Exposure of RNA Templates and Encapsidation of Spliced Viral RNA Are Influenced by the Arginine-Rich Domain of Human Hepatitis B Virus Core Antigen (HBcAg 165-173)

Sophie Le Pogam,† Pong Kian Chua, Margaret Newman, and Chiaho Shih*

Department of Pathology and Department of Microbiology and Immunology, WHO Collaborating Center for Tropical Diseases, and Center for Biodefense and Emerging Infectious Diseases, University of Texas Medical Branch, Galveston, Texas

Received 8 July 2004/Accepted 1 October 2004

Previously, human hepatitis B virus (HBV) mutant 164, which has a truncation at the C terminus of the HBV core antigen (HBcAg), was speculated to secrete immature genomes. For this study, we further characterized mutant 164 by different approaches. In addition to the 3.5-kb pregenomic RNA (pgRNA), the mutant preferentially encapsidated the 2.2-kb or shorter species of spliced RNA, which can be reverse transcribed into double-stranded DNA before virion secretion. We observed that mutant 164 produced less 2.2-kb spliced RNA than the wild type. Furthermore, it appeared to produce at least two different populations of capsids: one encapsidated a nuclease-sensitive 3.5-kb pgRNA while the other encapsidated a nuclease-resistant 2.2-kb spliced RNA. In contrast, the wild-type core-associated RNA appeared to be resistant to nuclease. When arginines and serines were systematically restored at the truncated C terminus, the core-associated DNA and nuclease-resistant RNA gradually increased in both size and signal intensity. Full protection of encapsidated pgRNA from nuclease was observed for HBcAg 1–171. A full-length positive-strand DNA phenotype requires positive charges at amino acids 172 and 173. Phosphorylation at serine 170 is required for optimal RNA encapsidation and a full-length positive-strand DNA phenotype. RNAs encapsidated in Escherichia coli by capsids of HBcAg 154, 164, and 167, but not HBcAg 183, exhibited nuclease sensitivity; however, capsid instability after nuclease treatment was observed only for HBcAg 164 and 167. A new hypothesis is proposed here to highlight the importance of a balanced charge density for capsid stability and intracapsid anchoring of RNA templates.

The hepatitis B virus (HBV) core antigen (HBcAg) is a 22-kDa protein with multiple functions, including interactions with the pregenomic RNA (pgRNA) and the polymerase during encapsidation (10, 31, 41) and with the viral DNA during reverse transcription and DNA elongation (18), polymerization to form the nucleocapsid or core particles (4, 13), importing of HBV DNA to the nucleus (51), and targeting to the endoplasmic reticulum for envelope formation (5). The C-terminal region of HBcAg contains a protamine-like domain, which is rich in arginine and presumably binds to HBV RNA and DNA during pgRNA encapsidation and DNA replication (13, 18, 35). This arginine-rich domain has been shown to be dispensable for core particle assembly but not for viral replication (2, 4, 13, 30).

The virion release of HBV from hepatocytes is a tightly regulated event. The current dogma indicates that the mature hepadnavirus genome is preferentially exported from the intracellular compartment (15, 34, 47, 48, 54, 57–59). Exceptions to this dogma have been observed for one naturally occurring HBcAg variant and two artificially engineered HBV mutants. One engineered mutant is defective in RNase H activity and is unable to degrade the RNA moiety of the RNA-DNA hybrid produced during reverse transcription (15, 37, 54). When such a DNA-RNA hybrid genome was secreted by this RNase H mutant, it could mimic a mature genome-like double-stranded DNA (dsDNA) signal, leading to the secretion of virions (15, 34; S. Le Pogam and C. Shih, unpublished results). The other exception is the engineered HBV mutant 164, which has a truncation of 19 amino acids from the C-terminal arginine-rich domain of HBcAg (30). This mutant was reported to be deficient in positive-strand DNA synthesis, yet it remained capable of secreting an immature genome containing predominantly ssDNA.

In addition to mutant 164, other hepadnaviral mutants with truncations at the C terminus of the core protein have been reported. For example, in the case of duck hepatitis B virus (DHBV) mutants which were progressively truncated at the C terminus of the core protein, four different phenotypes were described (57). Like mutant 164, the so-called class II mutants of DHBV are defective in positive-strand DNA synthesis. However, unlike mutant 164, class II mutants are defective in virion secretion. Furthermore, the capsid particles of class II mutants appear to be more stable and tend to disintegrate and become nuclease resistant, implying viral RNA maturation (22).

The accumulation of frequent mutations in HBcAg has been found in chronic carriers of HBV (16, 20, 42). The most frequent naturally occurring mutation of HBcAg occurs at amino acid 97 and has been well documented in the literature (see references cited in reference 46). This mutation at codon 97 often changes a hydrophobic isoleucine or phenylalanine to leucine, another hydrophobic residue. Such conservative struc-
nurial changes (F97L and I97L) nevertheless result in two distinct phenotypes. One phenotype is a host factor-independent replication advantage of the I97L mutant in Huh7 cells, but not in HepG2 cells (46). The other phenotype is that the 97L variant secretes an excessive amount of less mature genomes compared to wild-type HBV (58, 59). This immature secretion phenomenon is not caused by degradation of the mature genome during DNA sample preparations, by the instability of core particles (22, 32), or by any deficiency in viral reverse transcription (26, 58, 59). The mechanism for the immature secretion phenomenon remains to be elucidated. To date, immature secretion of hepadnaviruses in vivo has been observed in snow geese (6) and in one woodchuck that was treated with acyclovir (49). However, it has not been found in patients (F. M. Suk, M. H. Lin, P. K. Chua, and C. Shih, unpublished results). Compensatory mutations of HBcAg, PST and P130T,
were found to offset the immature secretion phenotype caused by the I97L mutation (8, 60).

