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# Posttranscriptional Regulation of Hepatitis B Virus Replication by the Precore Protein

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**Hepadnaviruses encode two core-related open reading frames. One directs the synthesis of the p21 core protein, which subsequently becomes a structural component of the viral nucleocapsid. The other produces a p25 precore protein that is targeted by a signal peptide to a cell secretory pathway where N-terminal processing will create a p22 species. This molecule will be further modified at the C-terminal region to generate p17, and the truncated protein is secreted from the cell as hepatitis B e antigen (HBeAg). The function of the precore gene in the biology of hepadnaviruses is unknown. We found that ablation of the precore gene resulted in the generation of a hepatitis B virus (HBV) species with a high-replication-level phenotype. More important, expression in *trans* of physiologic levels of p25 restored viral replication to wild-type levels. Moreover, transient or stable overexpression of the precore gene resulted in striking inhibition of HBV replication. The molecular species responsible for this viral inhibitory effect was identified as the p22 nonsecreted HBeAg precursor protein. By sucrose gradient sedimentation analysis, we determined that expression of p22 leads to the formation of nucleocapsids similar to those made with wild-type p21 core protein. Immunoprecipitation experiments revealed that the p21 and p22 physically interact and form hybrid nucleocapsid structures devoid of pregenomic viral RNA. These experiments suggest that expression of the precore gene may be important in the regulation of HBV replication and describe a possible molecular mechanism(s) for this effect.**

Hepatitis B virus (HBV) is the prototype member of the hepadnavirus family, a group of enveloped DNA viruses that primarily infect the liver. HBV infection may lead to significant liver disease, ranging from acute liver failure to chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (23). The HBV genome consists of a partially double-stranded 3.2-kb DNA molecule arranged in a relaxed circular conformation. Sequence analysis of the genome reveals that HBV encodes four partially overlapping open reading frames (ORF) that direct the synthesis of at least seven viral gene products. This expansion of coding capacity is due to the presence of multiple in-frame initiation codons within the precore-core and envelope ORFs, which generate proteins with a common carboxy terminus but extended amino-terminal regions. This phenomenon is exemplified by the precore-core ORF. The genomic promoter of HBV directs the synthesis of two types of 3.5-kb transcripts that differ at their 5′ initiation sites. The shorter transcript is initiated 5 nucleotides downstream from the precore initiation codon (49). It serves as the mRNA for the core and the polymerase genes (10). This mRNA species following encapsidation into viral nucleocapsids represents the template for reverse transcription to generate viral DNA (3, 19). The second transcript has a 5′-end extension that direct the synthesis of the precore gene product, but this molecular species is not packaged into viral nucleocapsids (29, 50).

The 21-kDa viral core protein assembles into a 180-subunit nucleocapsid structure (4, 14). This molecule is also involved in nucleic acid binding and promotes viral replication (18, 30). Translation of the precore gene results in a core-related polypeptide designated p25. The precore protein has a 29-amino-acid (aa) amino-terminal extension. In this regard, the first 19 aa acts as a signal peptide and directs the protein into the secretory pathway of the cell (6, 35). The 19-aa peptide is subsequently cleaved to generate a p22 intermediate protein product that is either translocated to the endoplasmic reticulum or released back into the cytoplasm (15). In the endoplasmic reticulum, p22 is cleaved at the carboxy terminus in an arginine-rich domain to create a 17-kDa soluble hepatitis B e antigen (HBeAg) that will be secreted from the cell (23).

The function of HBeAg in the biology of HBV infection is unknown. HBeAg is found in the serum of HBV-infected individuals, where it generally correlates with high levels of viremia. HBV titers have been found to decrease in serum when there is a detectable anti-HBeAg immune response (23). It has been proposed that the precore protein or the processed shorter p22 form may play a role in virion morphogenesis (6, 35). There is also evidence to suggest that HBeAg may function as a circulating protein that blocks cytotoxic T-cell activity against HBV core-associated epitopes (25). Finally, it has been demonstrated that HBeAg determinants are expressed on the surfaces of infected hepatocytes and present HBeAg and hepatitis B core antigen (HBcAg) epitopes in the context of HLA class I molecules to the host immune response (41).

However, a functional precore gene appears not to be essential for viral replication, particularly in animals experimentally infected with the related duck hepatitis B virus (9, 40) and woodchuck hepatitis virus (11). With respect to HBV, genomes defective in HBeAg synthesis are frequently found in individuals with chronic infection. The most common mutation detected is a TGG-to-TAG transition that introduces an amber termination signal at codon 28 in the precore ORF. This naturally occurring HBV mutant has been associated with fulminant hepatitis and high levels of viral replication (8, 21, 34) as well as with chronic infection (5, 7, 28, 32, 46). However, the question of whether HBeAg-negative HBV genomes are associated with a more severe form of chronic liver disease is still controversial. In this respect, one study demonstrated that

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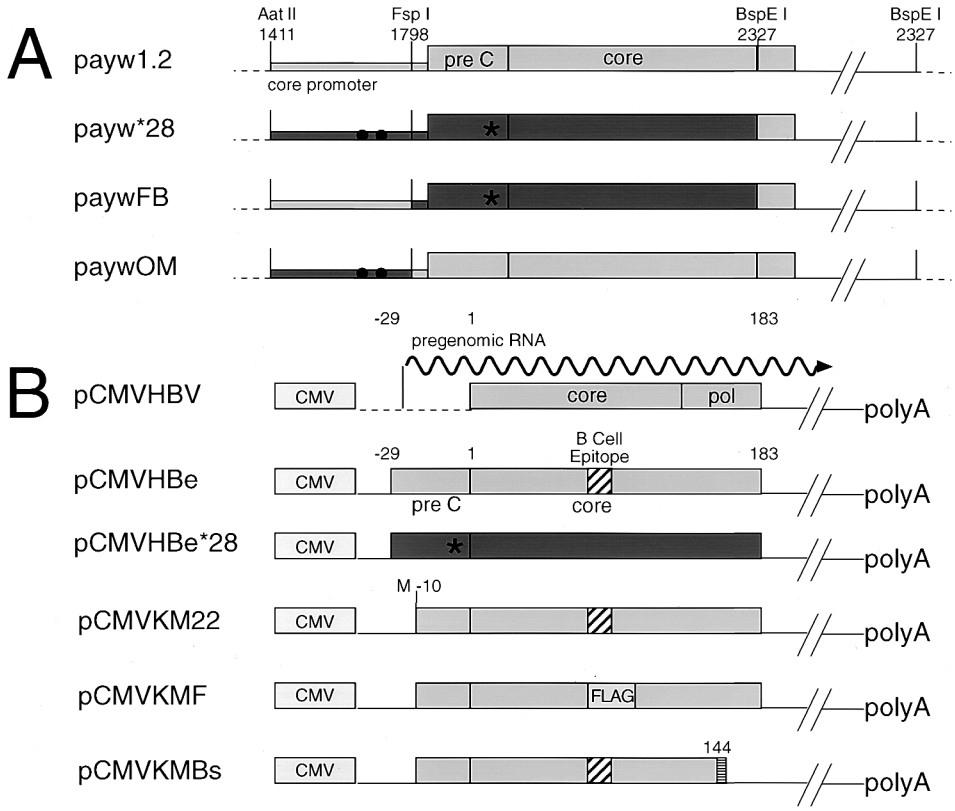


FIG. 1. Genetic organization of the plasmids used in this study. (A) payw1.2, payw*28, paywFB, and paywOM are more-than-one-genome-length constructs that express HBV pregenomic RNA from the homologous promoter. HBV DNA derived from restriction sites *Aat*II (1411) to *Bsp*EI (2327) was cloned into the *Aat*II-*Sma*I sites of pGEM7 Zf(+), as represented by the dotted lines. The core promoter region and precore and core ORF are depicted. The darker areas correspond to DNA exchanged from a naturally occurring HBV mutant carrying the amber termination signal at codon 28 in the precore ORF (*) and point mutations in the core promoter (●). Relevant restriction sites used to define the exchanged fragments are indicated. (B) pCMVHBV expresses the pregenomic RNA. pCMVHBe expresses the entire precore and core ORF, whereas pCMVHBe*28 contains the amber mutation at codon 28 in the precore ORF (*). The position of the first amino acid in precore ORF is indicated as −29. pCMVKM22 expresses a cDNA with an engineered ATG in front of serine$^{-10}$ in the precore ORF. The pCMVKMF construct is identical to pCMVKM22 except that a FLAG epitope has been engineered into the loop (hatched area) of the core protein. The pCMVKMBs construct expresses a protein that stops at proline$^{144}$ of the core molecule. A polyadenylation signal was provided by the pcDNA3 vector in all plasmids except pCMVHBV, where the polyadenylation site is derived from the endogenous HBV sequence.

transfection of an HBeAg-negative genome into human HCC cells resulted in increased viral replicative forms compared to wild-type HBV (20). In the present study, we investigated if the HBV precore gene influences viral replication following transient and stable transfection into HCC cells.

