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Insertions within the Hepatitis B Virus Capsid Protein Influence Capsid Formation and RNA Encapsidation

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Hepatitis B virus (HBV) capsid proteins, termed core proteins, with two- to four-amino-acid insertions were assessed for capsid formation, RNA encapsidation, and the ability to support reverse transcription of the pregenome by the polymerase molecule. Velocity sedimentation analysis of insect cell-expressed recombinant core proteins revealed that only two of the nine insertion mutant proteins formed capsids with the tight banding patterns of wild-type capsids. The remaining mutant core proteins were spread over the gradients, suggesting aggregate formation, or at the top of the gradients, suggesting lack of stable capsid formation. The mutant capsid proteins were coexpressed in Huh7 cells with an HBV genome lacking a functional core gene to test for *trans* complementation of HBV replication. Three of the mutant core proteins formed capsids containing HBV RNA, but only two of these contained reverse-transcribed HBV DNA. While the core protein has shown resiliency in capsid formation following insertion of foreign residues into the major B-cell epitope, several of the small insertions severely reduced the efficiency of capsid formation and inhibited capsid function.

Hepatitis B virus (HBV), the type member of the family *Hepadnaviridae*, causes acute and chronic hepatitis in humans. Chronic HBV infection is a risk factor for development of cirrhosis and hepatocellular carcinoma (7). The mature HBV virion, the Dane particle, consists of an enveloped capsid formed by a single capsid protein, the 183-amino-acid (strain *ayw*) core protein. The capsid contains the partially double-stranded, noncovalently closed circular DNA genome which is reverse transcribed from the pregenomic RNA by a viral reverse transcriptase, pol (33), which is covalently bound to the 5′ end of first-strand DNA. Recombinant core proteins expressed in bacteria (8, 13), *Saccharomyces cerevisiae* (25), *Xenopus* oocytes (40), and insect cells (17, 35) spontaneously associate to form capsids in the absence of additional HBV proteins. HBV capsids assembled in *S. cerevisiae* (21) and bacteria (30) are nonphosphorylated, while those expressed in insect cells are phosphorylated in vivo and can be phosphorylated in vitro by the encapsidated kinase (17). Bacterially expressed HBV core proteins contain cellular RNA (8), while those expressed in insect cells are devoid of RNA (17). The polymerase molecule is required for pregenomic RNA encapsidation in permissive cells (2, 15). Encapsidation of the proposed pregenome-pol complex recognized by the core protein (3) may be influenced by modification of the core protein and by additional factors which lend specificity to this reaction.

Core protein domains necessary for capsid formation and encapsidation of the pregenome-pol complex have not been fully defined. Recent studies have demonstrated that the carboxy termini of the HBV and related duck hepatitis B virus (DHBV) core proteins influence RNA encapsidation and reverse transcription of the pregenome by the pol protein (6, 26, 30, 38). Sizable portions of the carboxy terminus can be removed without inhibiting capsid formation, although pregenome encapsidation is affected (6, 26). The HBV capsid structure has been indirectly investigated by fusing additional sequences to the core gene to use the HBV capsid to display foreign epitopes. Foreign sequences have been fused to the amino terminus, at a number of sites within the carboxy terminus, and at selected internal sites. The region around amino acid 80, the major B-cell antigenic site (28), tolerates insertion of foreign epitopes without loss of particle formation (9, 12, 31), while insertion at amino acid 30 prevents capsid formation (10).

In this study, we investigated the influence of two- to four-amino-acid insertions within the HBV core protein on capsid formation, RNA encapsidation, and reverse transcription. Insertions at several sites within the core protein altered the mobility of recombinant capsids on sucrose gradients, suggesting dense aggregate formation or a decrease in stable particle formation. Mutant core proteins supplied in *trans* were assayed for complementation of HBV replication of a core gene-negative HBV genomic clone in Huh7 hepatoma cells. RNase protection assays revealed that only two of the insertion mutants and the single-point mutant formed cytoplasmic core particles containing HBV RNA, suggesting decreased encapsidation. The general disruption of capsid function was confirmed by endogenous polymerase assays on capsids pelleted from media, with one insertion mutant and the single-point mutant forming capsids supporting polymerase activity. Overall, the HBV capsid appears to be very sensitive to insertions within the first 131 amino acids as measured by capsid formation and function.

