsid proteins are primarily designed to provide a protein shell for the virus genome; therefore, its constitutent core protein may generally exhibit affinity for nucleic acid and also have the property of dimerization or oligomerization, which is well-known for most viruses, if not all. In this study, we have no evidence that phosphorylation regulates the nucleic acid-binding ability or specificity of core protein. However, we have evidence to suggest that phosphorylation may modulate virus capsid formation. If we consider the p44 species to be the dimer form of the full-length core protein, p22, the results suggest that in marked contrast to the monomer form, p22, the dimer exhibits a drastic increase in the Ser-53–to–Ser-116 ratio of phosphorylation (23:52 versus 3:51; Fig. 6B). This suggests that the interaction of core protein imposes phosphorylation preferentially on Ser-53. Alternatively, phosphorylation at Ser-53 may modulate dimer formation or oligomerization of core protein. Similar examples which suggest that phosphorylation controls the dimeric state or vice versa are the transcriptional antiterminator protein BglG for the *bgl* operon in *E. coli* (3) and transcriptional factors Fos and Jun (1). In the former case, the phosphorylation and dephosphorylation of BglG regulate its activity by controlling its dimeric state (3). In the latter cases, phosphorylation of Fos and Jun by several protein kinases (e.g., PKA, PKC, casein kinase II, and cdc2 kinase) is affected by dimerization and binding to DNA (1).

In conclusion, this study raises the possibility that two signal pathways, one which is cAMP dependent (PKA) (58, 59) and another which is growth promoting and tumor promoter inducible (PKC) (45), participate in the activity of HCV core protein. Besides being a component in the virion, core protein is likely to act as a nuclear target at which other distinct signalling pathways may converge and/or cross-talk. Further studies are required to establish if a functional effect of phosphorylation on HCV virion assembly and replication exists and whether there are some other kinases which control these processes in discriminating the multiple states of core protein within cells. Analysis of the in vivo functions of HCV core protein phosphorylation will contribute to a better understanding of the role of these events in the virus life cycle as well as its pathogenesis in host cells.

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