## MATERIALS AND METHODS

**Plasmid constructs.** The design of the plasmid vectors utilized in this study is depicted in Fig. 1. The plasmids payw1.2 and pCMVHBV express HBV pregenomic RNA under the control of the endogenous or the cytomegalovirus (CMV) immediate early (IE) promoters, respectively. These vectors allow HBV replication to occur in HCC cells and in cells of nonhepatic origin (12, 43). The payw1.2 construct contains more than one genome length of HBV (13) and carries the genomic fragment between the *Aat*II (nucleotide [nt] position 1411, where nt 1 [underlined] is by convention located at GAATTC in the unique *Eco*RI site) and the *Bsp*EI (nt 2327) sites; the 3′-end *Bsp*EI site was blunted by Klenow DNA polymerase I to allow cloning into *Aat*II and *Sma*I restriction sites of the pGEM7 Zf (+) vector (Promega Corporation, Madison, Wis.).

The plasmid payw*28 has a 0.9-kb *Aat*II-*Bsp*EI fragment derived from the plasmid pC*28 (47). This construct carries the nonsense TGG-to-TAG mutation that introduces a stop codon in the precore ORF at codon 28. This mutant DNA fragment also carries 14 other nucleotide substitutions in the precore-core ORF. In particular, it carries the sequence ATGAT (wild-type ayw has AAGGT), which has been found in naturally occurring HBV mutants either alone or in combination with the stop codon 28 mutation in the precore ORF (33). By exchanging the *Fsp*I (1798)–*Bsp*EI (2327) fragment in payw1.2, we generated a plasmid, paywFB, that contains only the stop codon at position 28 in the precore ORF. The counterpart paywOM construct contains the *Aat*II-*Fsp*I fragment from pC*28 and therefore has the sequence ATGAT (1759 to 1763) but lacks the stop at codon 28 in the precore ORF.

Plasmid pCMVHBe expresses the precore ORF under the control of the CMV IE promoter. The precore ORF was obtained from the plasmid paywSP2 (unpublished data), which harbors a deletion in the payw1.2 genome between nt 2471 and 486. This deletion ablates the HBV pre-S1 and pre-S2 genes and a large portion of the HBV polymerase genes but leaves the precore-core ORF intact. The paywSP2-derived *Fsp*I (1798)–*Spe*I (677) fragment was cloned into the *Eco*RV and *Xba*I sites of the pcDNA3 polylinker (Invitrogen Co., San Diego, Calif.). As a control, we generated the plasmid pCMVHBe*28, containing, in the same unit, a stop codon at position 28 in the precore-core ORF. To make this construct, a plasmid designated paywSP2*28 was generated by cassette exchange of the 0.9-kb *Aat*II-*Bsp*EI fragment obtained from pC*28 and inserted into the backbone of paywSP2. From this plasmid, a *Fsp*I-*Spe*I DNA fragment was obtained and cloned into the *Eco*RV and *Xba*I sites of the pcDNA3 expression vector. This vector does not allow the synthesis of either p25 precore or p21 core proteins, as shown by in vitro translation experiments in a wheat germ extract system (data not shown).

Plasmid pCMVKM22 expresses the precore protein lacking the signal peptide sequence under the control of the CMV IE promoter. The coding sequence was modified by PCR amplification using the sense primer KM22 (5′ CGGGGTAC C**ATG**TCCAAGCTGTGCCTTGGGTG 3′) which engineers a *Kpn*I restriction site (underlined) upstream from an artificial ATG codon (bold), and SP6 as the antisense primer. The DNA template employed was a *Sma*I-linearized pCMVHBe plasmid. We carried out 25 cycles of PCR amplification (1 min at 95°C, 1 min at 40°C, and 1 min at 72°C) in a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM (each) deoxynucleoside triphosphates, 2 mM $MgCl_2$, 50 pM each primer, and 2.5 U of *Taq* DNA polymerase/100 μl of reaction mixture (Roche Molecular Systems, Inc., Branchburgh, N.J.). The amplified DNA was cut by *Kpn*I and *Apa*I restriction enzymes and cloned into the unique

*Kpn*I-*Apa*I sites in the pcDNA3 polylinker. This plasmid directs the synthesis of a protein that has serine$^{-10}$ as the second amino acid of the p22 precore protein. A carboxy-terminal truncated version of pCMVKM22 was generated by digestion with the single *Bsp*EI cutter and filling-in of the staggered ends by Klenow DNA polymerase I followed by plasmid self-ligation to generate the vector pCMVKMBs. Expression of this vector generates a protein of 18 kDa; the amino acid sequence following the core proline$^{144}$ is AlaGlyAspTyrCysCysAmber. Moreover, to differentially immunoprecipitate the wild-type p21 core protein from p22 synthesized by pCMVKM22, a derivative was generated (pCMVKMF) that contained an in-frame FLAG epitope (Eastman Kodak Co., New Haven, Conn.) engineered into the B-cell epitope loop at aa 74 to 83 (45) of the core protein, which was substituted for wild-type aa 78 to 81. This modification was accomplished by PCR with a reverse primer containing an *Xba*I site, the FLAG sequence, and the HBV-specific sequence (5′ GCTCTAGACTTGTCATCGT CGTCCTTGTAATCTTCCAAATTAACACCCACCCAGG 3′) and with the above-described KM22 sense primer. The DNA template employed was a *Sma*I-linearized pCMVKM22 plasmid, and PCR was carried out as described above. The PCR product was digested with *Xba*I, and the 166-bp fragment was gel purified and cloned into *Xba*I-digested pCMVKM22.

The retroviral pBabepuro vector (27) was used to express the precore ORF under the transcriptional control of the retroviral long terminal repeat. For this purpose, the pCMVHBe plasmid was digested with *Bam*HI, which cuts 5′ to the precore cDNA start in the pCMVHBe polylinker and again at nt 486 in the ayw genome. The *Bam*HI insert was then cloned into the *Bam*HI site of the pBabepuro polylinker and controlled for correct orientation to generate the plasmid pBP HBe. Finally, the plasmid pCMV Luc(+) was utilized to monitor the transfection efficiency in HCC cells. This plasmid contains the firefly luciferase gene under the control of the CMV IE promoter and inserted into the pcDNA3 expression vector.

Assessment of the correct design of the plasmids was ascertained by restriction digest mapping and by direct DNA sequencing. The restriction enzymes were provided by New England BioLabs (Beverly, Mass.). Sequencing reactions were carried out with the Sequenase version 2.0 enzyme (U.S. Biochemicals, Cleveland, Ohio). Plasmid DNAs were grown in *Escherichia coli* JM109 cells and purified by a commercially available kit by using the manufacturer's instructions (Wizard Maxiprep kit; Promega Co.).

**Tissue culture.** The HepG2 and HuH-7 lines were utilized, since they will support a complete viral replication cycle and produce infectious virions following transient transfection with HBV containing plasmids (1). The highly transfectable HEK 293 human embryo kidney-derived cell line (16) was also utilized, since it efficiently supports viral replication following transfection with a plasmid expressing the HBV pregenome under the control of CMV IE promoter (pCMVHBV) and to a lesser extent the payw1.2 construct driven by the endogenous viral promoter.