MATERIALS AND METHODS

Cell culture and transfections. Maintenance of the human hepatocellular carcinoma cell line Huh7 has already been described (6). Plasmid DNA was introduced into Huh7 cells with the lipofectin reagent (Life Technologies, Gaithersburg, Md.). Briefly, 15 μg of each plasmid in medium lacking serum, 30 μg total in cotransfection experiments, was mixed with 50 μl of lipofectin and added to 100-mm-diameter dishes of 3×10^6 cells. Following 6 h of incubation at 37°C, the cells were washed three times with phosphate-buffered saline (PBS) and overlaid with Dulbecco modified Eagle medium-F12 medium (1:1) plus 10% fetal bovine serum, 2 mM glutamine, and 50 μg of gentamicin per ml. Cultures were maintained for 6 days with a medium change at day 3.

The maintenance and infection of *Spodoptera frugiperda* insect cells, clone Sf9, have been described previously (17). Generation of recombinant baculoviruses was performed essentially by the methods of Summers and Smith (34).

Plasmid constructs. HBV subtype *ayw* sequences are numbered as described

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by Galibert and coworkers (14). The HBV core gene was cloned as a 578-bp *Sty*I fragment, with *Xho*I linkers added to each end, into a pUC18 derivative lacking about 350 bases on either side of the multiple cloning site. This smaller pUC18 derivative was made to facilitate partial digestion of the core gene at a number of sites and to remove the *lac* operator. Digestion of the core gene with *Hinc*II or *Alu*I generated blunt ends, while digestion with *Hin*fI or *Dde*I left three-base 5′ overhangs which were made blunt with the Klenow fragment of DNA polymerase, adding an extra codon in each instance. Four amino acids were introduced at the *Hinc*II site by inserting a double *Pst*I site linker, CTGCAGCTGC AG. Insertions at the other sites were identified by screening for the presence of a *lac* operator to induce β-galactosidase activity. The double *Pst*I site linker was ligated to each end of the *lac* operator fragment from pUC18 and then digested with *Pvu*II, whose recognition site (CAGCTG) is found in the linker. The *lac* operator fragment flanked by *Pvu*II-digested linkers was blunt end ligated into the core gene at the sites listed above. HB101 cells were transformed with the ligated plasmids, and *lac*-positive colonies were identified by blue color in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Plasmids with the *lac* operator at the appropriate site were digested with *Pst*I to remove the operator and then ligated, leaving a six-base, CTGCAG (*Pst*I site), insertion. Because the insertions occur at different sites within a codon, identical residues were not added at each site (see Fig. 1).

A single point mutation within the core gene was generated for use in this study. One of the two lysine residues present in the HBV core protein, Lys-96, was converted to an arginine with the Muta-gene mutagenesis kit (BioRad, Richmond, Calif.) and the oligonucleotide GGGCCTAAGGTTCAGGC, bases 2181 to 2197 (the altered base is underlined). All mutations were confirmed by dideoxy sequencing (29).

Core genes containing point and insertion mutations were cloned into baculovirus expression vector pVL941 (19) for expression in Sf9 cells. All core genes were cloned out of frame with respect to the mutant polyhedrin initiation codon (AUU) in this vector, as we have shown that the mutated initiator codon can be used in insect cells, generating fusion proteins (5). For expression in mammalian cells, the mutated genes were cloned into a derivative of pRc/CMV (Invitrogen, San Diego, Calif.) containing the cytomegalovirus immediate-early promoter.

HBV core proteins expressed in Huh7 cells. Huh7 cells transfected with mutant core genes expressed from the human cytomegalovirus immediate-early promoter were incubated for 3 days posttransfection. Two hours prior to labeling, the medium was removed and replaced with labeling medium, William's E medium lacking methionine and cysteine (Life Technologies) and supplemented with 2% dialyzed fetal bovine serum. The cells were labeled for 4 h with 100 μCi of [^{35}S]Met-Cys (Expre^{35}S^{35}S; NEN, Boston, Mass.) per ml in labeling medium. The cells were lysed in PBS–1% Nonidet P-40, and the labeled core protein was immunoprecipitated with a mixture of mouse anti-core protein and anti-precore protein sera made against insect cell-derived HBV core and precore proteins (17). Immunoprecipitates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 15% polyacrylamide gel and detected by autoradiography. Similarly labeled HBV core proteins were examined for sedimentation through 30% sucrose in PBS–1 mM EDTA onto 70% sucrose in PBS–1 mM EDTA–10 mg of phenol red per liter by centrifugation at 50,000 rpm for 6 h in an SW55 rotor (Beckman, Palo Alto, Calif.). The interface between the 30 and 70% sucrose layers was collected, and labeled core protein from this fraction was immunoprecipitated with a rabbit anti-core protein antibody prepared against insect cell-expressed core protein. The immunoprecipitates were separated by SDS-PAGE and viewed by autoradiography.