HepG2, HuH-7, and HEK 293 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Ten million cells, seeded in a 10-cm-diameter dish, were transiently cotransfected by means of the calcium phosphate procedure ($CaPO_4$ transfection kit; 5′-3′, Inc., Boulder, Colo.) with 10 μg of a construct expressing wild-type HBV together with different amounts of the other plasmid constructs. Plasmid pGEM7 Zf(+) was added to keep the final amount of transfected DNA constant. The HepG2215 cell line constitutively produces infectious virions (44). Two pools of cells called HepG2215BP and HepG2215BPHBe were derived from this parental cell line by infection with the parental Babepuro and BabepuroHBe retroviral stocks, respectively (26). Transfection efficiency was monitored by adding 1 μg of pCMV Luc(+) to the transfection reaction mixtures, and approximately 1/100 of the cell lysate was subjected to the luciferase assay (2). After transfection, cells were harvested at 2, 3, and 5 days for RNA, protein, and DNA analyses, respectively.

**Analysis of viral DNA replication.** HBV DNA replication following transient transfection of cell lines was assessed by Southern blot analysis of viral DNA extracted from purified intracellular core particles as described by Pugh et al. (37). The same technique was used to evaluate HBV DNA replication in the HepG2215BP and HepG2215BPHBe cell lines. In these experiments, $5 \times 10^6$ cells were seeded in a 10-cm tissue culture dish. The cells were harvested and counted and the capsid-associated viral DNA was measured after 3 days of culture as described above. DNA was fractionated on agarose gels in 1× TAE buffer and transferred onto Hybond N+ (Amersham International, Little Chalfont, United Kingdom) for Southern blot analysis. HBV DNA was detected by hybridization with a randomly primed $^{32}$P-labeled full-length HBV probe. Prehybridization, hybridization, and washings were performed as previously reported (24).

**Detection of viral proteins.** Human HCC cells transfected with the various expression vectors were lysed at 4°C by the addition of 500 μl of a mixture containing 10 mM Tris-Cl, 100 mM NaCl, and 1 mM EDTA (TNE), 1% Nonidet P-40 (NP-40), and protease inhibitors (Boehringer Mannheim Corp., Indianapolis, Ind.). The cellular lysates were cleared of nuclei and cellular debris by centrifugation at 10,000 × *g* for 1 min. Cell lysates were mixed with Laemmli sample buffer, boiled for 5 min, and electrophoresed through a sodium dodecyl sulfate–15% polyacrylamide gel (Protogel; National Diagnostics, Atlanta, Ga.). The separated proteins were then blotted onto Immobilon-P membranes (Millipore Co., Bedford, Mass.) (17). HBcAg and HBeAg were detected by a rabbit polyclonal antiserum raised against recombinant core protein (Dako Co., Carpinteria, Calif.) as described elsewhere (39). The same antibody was used for immunoprecipitation of core nucleocapsids at a 1:250 dilution in TNE–1% NP-40 buffer. The anti-FLAG antibody was used for both immunoprecipitation and Western blot analysis. Bound antibody was detected by horseradish peroxidase-labeled goat anti-rabbit or anti-mouse immunoglobulin G antibodies and revealed by the chemiluminescence method (SuperSignal; Pierce, Rockford, Ill.). Exposure was performed with NEN reflection films (Dupont Company, Boston, Mass.) for 5 to 20 s. Detection of HBeAg in the transfected-cell supernatants was performed by a specific radio immunoassay kit for HBeAg (EBK $^{125}$I RIA KIT; Incstar Corporation, Stillwater, Minn.). Measurement of hepatitis B surface antigen (HBsAg) in the cell culture supernatants was performed with a radioimmunometric assay as described previously (24).

**Viral nucleocapsid isolation.** Transiently transfected HEK 293 cells were lysed, and 200-μl aliquots of the clarified cell lysates were ultracentrifuged at 500,000 × *g* through 2 ml of a 20% sucrose-TNE (wt/wt) cushion for 1 h at 4°C by using a TLA 100 rotor (Beckman Instruments, Palo Alto, Calif.). Under these conditions, viral core particles are pelleted whereas free core protein and soluble HBeAg remain in the supernatant (52). The pelleted material was directly analyzed by Western blot analysis or again subjected to ultracentrifugation on sucrose gradients. The resuspended pellet was layered onto 2 ml of a 25 to 60% (wt/wt) sucrose–TNE–1% NP-40 mixture. Gradients were established by ultracentrifugation at 55,000 rpm (corresponding to 200,000 × *g*) with a TLS55 rotor for 1 h at 4°C. Fifteen 150-μl aliquots were collected and proteins were concentrated as described previously (22). The resuspended pellet was electrophoresed through a sodium dodecyl sulfate–15% polyacrylamide gel followed by Western blot analysis.

**Extraction and analysis of viral RNA.** Total RNA was extracted at 2 days after transient transfection as described previously (39). Core particles derived from the cytoplasm of transfected cells were immunoprecipitated with anticore antibodies, and protein A-Sepharose was added. The precipitate was washed with lysis buffer and encapsidated viral RNA was extracted as previously described (38) followed by gel electrophoresis and Northern blot analysis of total and encapsidated viral RNA (2).

**Generation of recombinant retroviral vectors and infection of HCC cell lines.** The plasmids pBabepuro and pBabepuroHBe were used to generate recombinant retroviruses (26). Characterization of the packaging cell lines Bosc 23 and PA317 (American Type Culture Collection, Rockville, Md.) as well as the conditions for their maintenance and infection have been described (26, 36). Briefly, the pBabepuro and pBP HBe plasmids were transiently transfected into the ecotropic packaging cell line Bosc23. Two days later, the culture supernatant was harvested, and polybrene was added (final concentration, 8 μg/ml) and used to infect the amphotropic packaging cell line PA317. Two days after infection, 2 μg of puromycin per ml was added to the culture medium. The medium derived from drug-resistant cells contained recombinant amphotropic retroviruses, and it is important to note that PA317 cells infected with pBP HBe were shown to have HBeAg in the supernatant as well. Viral titers were measured as described elsewhere (2). HepG2215 cells were infected with $10^6$ CFU of recombinant retrovirus per ml. After 2 weeks of drug selection, pooled puromycin-resistant clones were expanded and designated HepG2215BP and HepG2215BPHBe, respectively.

## RESULTS

**A stop codon in the precore ORF generates a high-replication-level viral phenotype.** Increased levels of HBV replication were observed in HCC cells transiently transfected with payw*28 vector containing a stop signal at codon 28 in the precore ORF. Southern blot analysis of purified HBV core particles demonstrated that transfection of payw*28 alone produced a severalfold increase in HBV DNA replicative forms compared to the wild-type payw1.2 construct (Fig. 2). The difference between the two plasmids resides only in mutations present in the precore promoter and precore ORF. To further analyze the specific mutation(s) responsible for this phenotype, a selected DNA fragment was cassette exchanged into parental payw1.2. This paywFB construct now carries the amber mutation at codon 28 of the precore ORF and produces a high-replication-level phenotype comparable to that of the mutant payw*28 genome. paywOM, a construct that carries a 2-nt substitution in a putative transcription factor binding site of the precore promoter region (51), yielded substantially the wild-type level of HBV replication (Fig. 2). To ascertain that the difference in HBV replicative pattern was not related to the cell type used for transfection, experiments were performed with both HepG2 and HuH-7 HCC cell lines, and similar results were obtained, as shown in Fig. 2.

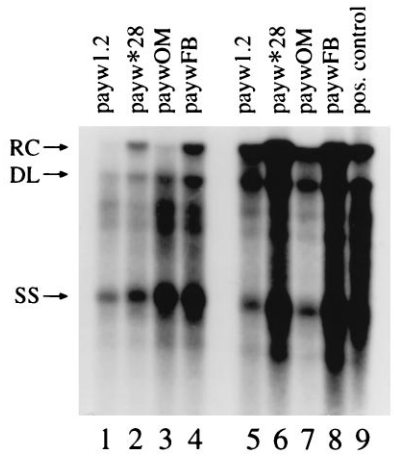


FIG. 2. A stop signal at codon 28 in the precore ORF leads to high-level viral replication. A Southern blot of HBV DNA extracted from intracellular core particles following transient transfection of HepG2 cells (lanes 1 to 4) and HuH-7 cells (lanes 5 to 8) is shown. DNA of wild-type HBV virions extracted from HepG2215 cells was used as a positive (pos.) control. The arrows on the left indicate the relaxed circular (RC), double-stranded linear (DL), and single-stranded (SS) HBV DNA species.

**Precore protein expression inhibits HBV replication.** Next, the effect of the precore gene product on HBV replication was evaluated. The wild-type payw1.2 HBV-expressing construct was cotransfected with a plasmid expressing the full-length precore protein (pCMVHBe). Under these conditions there was striking reduction of wild-type HBV replication. In contrast, this level of wild-type HBV DNA replication was not affected when the payw1.2 construct was cotransfected with a plasmid (pCMVHBe*28) incapable of producing both the precore and core proteins (Fig. 3A). The same inhibition of HBV replication was observed when pCMVHBV was cotransfected with pCMVHBe (see Fig. 5B). The pCMVHBV construct expresses the pregenomic RNA but does not express a precore transcript (HBeAg is absent in supernatant derived from transfected cells) due to the positioning of the transcription initiation site downstream from the CMV IE promoter. In order to assess experimental variability, all transfections were repeated at least five times, with comparable results. From these experiments it appears that the lack of a functional precore gene results in enhanced HBV replication and that expression of the HBV precore gene product results in striking inhibition of HBV DNA replication.

Precore gene expression did not appear to have an adverse effect on transfected cells at the plasmid concentrations used in this study. Indeed, HCC cells were routinely analyzed by light microscopy, and no evidence of cytotoxicity was observed (data not shown). Moreover, we evaluated whether HBV precore gene expression negatively influences the activity of a panel of transcriptional elements controlling the expression of the firefly luciferase gene, including HBV precore promoter and enhancer I, the HBV core promoter, AP1 responsive elements, the Rous sarcoma virus long terminal repeat, the simian virus 40 promoter and enhancer, and the CMV IE promoter. These elements were not significantly influenced by cotransfection of pCMVHBe at a DNA molar ratio varying from 20:1 to 1:1 (data not shown).

**Inhibition of HBV replication in HepG2215 cells by a retrovirus expressing the precore protein.** It was of interest to determine if the expression of the precore ORF in a different experimental system would also inhibit HBV replication. Replication of HBV DNA was substantially reduced in HepG2215BPHBe cells compared to that in empty retroviral vector-infected HepG2215BP cells (Fig. 3B). Densitometric scanning analysis estimated that the HBV DNA content of these cells was reduced by at least 90% compared to that in the mock virus-infected cells. The cell culture supernatants were assessed for the HBsAg levels, and there was no difference compared to the levels found in the parental HepG2215 cell line. Finally, the retrovirus-infected HepG2215 cells appeared to be identical to the noninfected parental cell line with respect to growth rate and cellular morphology (data not shown).

**Overexpression of the HBV precore gene leads to alteration of the nucleocapsid structure.** It is possible that the precore protein was acting as a dominant negative factor on the nucleocapsid assembly process, as had been previously observed with a mutant core-surface envelope protein (39). Thus, the capsid structure formed in HuH-7 cells transfected with pCMVHBV alone, with pCMVHBV and pCMVHBe in combination (at a 1:1 molar ratio), and with pCMVHBe alone was evaluated. A single 21-kDa band corresponding to the wild-type HBV core protein was detected in cells transfected with pCMVHBV alone (Fig. 4A). It was of interest that a fainter core-immunoreactive band with a lower electrophoretic mobility, of about 22 kDa, was also found in cells cotransfected with pCMVHBV and pCMVHBe. The same p22 core-immunoreactive protein was detected in cells transfected with the pCMVHBe alone. No HBV core-immunoreactive bands were detected in the lysate derived from the mock DNA-transfected cells. The cell lysate-derived material retained on top of the sucrose cushion was analyzed by West-

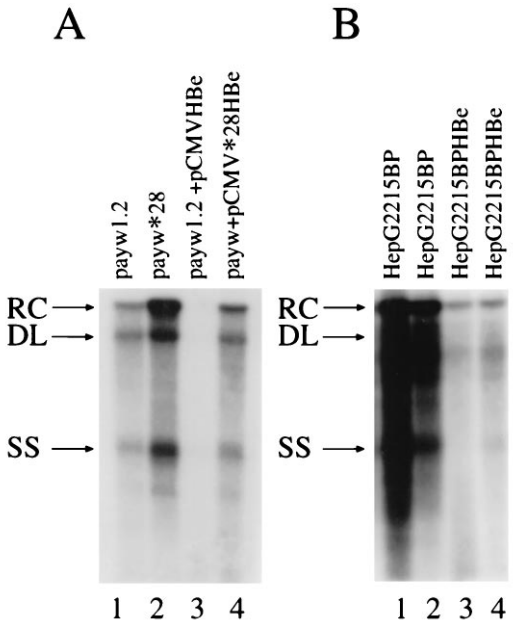


FIG. 3. Expression of the precore ORF inhibits HBV replication. (A) Southern blot analysis of HBV DNA extracted from intracellular core particles following transfection of HuH-7 cells with payw1.2, illustrating the level of wild-type HBV replication, with payw*28, a plasmid carrying a stop signal at codon 28 of the precore gene, with equal amounts of payw1.2 and pCMVHBe, or with payw1.2 and pCMVHBe*28. (B) Southern blot analysis of intracellular core particles derived from HepG2215 cells infected with a recombinant retrovirus that expresses the precore gene. HBV DNA was extracted from two independent pools of HepG2215 cells stably transduced with the parental Babepuro retrovirus illustrating the level of HBV replication (lanes 1 and 2) or from two independent pools of HepG2215 infected with a Babepuro retrovirus expressing the HBV precore gene (lanes 3 and 4).

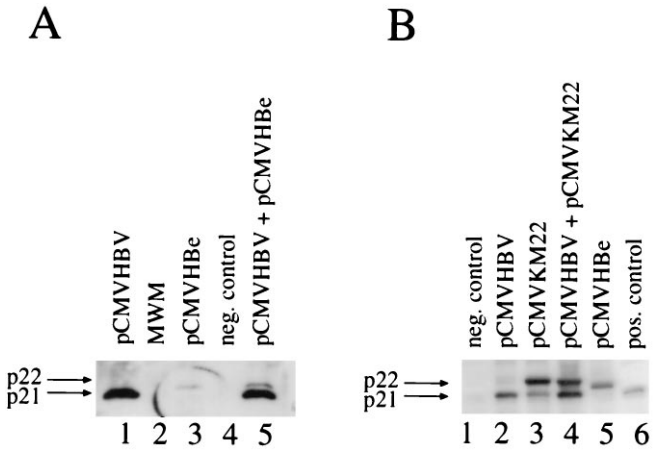


FIG. 4. Processed precore protein assembles into nucleocapsids. (A) Western blot analysis of pellets derived from transfected HuH-7 cells. The 21-kDa core protein was found in viral nucleocapsids following transfection with pCMVHBV. The 22-kDa core-reactive polypeptide was found in cells transfected with pCMVHBe. Cells cotransfected with the pCMVHBV and pCMVHBe constructs showed both the p21 and p22 core-related polypeptides. Mock-transfected cells served as a negative (neg.) control. MWM, molecular weight markers. (B) Western blot analysis of pellets from HEK 293 cells transfected with various constructs. The p21 core protein was derived from nucleocapsids following transfection with pCMVHBV; cells transfected with pCMVKM22 showed core-related polypeptides of 22 kDa and a weaker, 21-kDa, band. p21 and p22 core-related peptides were detected in cells cotransfected with pCMVHBV and pCMVKM22. Cells transfected with pCMVHBe showed a 22-kDa core-reactive polypeptide. The negative (neg.) control was mock-transfected cells, and the positive (pos.) control was derived from HepG2215 cells.

ern blot analysis. There were no p21 or p22 HBV core-reactive proteins when the material was studied at a protein concentration comparable to that in the lanes loaded with the resuspended pellets (data not shown). Finally, the 17-kDa secreted form (HBeAg) of the precore protein was not detected in the cytoplasm of transfected cells by Western blot analysis.