HBV core particles expressed in insect cells. Baculovirus-infected Sf9 cells were scraped into the medium and sonicated to release expressed HBV core protein. Cell debris was pelleted at 13,000 × *g*, and the HBV core proteins in the clarified lysate were separated by velocity sedimentation on 10 to 50% linear sucrose gradients in PBS–1 mM EDTA. The gradients were centrifuged in an SW55 rotor at 40,000 rpm for 2 h at 4°C. The 5-ml gradients were divided into 500-μl fractions, and 20 μl from each fraction was separated by SDS-PAGE on a 15% polyacrylamide gel. The core proteins were detected by immunoblotting with a rabbit anti-core protein antibody and ^{125}I-labeled protein A as previously described (6).

RNase protection assay. Mutant core proteins which pelleted onto the 70% sucrose cushion were coexpressed with plasmid p1.3HBcm, which contains 1.3 copies of an HBV genome with the core gene initiator AUG altered to AUA to prevent core protein expression (6). Complementation by the wild-type (WT) or mutant core proteins in *trans* was assessed initially by an RNase protection assay to detect encapsidated HBV RNA. Cytoplasmic HBV particles taken from cultures at 6 days posttransfection were immunoprecipitated as previously described (6), except that 1% sodium deoxycholate was added to the PBS–1% Nonidet P-40 used to wash immunoprecipitates. Immunoprecipitated particles were treated with 15 U of micrococcal nuclease per ml in the presence of 3 mM $CaCl_2$ at 37°C for 30 min. Immunoprecipitated core particles bound to protein A agarose beads were washed to remove the micrococcal nuclease, and RNA from the immunoprecipitated core particles was isolated by acid-phenol guanidinium thiocyanate extraction followed by ethanol precipitation (11).

An RNA probe for the RNase protection assay was generated by using T7 phage polymerase to transcribe an antisense copy of the 3′ end of the core gene, bases 2463 to 2170, in accordance with previously published procedures (24). The predicted 323-base probe was purified on a 4% polyacrylamide gel containing 8 M urea. The probe was eluted from the gel into a solution of 0.5 M ammonium acetate–1 mM EDTA–0.2% SDS and used in the protection assay. Sample RNA was ethanol precipitated in the presence of 10^6 cpm of an HBV core RNA probe and 10 μg of tRNA. Samples suspended in 20 μl of 80% formamide–100 mM sodium citrate (pH 6.4)–300 mM sodium acetate–1 mM EDTA were heated to 95°C, slowly cooled, and incubated overnight at 42°C. Single-stranded RNA was digested with a mixture of RNases A and T1 at 3.3 μg/ml and 66 U/ml, respectively (Ambion, Austin, Tex.), for 30 min at 37°C. The reactions were extracted with a mixture of 4 M guanidinium thiocyanate (11), phenol, and 2 M sodium acetate (pH 4.0) (5:10:1) plus 10 μg of tRNA per tube. One-tenth of a volume of $CHCl_3$ was added to separate the phases, and the protected probe was precipitated from the aqueous phase by addition of 1 volume of isopropanol. The protected probe was separated on a 4% polyacrylamide–8 M urea gel and detected by autoradiography.

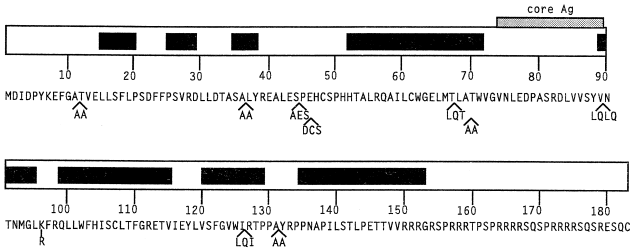

FIG. 1. Insertions within the core gene. The core gene is represented as large rectangles, and the amino acid sequence is shown below in the single-letter code. Residues forming the core insertion mutations are shown below the sequence. Filled boxes within the core gene represent the locations of predicted β strands (1). The box over the core gene denotes the location of the major B-cell epitope (28) believed to be in an external loop domain. The vertical line below core residue 96 denotes the single core point mutation of Lys to Arg. Ag, antigen.

Endogenous polymerase assay. Mature core particles were pelleted from tissue culture medium adjusted to 1% Nonidet P-40 through a 5-ml 30% sucrose cushion in PBS with a Beckman SW28 rotor run at 26,000 rpm for 16 h at 4°C. Pelleted particles were treated with micrococcal nuclease as described above. After nuclease treatment, all buffers were supplemented with 20 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). HBV DNA was detected with an endogenous polymerase assay (16) to complete the positive-strand DNA in the presence of a radiolabeled nucleotide as previously described (6). Briefly, [α-^{32}P]dCTP was added to the core particles with unlabeled dGTP, dATP, and TTP in transcription buffer. The samples were incubated at 37°C for 1.5 h, and then unlabeled dCTP was added and the incubation was continued for an additional 30 min to complete the reaction. Sample treatment with SDS and proteinase K for 2 h at 37°C was followed by phenol extraction and ethanol precipitation. Precipitates were suspended in water and separated on a 1% agarose gel. Following electrophoresis, the gel was dried and exposed for autoradiography.

RESULTS

To better understand the HBV core protein structure and the effect of small insertions on core protein functions, a series of mutant core genes was constructed for expression in insect and mammalian cells. Linkers inserted at restriction sites within the core gene added two to four amino acids to the expressed core proteins. The residues added varied depending on the codon position at the site of linker insertion. In addition to the insertion mutations, a single-point mutation at amino acid 96 was generated. Inserted residues are shown relative to the WT HBV core amino acid sequence, the predicted locations of β strands (1), and the major B-cell epitope site (28) in the core protein in Fig. 1.

HBV capsid formation. WT and mutant HBV core proteins were expressed in insect cells by using a baculovirus expression vector. Capsid formation by the mutant core proteins was assessed by velocity sedimentation. Recombinant baculovirus-infected Sf9 cells were sonicated, and the clarified lysates were separated on linear 10 to 50% sucrose gradients. Immunoblotting of aliquots from each gradient fraction revealed that most of the mutant core proteins migrated differently from the WT core protein (Fig. 2). Centrifugation conditions were chosen such that the WT core protein migrated near the middle of the

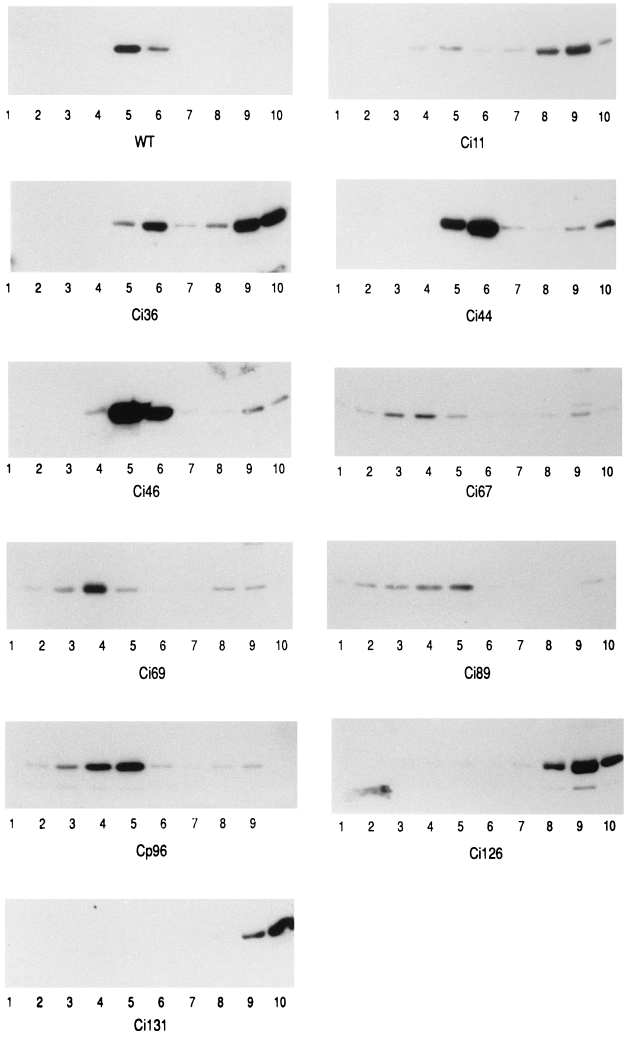

FIG. 2. Velocity sedimentation analysis of mutant core proteins. Mutant core proteins were released by sonication from Sf9 cells at 48 h postinfection with recombinant baculovirus vectors. Lysates were clarified and applied to 5-ml 10 to 50% linear sucrose gradients which were centrifuged in an SW55 rotor at 40,000 rpm for 2 h. Gradients were collected as 500-μl fractions, and 20-μl aliquots were separated on 15% polyacrylamide gels. Proteins were transferred to a nylon membrane and incubated with a 1:500 dilution of rabbit anti-core protein serum and then with ^{125}I-labeled protein A. After washing, the filters were dried and exposed to Kodak XAR-5 film for autoradiography.