**Expression of the p22 nonsecreted HBeAg precursor protein inhibits HBV DNA replication.** The size of the p22 protein closely resembles that of the processed nonsecreted HBeAg precursor protein (15, 31, 42, 45). Thus, selective expression of this protein was studied with respect to effects on HBV replication. pCMVKM22 will direct the synthesis of a precore-related peptide that lacks the signal peptide sequence. HuH-7 and HEK 293 cells were transiently transfected with pCMVHBV alone, pCMVHBV plus pCMVHBe, or pCMVHBV plus pCMVKM22, and the level of HBV replication was determined. The results obtained from HuH-7 and from HEK 293 cells were equivalent. Transfection of pCMVHBV in HEK 293 cells resulted in 20-fold-higher levels of HBV DNA than transfection in HuH-7 cells; the patterns of replicative forms in the two cell lines were indistinguishable. Cotransfection of pCMVHBV with pCMVHBe resulted in substantial reduction of viral replication. More striking was the experiment where pCMVHBV was cotransfected with pCMVKM22. Under these conditions, HBV replication was almost completely inhibited (Fig. 5B). This observation suggests that p22 was a more potent and direct inhibitor of HBV DNA replication than its immediate p25 precursor protein. Titration experiments were then performed, and pCMVHBe was found to maximally inhibit viral DNA synthesis when transfected at a 1:1 molar ratio with pCMVHBV. In contrast, maximal inhibition exhibited by pCMVKM22 was observed at a DNA molar ratio of 1:15 (data not shown). Therefore, based on a molar ratio of viral DNA, the pCMVKM22 construct was about 15-fold more potent in inhibiting HBV replication than the pCMVHBe construct.

The pCMVKM22 construct expressed a protein that was found in core-like particles when transfected either alone or in combination with pCMVHBV (Fig. 4B). Analysis of the pellet derived from cells transfected with these constructs demonstrated two protein bands of the same intensity. A minor band corresponding in size to the wild-type p21 core protein was evident in cells transfected with pCMVKM22. We speculate that this protein may have originated from the use of the core AUG codon of the KM22 mRNA (31). Analysis of pellets of cells transfected with pCMVHBe alone revealed that the core-reactive band migrated slightly faster than the KM22 polypeptide but slower than the wild-type p21 core protein (Fig. 4B). This may be due to the methione residue engineered into the KM22 protein, which would add an extra 130 Da to the polypeptide chain, or the faster-migrating p22 could represent a partially processed carboxy-terminal form of the translocated precore protein (31, 42).

**The p22 protein inhibits HBV replication by interfering with pregenomic RNA encapsidation.** The inhibition of viral replication may be due to the lack of pregenomic RNA encapsidation into the nucleocapsids. To evaluate this possibility, viral RNA was extracted from nucleocapsids immunoprecipitated from HEK 293 cells transfected with pCMVHBV or pCMVKM22 alone or in combination. Total cytoplasmic and capsid-containing viral RNA was compared by Northern blotting (Fig. 6). This experiment demonstrated that encapsidation of 3.5-kb pregenomic RNA was abolished when the pCMVKM22 was cotransfected with pCMVHBV (Fig. 6, lane 6). Inside the wild-type viral nucleocapsids, pregenomic RNA as well as a smear of specific viral RNA was present (Fig. 6, lane 4), probably due to the RNAse H activity of the viral polymerase. The transcript expressed by pCMVKM22 was not encapsidated as predicted (3, 19). Moreover, there was no evidence of inhibition of transcription of the pregenomic

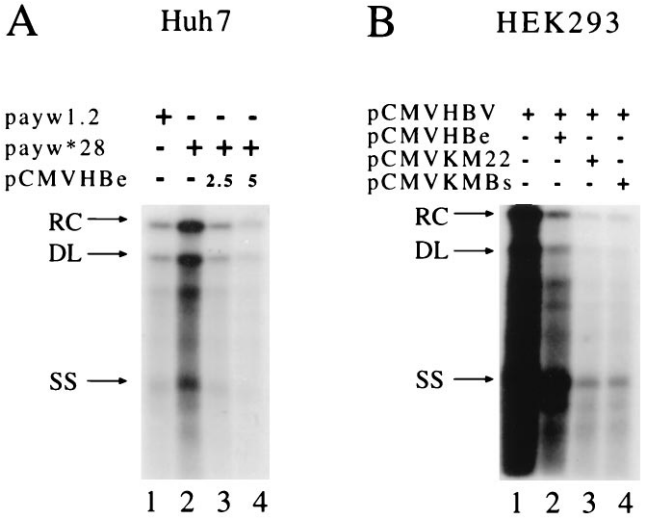


FIG. 5. Inhibition of HBV replication by precore proteins. A Southern blot of HBV DNA derived from nucleocapsids is shown. (A) HuH-7 cells transfected with payw1.2 or payw*28 alone or in combination with pCMVHBe (amounts are in micrograms). Wild-type HBV replication was obtained following transfection with payw1.2. An enhanced replication phenotype was exhibited by the payw*28 mutant. (B) HEK 293 cells transfected with the wild-type pCMVHBV alone or in combination with pCMVHBe, pCMVKM22, and/or pCMVKMBs. The arrows on the left indicate the relaxed circular (RC), double-stranded linear (DL), and single-stranded (SS) DNA species.

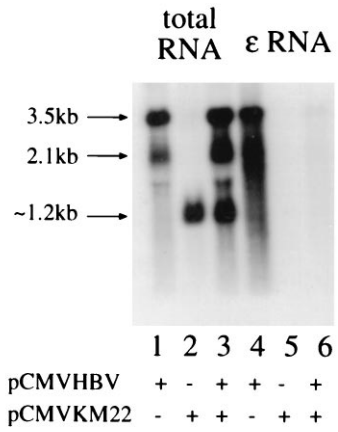


FIG. 6. Expression of the processed precore protein inhibits pregenomic RNA encapsidation in HEK 293 cells. A Northern blot of total cellular RNA and viral RNA isolated from nucleocapsids is shown. One half of the cells present in a 10-cm tissue culture dish was used for total RNA extraction, while the other half was employed for immunoprecipitation of viral capsids and extraction of encapsidated RNA (ε RNA). About 1/40 of the total RNA and all the encapsidated RNA were analyzed. For lane 4, HBV RNA was derived from nucleocapsids immunoprecipitated by anti-HBc following transfection with pCMVHBV. Lane 5 shows a lack of encapsidation of viral RNA following transfection with pCMVKM22 and lane 6 shows greatly reduced levels of encapsidated RNA following cotransfection with pCMVHBV and pCMVKM22. The arrows on the left indicate the relative sizes of the 3.5-kb pregenomic RNA and subgenomic transcripts. Note that pCMVKM22 expresses a 1.2-kb transcript.

RNA by the pCMVKM22-derived transcript (Fig. 6, lanes 1 through 3). The same type of analysis performed on RNA extracted from polyethylene glycol-precipitated nucleocapsids revealed similar results (data not shown).

**Comparison of the relative inhibitory activities of precore proteins on HBV DNA replication.** It was of interest to determine if the expression of precore gene reverses the high-replication-level phenotype exhibited by the payw*28 construct. Southern blot analysis of HCC cells transiently transfected with payw*28 together with increasing amounts of pCMVHBe demonstrated that only 2 μg of pCMVHBe was required to reduce the high level of viral replication exhibited by the mutant payw*28 (Fig. 5A) to wild-type HBV levels. Transfection of increasing amounts of pCMVHBe further depressed both payw*28- and wild-type payw1.2-generated HBV DNA replicative forms. This inhibitory effect was shown to be dependent on the presence of a functional wild-type precore gene, since expression of pCMVHBe*28 does not affect wild-type HBV DNA replication (Fig. 3A and data not shown).

The question of the relative potency of precore-related proteins with respect to inhibition of HBV DNA replication in the HEK 293 cells was studied, since this system has a very high wild-type HBV replication capacity (Fig. 5B). When pCMVHBV was cotransfected along with pCMVHBe, inhibition of viral replication was about 10 times less than that observed with the pCMVKM22 or pCMVKMBs construct. The pCMVKMBs protein product (p18) was capable of exerting the same degree of inhibition on viral replication as the p22 species produced from the parental pCMVKM22 construct. This experiment demonstrates that the domain responsible for the inhibition of viral replication was not located within the arginine-rich carboxy-terminal region encoded by the core ORF.

**Expression of p22 precore protein results in the formation of hybrid nucleocapsids.** Previous experiments had demonstrated that p22 protein was found in the pellet either alone or in combination with wild-type p21 core after sedimentation through a 20% (wt/wt) sucrose cushion. It became of interest, therefore, to determine if p22 was forming hybrid nucleocapsids with p21. The pellet derived from HEK 293 cells transiently transfected with pCMVHBV, pCMVKM22, or pCMVHBV plus pCMVKM22 was resuspended and loaded onto 20 to 60% (wt/wt) sucrose gradients and ultracentrifuged at 200,000 × *g* for 1 h. Following this sedimentation procedure, 15 150-μl fractions were sequentially removed from the top of the gradient and half of each fraction was analyzed by Western blot analysis for the presence of core-immunoreactive proteins. Under these experimental conditions, mature core particles have been found predominantly in fractions 4 to 10 (52).

HEK 293 cells transfected with the wild-type HBV-expressing construct demonstrated the expected sedimentation pattern for core protein. The majority of the core polypeptides assembled into native nucleocapsids were found to reside in fractions 4 to 9 (Fig. 7A). When wild-type HBV and p22-expressing constructs were transfected together, p22 cosedimented in the same fractions as native nucleocapsids (Fig. 7C). It was also of interest that p22 expressed from the pCMVKM22 construct alone also sediments as particulate nucleocapsids in the same fraction as wild-type p21. Indeed, the sedimentation pattern of p22 overlaps with that of the wild-type nucleocapsids. The same results were obtained when the pCMVHBe was transfected alone or in combination with pCMVHBV, but the intensity of the protein band signal was weaker (data not shown). These results suggest that hybrid capsid-like particles consisting of p21- and p22-derived core proteins are assembled in HEK 293 following transient transfection.

Since the p22 protein makes nucleocapsids, it was not possible to address the question of whether heterodimers or aggregates of other multiplicities with p21 were also formed. Moreover, since p22 and p21 were indistinguishable by the antibodies employed, a FLAG epitope was introduced into the B-cell immunodominant loop of the p22 core protein derived from plasmid pCMVKM22. This pCMVKMF construct expressed a pF22 protein that has the sequence $\underline{D^{78}YKDDDDKS^{81}}$ (FLAG epitope is underlined) instead of $D^{78}PAS^{81}$. The pF22 protein also assembles into nucleocapsid particles as well as the p22 parental protein (Fig. 8B, lane 4). Western blot analysis of the pellet derived from HEK 293 cells transfected with pCMVKMF demonstrated that pF22 was not

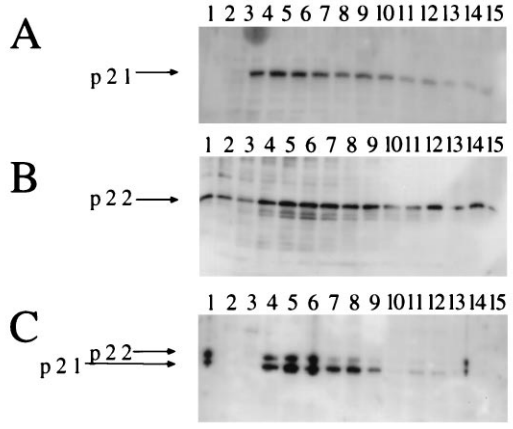


FIG. 7. Western blot analysis of various sucrose gradient fractions (lanes 1 to 15) showing nucleocapsid formation in HEK 293 cells transiently transfected with pCMVHBV (A), pCMVKM22 (B), or pCMVHBV and pCMVKM22 (C).

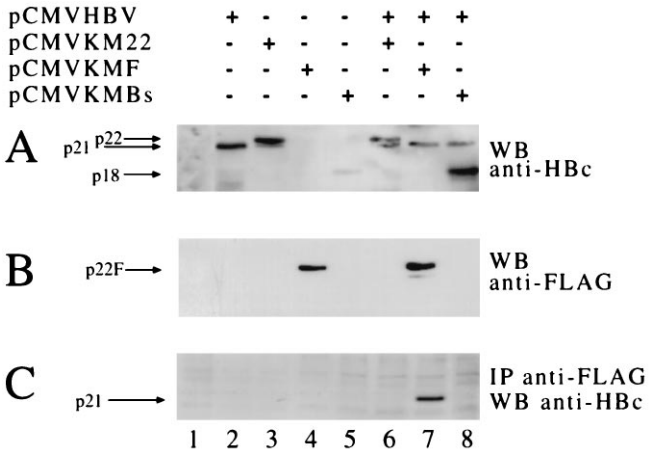


FIG. 8. Western blot (WB) and immunoprecipitation (IP)-Western blot analyses demonstrating that p22 physically interacts with wild-type p21 core protein in HEK 293 cells. (A and B) Western blot analysis with anti-HBc antibodies (A) and anti-FLAG M2 monoclonal antibody (B). (C) Cell lysates were immunoprecipitated with anti-FLAG monoclonal M2 antibody, and Western blot analysis was performed with polyclonal anti-HBc antibodies. Lanes 1 show results for nontransfected cells.

detectable by the polyclonal anticore antibodies, suggesting that the immunoreactivity was directed against the same antigenic loop where the FLAG epitope was inserted (Fig. 8A, lane 4). When the resuspended pellet derived from cells cotransfected with pCMVHBV and pCMVKMF was immunoprecipitated by the anti-FLAG antibody and the immunoprecipitate was analyzed by Western blot analysis with the polyclonal anticore antibodies, the wild-type p21 core protein was detected (Fig. 8C, lane 7). Thus, wild-type p21 core and pF22 proteins coimmunoprecipitate, and this observation suggests that the two peptide species physically interact. Taken together with the sedimentation properties of p21 and p22, the results indicate that these two polypeptides will assemble to form hybrid nucleocapsids.

Finally, it was interesting that the p18 protein expressed from pKMBs traverses the sucrose cushion poorly (Fig. 8A, lane 5). This finding is in agreement with previous studies demonstrating that a core protein truncation upstream from aa 144 results in formation of a less stable nucleocapsid structure (4, 14). However, when this expression vector was cotransfected with pCMVHBV not only was there almost total inhibition of HBV replication (Fig. 5B, lane 4) but also p18 was detected at a much higher level in the pelleted material together with wild-type p21 core protein. Thus, although partially deficient with respect to self-assembly into nucleocapsids, p18 was still capable of incorporation into nucleocapsids along with p21.

## DISCUSSION

The biological function of the precore protein has been difficult to define. It has been established that precore proteins are not required for viral replication in both avian and mammalian species (5, 7, 9, 11, 40, 46). Therefore, it was surprising that transient expression of the HBV precore gene resulted in inhibition of viral DNA synthesis. In the present study, we determined if ablation of the precore gene product influences HBV replication in HCC cells. Our findings indicate that the absence of a functional precore gene results in approximately a 10-fold increase in the level of HBV replication as previously reported by Lamberts et al. (20). Mutations in the precore promoter region have also been proposed to affect the replication of HBV (33). In this regard, a modest increase in HBV replication (Fig. 2, lanes 3 and 7) was observed when HCC cells were transfected with an HBV construct containing precore promoter mutations upstream of an intact precore gene. Thus, the high-replication-level phenotype exhibited by HBeAg-negative mutant viruses and commonly found in patients with chronic HBV infection may be due, in part, to the absence of the precore gene product. Indeed, when HBeAg was provided in *trans*, replication of the mutant genome was reduced to levels observed with wild-type HBV. It is noteworthy that the precore gene overlaps with ε, a structure required for encapsidation of pregenomic RNA and DNA priming. Point mutations in this structure could interfere with HBV replication. This appears not to be the case, since it has been shown that the amber termination signal introduced at aa 28 does not affect pregenomic RNA encapsidation (29) and HBV replication (unpublished data).