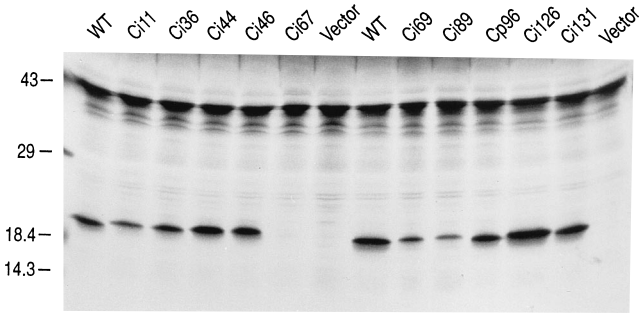

FIG. 3. Expression of mutant core proteins in mammalian cells. Huh7 cells were transfected with plasmids encoding WT and mutant core genes, and the cultures were incubated for 3 days at 37°C. Control cells were transfected with the cytomegalovirus vector lacking the core gene. Transfected cells were labeled at 3 days posttransfection with 100 μCi of [^{35}S]Met-Cys per ml for 2 h and then lysed with PBS–1% Nonidet P-40. The labeled core proteins were immunoprecipitated with a mixture of mouse anti-core protein and anti-precore protein sera bound to protein A agarose, boiled in SDS disruption buffer, and separated on a 15% polyacrylamide gel. The gel was dried and exposed for autoradiography. Marker protein molecular sizes are in kilodaltons.

gradient, typically in fractions 5 and 6, demonstrating that most of the core protein was present as intact capsids. Proteins Ci11 and Ci36 were found primarily at the top of the gradient, suggesting inefficient capsid formation, although core protein was detected in fraction 6 of Ci36, suggesting some capsid formation by this mutant protein. Mutant proteins Ci44 and Ci46 had mobilities similar to that of the WT core protein, presumably efficiently forming cores. Proteins Ci67 and Ci69 were found primarily in denser fractions than the WT core protein, suggesting large aggregate or denser capsid formation. The Ci89 and Cp96 core proteins were found in denser fractions, but the greatest amount was found in fraction 5, where WT capsids migrated. Mutant proteins Ci126 and Ci131 were found primarily in the top fractions, suggesting decreased capsid formation and/or stability.

Core protein expression in Huh7 cells. A single 22-kDa HBV-specific band was observed when WT or mutant HBV core proteins were expressed from Huh7 cells by using a vector containing the cytomegalovirus immediate-early promoter. The cells were labeled with [^{35}S]Met-Cys for 2 h, and core proteins were immunoprecipitated from the cell lysates (Fig. 3). All of the core proteins appeared to be of the expected sizes and to be expressed at similar levels, except Ci67, which failed to be expressed or accumulate to detectable levels in mammalian cells.

Assembly of core protein in Huh7 cells. Since core protein is expressed at a much lower level in mammalian cells than in insect cells and capsid formation is sensitive to the core protein concentration (18, 32, 41), it was necessary to confirm whether mutant core proteins can form high-molecular-weight assemblies in Huh7 cells. Metabolically labeled WT and mutant core proteins from transfected Huh7 cell lysates were assayed by pelleting through 30% sucrose onto a 70% sucrose cushion. The results (Fig. 4) generally were in good agreement with those obtained with baculovirus-expressed core proteins. Ci11 was not present at the interface, while Ci36 was present at a low level. Ci44 and Ci46 had WT levels of core protein at the interface, as expected from the velocity sedimentation data on baculovirus-expressed capsids. Ci67 again failed to show evidence of accumulation in mammalian cells and was not examined further. Ci69 was found at the interface, while Ci89, Ci126, and Ci131 were not present or were present at greatly reduced levels. Cp96 was present at nearly the WT level, suggesting that the single amino acid change was not significantly disruptive to capsid formation. For all samples except Ci67, core protein was immunoprecipitated from the cell lysate or from the 30% sucrose solution, demonstrating core protein synthesis (data not shown).

Encapsidation of HBV RNA. Core proteins capable of forming high-molecular-weight complexes (Fig. 4) were tested further for complementation in *trans* of plasmid p1.3HBcm, containing 1.3 copies of the HBV genome with a mutation altering the core initiator AUG to AUA (6). Huh7 cells cotransfected with the p1.3HBcm plasmid and each of the mutant core plasmids individually were tested for cytoplasmic core particles containing HBV RNA. Cytoplasmic HBV core proteins were immunoprecipitated from a cell lysate by using a rabbit anti-core protein serum, and the immunoprecipitates were analyzed for encapsidated RNA by an RNase protection assay with a probe complementary to the 3′ half of the core gene (Fig. 5,