We confirmed that overexpression of the precore gene results in a striking inhibition of wild-type HBV DNA replication (20). Moreover, constitutive expression of the precore gene resulted in a substantial reduction of HBV replication in HepG2215 cells as well. This effect was not due to inhibition of transcription of HBV pregenomic RNA since by Northern blot analysis, we found no evidence for reduced levels of pregenomic RNA in the cytoplasm of transfected cells. The possible molecular mechanism(s) of inhibition exhibited by these precore gene products was explored. It has been well established that the HBV pregenomic RNA is encapsidated together with the polymerase gene product into nucleocapsid structures as formed by multimeric assembly of p21 core proteins (3, 19). Following expression of the precore gene, the nucleocapsid particles were found to contain both wild-type p21 and a larger, 22-kDa, protein product. We are led to believe that this p22 species was a proteolytically cleaved product of the precore gene product (p25) following removal of the signal peptide sequence (15). Indeed, when a plasmid designed to express the nonsecreted p22 form of the precore protein was transfected into HCC cells along with a construct expressing wild-type HBV, p22 was identified in approximately the same proportion as p21 in the nucleocapsids. More important, formation of such particles was associated with striking inhibition of HBV replication. One attractive hypothesis is that the high degree of inhibition of HBV replication was, in fact, due to increased cellular levels of p22 and the formation of hybrid nucleocapsids.

The sedimentation properties of the viral nucleocapsids produced in the presence or absence of p22 protein were identical. Wild-type p21 core and p22 proteins were found in the same gradient fractions when their respective constructs were transfected into HCC cells either separately or in combination. More direct evidence that the two protein species coassembled into particles was provided by the finding that wild-type p21 and pF22 will coimmunoprecipitate with an anti-FLAG monoclonal antibody. These results suggest that the two proteins physically interact and probably assemble into hybrid particles.

With respect to a possible molecular mechanism for inhibition of viral replication, isolated nucleocapsids were found to be devoid of pregenomic RNA when p22 was coexpressed with p21. Assembly of replication-competent HBV nucleocapsids requires the specific interaction of at least three viral components, including the core and polymerase proteins and the pregenomic RNA. The most likely explanation to account for this packaging defect is that the hybrid nucleocapsids do not allow proper RNA encapsidation. Thus, p22 may interfere with the recognition of the pregenomic RNA–polymerase complex

by the p21 core protein. In this regard, expression of p18, a protein with the same amino terminus as p22 but formed as the result of a truncation of the arginine-rich region from the carboxy terminus (i.e., it most closely resembles the secreted 17-kDa HBeAg species), strikingly inhibited wild-type HBV replication as well. The core portion of this protein has been previously shown to have the minimal sequence requirement to form capsid-like structures (4, 14). Indeed, when p18 was expressed alone, nucleocapsid-like particles were far less efficiently formed than nucleocapsids made with wild-type p21 core protein. Nevertheless, following coexpression with p21, the 18-kDa protein was found at much higher levels in nucleocapsid-like structures. We speculate that p21 may have served as a framework and facilitated the assembly into nucleocapsids of a less stable but interacting hybrid subunit(s). Taken together, these findings suggest that the role of p22 and p18 in inhibiting HBV replication may be due to an altered protein structure created by the N-terminal region of the precore gene that renders the nucleocapsids unable to incorporate pregenomic RNA. However, it will be important in future studies to determine the amino acid sequence of p22 and/or p18 during natural HBV infection or as a product of pCMVHBe following transient-transfection experiments.

These studies indicate that wild-type HBV replication was inhibited by the processed form of the precore protein. Previous studies have described a mutant core-envelope fusion protein that exhibited a potent dominant negative phenotype with respect to HBV replication. In these studies, it was found that the formation of core particles was abolished as the molecular mechanism for the dominant negative effect (39). In the present investigation, we have identified a naturally occurring molecule with a 15-fold more potent inhibitory effect on viral replication and a different mechanism of action, since it occurs at the level of either RNA encapsidation or RNA-polymerase–core interaction. Whether this phenomenon occurs in vivo during natural HBV infection is of course unknown, but it is of interest that proteins of the size of p22 and p23 have been identified in the liver of woodchuck hepatitis virus-infected woodchucks (48). If this were the case with HBV, a large fraction of the nucleocapsids produced during active infection would predictably be devoid of pregenomic RNA and presumably replication incompetent. Further experiments are warranted to substantiate this hypothesis.

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## ADDENDUM

While this paper was under review, Buckwold et al. demonstrated that reduction in precore gene expression was accompanied by an increase in progeny virus production (6a) and Guidotti et al. demonstrated in transgenic mice that the precore protein can exert a dominant negative effect on HBV replication (16a). These findings also indicate that the precore protein may be important in the life cycle of HBV.

## REFERENCES

1. **Acs, G., M. Sells, R. M. Purcell, P. Price, R. Engle, M. Shapiro, and H. Popper.** 1987. Hepatitis B virus produced by transfected HepG2 cells causes hepatitis in chimpanzees. Proc. Natl. Acad. Sci. USA **84:**4641–4644.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. Wiley, New York, N.Y.
3. **Bartenschlager, R., and H. Schaller.** 1992. Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. EMBO J. **11:**3413–3420.
4. **Birnbaum, F., and M. Nassal.** 1990. Hepatitis B virus nucleocapsid assembly: primary structure requirements in the core protein. J. Virol. **64:**3319–3330.
5. **Brunetto, M. R., M. Stemmler, F. Schodel, H. Will, A. Ottobrelli, M. Rizzetto, G. Verme, and F. Bonino.** 1989. Identification of HBV variants which cannot produce precore-derived HBeAg and may be responsible for severe hepatitis. Ital. J. Gastroenterol. **21:**151–154.
6. **Bruss, V., and W. H. Gerlich.** 1988. Formation of transmembraneous hepatitis B e-antigen by co-translational *in vitro* processing of the viral precore protein. Virology **163:**268–275.

6a. **Buckwold, V. E., Z. Xu, M. Chen, T. S. B. Yen, and J.-H. Ou.** 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J. Virol. **70:**5845–5851.

7. **Carman, W. F., M. R. Jacyna, S. Hadziyannis, P. Karayiannis, M. J. McGarvey, A. Mekris, and H. C. Thomas.** 1989. Mutation preventing formation of the hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet **ii:**588–591.
8. **Carman, W. F., E. A. Fagan, S. Hadziyannis, N. Tassopulos, and H. C. Thomas.** 1991. Association of a precore variant of HBV with fulminant hepatitis. Hepatology **14:**219–222.
9. **Chang, C., G. H. Enders, R. Sprengel, N. Peters, H. E. Varmus, and D. Ganem.** 1987. Expression of the precore region of an avian hepatitis B virus is not required for viral replication. J. Virol. **61:**3322–3325.
10. **Chang, L. J., D. Ganem, and H. E. Varmus.** 1990. Mechanism of translation of the hepadnaviral polymerase (P) gene. Proc. Natl. Acad. Sci. USA **87:**5158–5162.
11. **Chen, H. S., M. C. Kew, W. E. Hornbuckle, B. C. Tennant, P. J. Cote, J. L. Gerin, R. H. Purcell, and R. H. Miller.** 1992. The precore gene of the woodchuck hepatitis virus genome is not essential for viral replication in the natural host. J. Virol. **66:**5682–5684.
12. **Fallows, D. A., and S. P. Goff.** 1995. Mutations in the ε sequences of human hepatitis B virus affect both RNA encapsidation and reverse transcription. J. Virol. **69:**3067–3073.
13. **Galibert, F., E. Mandart, F. Fitoussi, P. Tiollais, and P. Charnay.** 1979. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli. Nature (London) **281:**646–650.
14. **Gallina, A., F. Bonelli, L. Zentilin, G. Rindi, M. Muttini, and G. Milanesi.** 1989. A recombinant hepatitis B core antigen polypeptide with the protamine-like domain deleted self-assembles into capsid particles but fails to bind nucleic acid. J. Virol. **63:**4645–4652.
15. **Garcia, P. D., J.-H. Ou, W. J. Rutter, and P. Walter.** 1988. Targeting of the precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. J. Cell Biol. **106:**1093–1104.
16. **Graham, F. L., J. Smiley, W. C. Russel, and R. Nairn.** 1977. Characteristic of a human cell line transformed by DNA from human adenovirus 5. J. Gen. Virol. **36:**59–74.