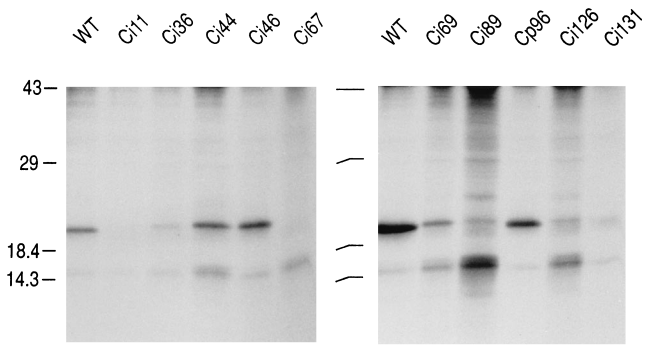

FIG. 4. Pelleting of core particles from Huh7 cells. Core proteins were labeled and extracted as described in the legend to Fig. 3 and pelleted through 30% sucrose onto a 70% sucrose cushion. The interface was collected, and the core proteins were immunoprecipitated with a rabbit anti-core protein antibody. The immunoprecipitated, labeled core proteins were separated and detected as described in the legend to Fig. 3. The numbers on the left are molecular sizes in kilodaltons.

bottom). Repeated experiments revealed that only mutant core proteins Ci44 and Cp96 encapsidated the pregenomic RNA at levels approaching that of the WT core protein tested in *trans*, while mutant core protein Ci46 contained reduced levels of HBV RNA (Fig. 5). While longer exposures revealed that mutant core proteins Ci36 and Ci69 contained very low levels of HBV RNA, these mutant proteins are greatly defective for encapsidation. Thus, the HBV capsid appears to be very sensitive to small insertions at several sites when assayed for the ability to encapsidate HBV RNA. Since Ci44 and Ci46 can be pelleted with similar efficiencies (Fig. 4), the packaging defect must be due to more than just inefficient capsid formation.

HBV polymerase activity. The partially double-stranded DNA of mature HBV particles can be completed with radiolabeled nucleotides in vitro, forming the basis of an endogenous polymerase assay (16). Addition of radiolabeled nucleotides to the HBV genome renders this a very sensitive test for the presence of a functional polymerase in a capsid. Each of the samples from Fig. 5 was assayed for the ability to provide core protein in *trans* to produce secreted particles with endogenous polymerase activity. Huh7 cells were cotransfected with the p1.3HBcm plasmid and each mutant core gene, and at 6 days posttransfection, tissue culture medium was removed and analyzed for the presence of HBV DNA-containing core particles. Core particles were pelleted from the media through a 30% sucrose cushion and assayed for endogenous polymerase activity. Products were separated on a 1% agarose gel and detected by autoradiography. Only WT core protein and mutant core proteins Ci44 and Cp96 were conclusively positive in this assay (Fig. 6). However, proteins Ci44 and Cp96 were less efficiently provided in *trans* than was the WT core protein, as the endogenous polymerase signal from these mutant proteins was consistently reduced relative to that of the WT core protein. The Ci44 endogenous polymerase reaction depicted showed a decrease in the formation of relaxed circular DNA. Relaxed circular DNA was synthesized in other experiments by the mutant producing Ci44, but it always showed reduced overall DNA synthesis. Thus, it appears that insertion of two to four amino acids at several sites within the HBV core protein greatly decreases RNA encapsidation and the ability to provide the appropriate environment for reverse transcription of the RNA by the viral polymerase.

DISCUSSION

While relatively simple in that it is formed from a single 22-kDa protein, the HBV capsid must assemble to permit RNA encapsidation and reverse transcription by pol. The core protein has been shown in a variety of exogenous systems (8, 13, 17, 23, 25, 40) to spontaneously form capsids morphologically resembling WT capsids found in cells permissive for HBV replication in which all HBV proteins are produced. Core assembly is cooperative, proceeding as a sufficient concentration is reached (18, 32, 41). Core assembly in an in vitro translation system has been shown to be chaperonin dependent at low protein concentrations (18), suggesting possible differences in assembly between high-level expression systems and permissive cells. This study examined capsids formed in insect and Huh7 cells and found good agreement between insect cell-derived core proteins that migrated as capsids on sucrose

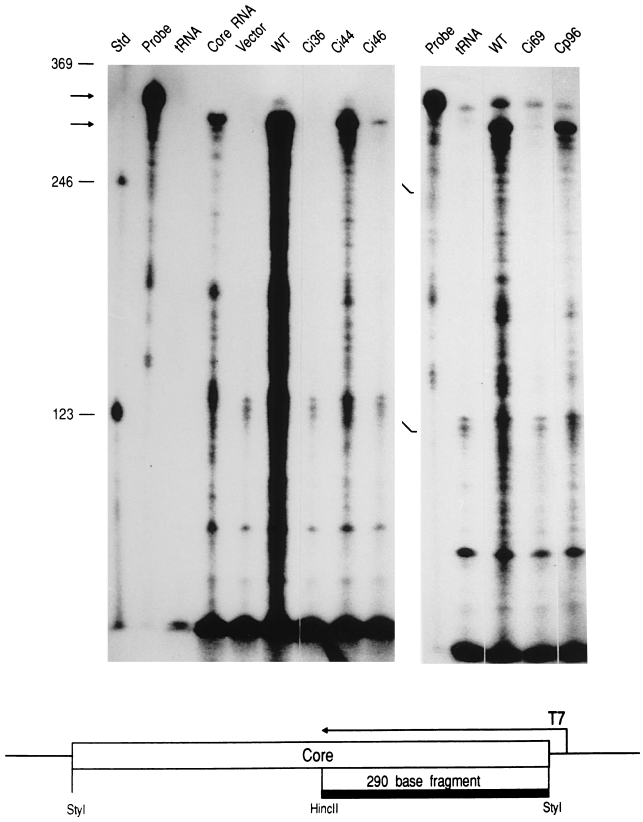

FIG. 5. Encapsidation of HBV RNA by mutant core proteins. Packaging of HBV RNA into immature core particles was assayed by using an RNase protection assay. Huh7 cells cotransfected with a plasmid encoding a core gene-negative HBV genome and plasmids encoding mutant core proteins were harvested as described in the legend to Fig. 3. Immunoprecipitated cytoplasmic HBV capsids were treated with micrococcal nuclease. Nucleic acid was extracted from the core particles and was treated with RNase-free DNase, phenol-chloroform extracted, and ethanol precipitated. Purified RNA was hybridized with the antisense core probe shown in the bottom panel. Samples were treated with RNases A and T1, and the fragments protected from RNase digestion were analyzed on a 4% denaturing polyacrylamide gel. Lane Std contained denatured [α-^{32}P]dCTP-labeled 123-bp DNA standards. The probe lane contained 1/100 of a tRNA control sample similarly treated but without added RNases. The core RNA lane contained 14 ng of total cell RNA from Sf9 cells infected with a baculovirus expressing the HBV core protein as a positive control. The vector lane contained RNA from Huh7 cells transfected with pHB1.3cm and the cytomegalovirus expression plasmid lacking an HBV core gene (pRc/CMV). The numbers on the left are molecular sizes in bases.

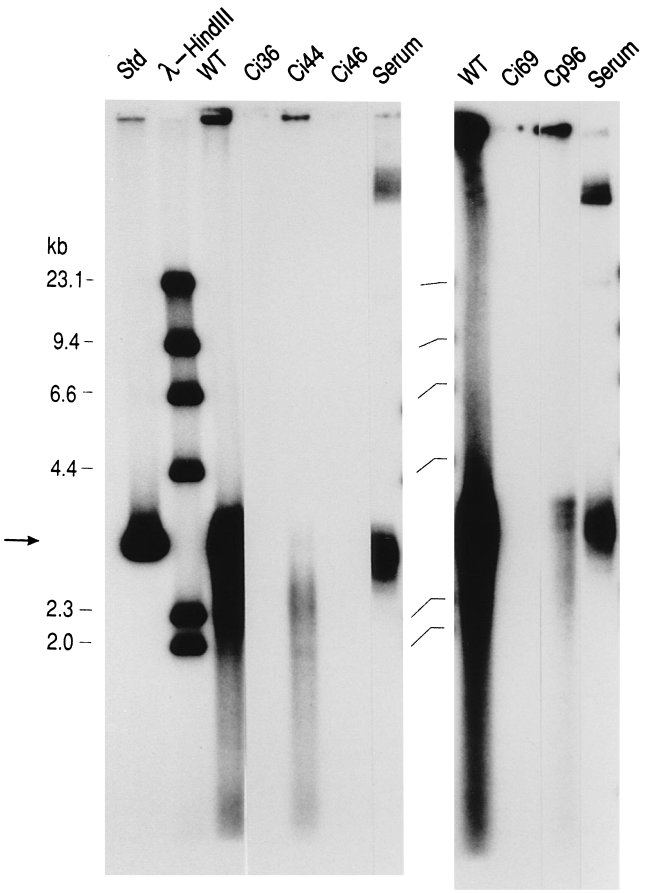

FIG. 6. Endogenous polymerase assay for HBV DNA within WT and mutant capsids. Huh7 cells were transfected and harvested as described in the legend to Fig. 5. Mature core proteins were pelleted from the medium through a 30% sucrose cushion at 26,000 rpm for 16 h in an SW28 rotor. Pelleted core particles were treated with micrococcal nuclease and used in an endogenous polymerase assay. DNA purified from the samples was electrophoresed on a 1% agarose gel which was dried and exposed for autoradiography. Lane Std contained an [α-^{32}P]dCTP-labeled linear double-stranded DNA similar in size to the linear 3,182-bp HBV DNA. The λ-*Hin*dIII lane contained [α-^{32}P]dCTP-labeled, *Hin*dIII-digested lambda phage DNA. The serum lane represents a 50-μl serum sample from a chronically HBV-infected chimpanzee used in the endogenous polymerase assay as a positive control. The arrow indicates full-length, double-stranded HBV DNA.

gradients and those which could be pelleted from an Huh7 cell lysate.