16a. **Guidotti, L. G., B. Matzke, C. Pasquinelli, J. M. Shoenberger, C. E. Rogler, and F. V. Chisari.** 1996. The hepatitis B virus (HBV) precore protein inhibits HBV replication in transgenic mice. J. Virol. **70:**7056–7061.

17. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor, Cold Spring Harbor, N.Y.
18. **Hatton, T., S. Zhou, and D. N. Standring.** 1992. RNA- and DNA-binding activities in hepatitis B virus capsid protein: a model for their role in viral replication. J. Virol. **66:**5232–5241.
19. **Hirsch, R., J. Lavine, L. J. Chang, H. E. Varmus, and D. Ganem.** 1990. Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for reverse transcription. Nature (London) **344:**552–555.
20. **Lamberts, C., M. Nassal, I. Velhagen, H. Zentgraf, and C. H. Schroder.** 1993. Precore-mediated inhibition of hepatitis B virus progeny DNA synthesis. J. Virol. **67:**3756–3762.
21. **Liang, T. J., K. Hasegawa, N. Rimon, J. R. Wands, and E. Ben-Porath.** 1991. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N. Engl. J. Med. **324:**1705–1709.
22. **Lingappa, J. R., R. L. Martin, M. L. Wong, D. Ganem, W. J. Welch, and V. R. Lingappa.** 1994. A eukaryotic cytosolic chaperonin is associated with a high molecular weight intermediate in the assembly of hepatitis B virus capsid, a multimeric particle. J. Cell Biol. **125:**99–111.
23. **McLachlan, A. (ed.).** 1991. Molecular biology of the hepatitis B virus. CRC Press, Boca Raton, Fla.
24. **Melegari, M., S. Bruno, and J. R. Wands.** 1994. Properties of hepatitis B virus pre-S1 deletion mutants. Virology **199:**292–300.
25. **Milich, D. R., J. E. Jones, J. L. Hughes, J. Price, A. K. Raney, and A. McLachlan.** 1990. Is a function of the secreted hepatitis B e antigen to

induce immunologic tolerance in utero? Proc. Natl. Acad. Sci. USA **87:**6599–6603.
26. **Miller, A. D., D. G. Miller, J. V. Garcia, and C. M. Lynch.** 1993. Use of retroviral vectors for gene transfer and expression. Methods Enzymol. **217:** 581–599.
27. **Morgenstein, J. P., and H. Land.** 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. **18:**3587–3596.
28. **Naoumov, N. V., R. Schneider, T. Grotzinger, M. C. Jung, S. Miska, G. R. Pape, and H. Will.** 1992. Precore mutant hepatitis B virus infection and liver disease. Gastroenterology **102:**538–543.
29. **Nassal, M., M. Junker-Niepmann, and H. Schaller.** 1990. Translation inactivation of RNA function. Discrimination against a subset of genomic transcripts during hepatitis B virus nucleocapsid assembly. Cell **63:**1357–1363.
30. **Nassal, M.** 1992. The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand synthesis but not for virus assembly. J. Virol. **66:**4107–4116.
31. **Nassal, M., and A. Rieger.** 1993. An intramolecular disulfide bridge between Cys-7 and Cys61 determines the structure of the secretory core gene product (e antigen) of hepatitis B virus. J. Virol. **67:**4307–4315.
32. **Okamoto, H., S. Yotsumoto, Y. Akahane, T. Yamanaka, Y. Miyazaki, Y. Sugai, F. Tsuda, T. Tanaka, Y. Miyakawa, and M. Mayumi.** 1990. Hepatitis B virus with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. J. Virol. **64:** 1298–1303.
33. **Okamoto, H., F. Tsuda, Y. Akahane, Y. Sugai, M. Yoshiba, K. Moriyama, T. Tanaka, Y. Miyakawa, and M. Mayumi.** 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J. Virol. **68:**8102–8110.
34. **Omata, M., T. Ehata, O. Yokosuka, K. Hosoda, and M. Ohoto.** 1991. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N. Engl. J. Med. **324:**1699–1704.
35. **Ou, J.-H., O. Laub, and W. J. Rutter.** 1986. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proc. Natl. Acad. Sci. USA **83:**1578–1582.
36. **Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore.** 1990. Production of high titer helper free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA **90:**8392–8396.
37. **Pugh, J. C., K. Yaginuma, K. Koike, and J. Summers.** 1988. Duck hepatitis B virus (DHBV) particles produced by transient expression of DHBV DNA in a human hepatoma cell line are infectious in vitro. J. Virol. **62:**3513–3516.
38. **Roychoudhury, S., A. F. Faruqi, and C. Shih.** 1991. Pregenomic RNA encapsidation: analysis of eleven missense and nonsense polymerase mutants of human hepatitis B virus. J. Virol. **65:**3617–3624.
39. **Scaglioni, P. P., M. Melegari, and J. R. Wands.** 1994. Characterization of hepatitis B virus core mutants that inhibit viral replication. Virology **205:** 112–120.
40. **Schlicht, H. J., J. Salfeld, and H. Schaller.** 1987. The duck hepatitis B virus pre-C region encodes a signal sequence which is essential for synthesis and secretion of processed core proteins but not for virus formation. J. Virol. **61:**3701–3709.
41. **Schlicht, H. J., and H. Schaller.** 1989. The secretory core protein of human hepatitis B virus is expressed on the cell surface. J. Virol. **63:**5399–5404.
42. **Schlicht, H. J., and G. Wasenauer.** 1991. The quaternary structure, antigenicity, and aggregational behavior of the secretory core protein of human hepatitis B virus are determined by its signal sequence. J. Virol. **65:**6817–6825.
43. **Seeger, C., B. Baldwin, and B. C. Tennant.** 1989. Expression of infectious woodchuck hepatitis virus in murine and avian fibroblasts. J. Virol. **63:**4665–4669.
44. **Sells, M. A., M. L. Chen, and G. Acs.** 1987. Production of hepatitis B virus particles in HepG2 cells transfected with cloned hepatitis B virus DNA. Proc. Natl. Acad. Sci. USA **84:**1005–1009.
45. **Standring, D. N.** 1991. *In* A. McLachlan (ed.), Molecular biology of the hepatitis B virus, p. 145–169. CRC Press, Boca Raton, Fla.
46. **Tong, S. P., J. S. Li, L. Vitvitski, and C. Trépo.** 1990. Active hepatitis B virus replication in the presence of anti-HBe is associated with viral variants containing an inactive pre-C region. Virology **176:**596–603.
47. **Tong, S. P., J. S. Li, L. Vitvitski, and C. Trépo.** 1992. Replication capacities of natural and artificial precore stop codon mutants of hepatitis B virus: relevance of pregenome encapsidation signal. Virology **191:**237–245.
48. **Weimer, T., J. Salfeld, and H. Will.** 1987. Expression of the hepatitis virus core gene in vitro and in vivo. J. Virol. **61:**3109–3113.
49. **Will, H., W. Reiser, T. Weimer, E. Pfaff, M. Busher, R. Spregel, C. Cattaneo, and H. Schaller.** 1987. Replication strategy of human hepatitis B virus. J. Virol. **61:**904–911.
50. **Yaginuma, K., Y. Shirakata, M. Kobayashi, and K. Koike.** 1987. Hepatitis B virus (HBV) particles are produced in a cell culture system by transient transfection of transfected HBV DNA. Proc. Natl. Acad. Sci. USA **84:**2678–2686.
51. **Zhang, P., and A. McLachlan.** 1994. Differentiation-specific transcriptional regulation of hepatitis B virus nucleocapsid gene in human cell lines. Virology **202:**430–440.
52. **Zhou, S., and D. N. Standring.** 1992. Characterization of hepatitis B virus capsid particle assembly in xenopus oocytes. J. Virol. **66:**3086–3092.