A goal of this approach to the study of the HBV core protein was to identify regions of the protein which may play a role in capsid formation, pregenome encapsidation, and reverse transcription by the pol molecule. Computer alignments of anticipated protein secondary structures led Argos and Fuller (1) to infer that the HBV capsid has an eight-stranded β-barrel structure common to many simple RNA viruses (27). However, recent circular dichroism data suggest that there is too much α-helical content in the capsid to conform to this β-barrel structure (36). The HBV core protein has been manipulated by adding foreign epitopes to the amino or carboxy terminus and, to a limited extent, within the core protein proper. A number of groups (9, 31, 39) have used the core protein as an antigenic carrier by inserting HBV surface antigen domains into the major B-cell antigenic loop situated around amino acid 80 (28). Core proteins with insertions of up to 39 foreign residues at this site formed particles resembling authentic HBV capsids in *Escherichia coli*. The β-barrel structure predicts that the surface antigen insertions would fall within an exterior loop containing an extra 39 amino acids in the related DHBV core protein (22). Insertions are found at a comparable site in many of the structurally related picornavirus capsid proteins (20). Generation of capsid-like structures with large insertions within this antigenic loop suggests that the core protein is rather insensitive to insertions, in contrast to data in this report. The sensitivity of the HBV capsids to insertions is supported by a recent study in which DHBV core proteins with a single-amino-acid deletion or a four-residue insertion failed to form capsids in *E. coli* (37). This apparent contradiction is likely due to the site of insertion within the core protein, the amino acids inserted, and the assay employed to detect capsid formation. The efficiency of capsid formation (the fraction of the core protein found as capsids) also needs to be addressed for comparison.

In this study, small insertions were used in the hope of perturbing the capsid sufficiently to generate detectable phenotypes without totally destroying capsid function. Assuming that alterations to β strands would be potentially more disruptive than alterations to the less structurally conserved flanking regions, insertions may help determine which predicted structure more closely resembles that of the HBV capsid. While several core protein domains sensitive to insertions have been identified, data from this study do not eliminate the predicted β-barrel structure. Four of the insertions, in Ci11, Ci44, Ci46, and Ci131, are located outside of predicted β strands, but proteins Ci11 and Ci131 failed to efficiently form capsids. Insertions within predicted β strands yielded three phenotypes. Proteins Ci67 and Ci69 migrated as dense capsids or aggregates, much of proteins Ci36 and Ci89 migrated as capsids, while almost none of protein Ci126 migrated as capsids. Insertions which fail to block capsid formation may not fall within a β strand. Blockage of capsid formation may depend on the ability of the insertions to disrupt the β-strand structure, the interactions between dimers, or the interaction between dimers and the rest of the capsid. The number and type of residues inserted also would have an influence on capsid formation.

A number of mutant core proteins warrant further study. Insertion mutant proteins which show evidence of capsid formation but little or no RNA encapsidation, Ci36, Ci46, and Ci69, may provide information on capsid domains involved in stability and/or pol-RNA pregenome complex packaging (4). Mutant core proteins much of which migrate near the top of the sucrose gradient, Ci11, Ci36, Ci126, and Ci131, may provide information on core protein interactive domains involved in dimer or higher-order complex formation. The mutation in Cp96, with a conservative change of Lys-96 to Arg, resulted in a marked decrease in endogenous polymerase activity, suggesting that this is a sensitive region for DNA synthesis. By contrast, 39 carboxy-terminal residues can be deleted without preventing capsid formation, although smaller deletions of the basic carboxy terminus reduce encapsidation and endogenous polymerase activity (6, 8). A core mutant with 7 amino acids deleted was essentially WT in function, while one lacking 12 amino acids formed capsids which encapsidated RNA but failed to synthesize second-strand DNA as efficiently as WT virions. A mutant protein lacking 20 amino acids formed capsids which contained only the 5′ half of the pregenome, while capsids formed from a core mutant lacking 39 amino acid were devoid of pregenomic RNA (6). In contrast to carboxy-terminal deletions, the extreme sensitivity of the HBV capsid to internal insertions and point mutations suggests that a series of well-chosen mutations may lead to further identification of the

domains necessary for assembly, encapsidation, and endogenous polymerase activity.

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