To compare the virion secretion behaviors between the artificial HBcAg mutant 164 and the naturally occurring HBcAg I97L mutant, we have characterized mutant 164 in further detail. We report here the identification of multiple new phenotypes of the old mutant 164 (30). To our surprise, mutant 164 actually secretes viral genomes containing predominantly preferred sites (positions 487 and 2449, respectively) of plasmid 164. Briefly, the plasmid was amplified by the use of Turbo DNA polymerase and two complementary oligonucleotides carrying a mutation at position 487 (sense oligonucleotide, 5'-CGGCCAACCGGCTAATCAGTGCGACGAGATCTCAATCTCGG-3') and 5'-CGAAGGCTCAATAGCCGCGTCGACAG-3'.

**MATERIALS AND METHODS**

The preparation of HBV intracellular core particles and viral DNA as well as centrifugation analyses of viral secretion is detailed elsewhere (58, 59). For capsid preparation without nuclease treatment, we most often used an antibiotic for immunoprecipitation (see below). For capsid preparation with nuclease treatment, we used either the polyethylene glycol (PEG) method (for Fig. 1A, 1B, 2, 3E, 4B, 5C, and 6C) or the immunoprecipitation method (for Fig. 1C, 4A, 4B, and 5D) interchangeably. We found no difference in the results between these two methods in the presence of nuclease.

**Plasmid constructs.** (i) **p164.** Plasmid 164 was named pCHT-9/3091/164 by Nassal (30). Briefly, it contained a 1.1-unit-length HBV genome (ayw subtype [12]) under the control of a cytomegalovirus promoter, with a mutation changing the arginine-rich domain of HBcAg 165-171 can prevent ex-

**FIG. 2.** Heat denaturation and a positive-strand-specific probe demonstrated that PEG-precipitated intracellular mutant 164 core particles contained shorter single-stranded and double-stranded DNA replicative intermediates. Replicative intermediates were extracted from intracellular core particles at 5 days posttransfection and were heat denatured (5 min at 100°C) before gel electrophoresis. Southern blot analysis was performed with a double-stranded HBV DNA probe as described in the legend to Fig. 1 (left). The double-stranded HBV DNA probe was then removed, and the same nitrocellulose filter was rehybridized with a positive-strand-specific riboprobe (right). The minus-strand probe was not used because it gives virtually identical Southern patterns as the double-stranded probe.
FIG. 3. PCR cloning and sequencing analysis of viral DNAs present in mutant 164 virions. (A) Oligonucleotide primers used to PCR amplify virus particle-associated DNAs. Nucleotide positions are given according to the ayw subtype (12). (B) Nested PCR products were loaded in a 1.2% agarose gel and detected by ethidium bromide staining. M1 and M2, DNA molecular weight markers (λ DNA/HindIII and φX174 RF DNA/HaeIII, respectively); NC, negative control (H2O); PC, positive control (plasmid 164). (C) Amplified DNAs from mutant 164 within the 0.5- to 1.3-kb (a) and 0.2- to 0.4-kb (b) ranges were cloned into a TA cloning vector, and colonies were screened by sequencing. The cartoon summarizes all of the spliced DNAs obtained from the PCR products. Nucleotide positions at the intron-exon boundaries are given according to the ayw subtype (12). ε, packaging signal; SD, splice donor; SA, splice acceptor. (D) Mutations at the major splice acceptor site alone (164/487KO) or at both the major splice donor and major splice acceptor sites (164/487-2449KO) eliminated strong signals (*) presumably corresponding to the ssDNA and dsDNA replicative intermediates of mutant 164. The question mark indicates DNA signals of splice mutants which happened to comigrate with the ssDNA of WT HBV. (E) Heat denaturation experiments demonstrated that no ssDNAs of splice mutants (164/487KO and 164/487-2449KO) were really of the same size as the ssDNA of WT HBV.
For isolations of core-associated RNAs, cells were lysed 3 days after transfection (10 mM Tris, 1 mM EDTA, 50 mM NaCl, 0.25% NP-40, 8% sucrose). After incubation at 37°C for 15 min, the nucleus was pelletted and the supernatant was collected. Core particles were pelleted by immunoprecipitation with an anticore antibody and protein A and protein G beads. After overnight incubation, the beads were washed four times with phosphate-buffered saline, resuspended in core buffer (10 mM Tris, 6 mM MgCl₂, 8 mM CaCl₂), and treated or not treated with nuclease (30 U of micrococcal nuclease, 1 U of DNase I; Roche Co., Palo Alto, Calif.) for 1 h at 37°C. RNAs were then extracted from core particles by the use of TriReagent as instructed by the manufacturer (Sigma Co.). RNA samples were then analyzed by Northern blotting with a vector-free 32P-labeled full-length HBV or core-specific DNA fragment. The PEG method for the isolation of core particle-associated RNAs was conducted as described elsewhere (46).

**PCR analysis of virus particle-associated DNA.** Virus particle-associated DNA was extracted as previously described (58). Briefly, the cell culture medium was collected on days 5 and 7 posttransfection. After layering on a 20% sucrose cushion, isopycnic centrifugation in a cesium chloride gradient (20% to 50% [wt/vol]) was performed. Particles were separated according to their buoyant densities. After dialysis, the fractions corresponding to the Dane particles (fractions 10, 12, 14, and 16; density $d = 1.23$ g/cm³) were pooled together and the DNA was extracted. The DNA was then treated with the DpnI enzyme to avoid contamination by the input plasmid during PCR amplification. DpnI-treated DNA was used as a template to amplify the core and envelope genes with the primers 1861S (5'-ACTGTCAGGTCCTCAAGCT-3') and 990AS (5'-ACTTTCAAATCAATGG-3'). Amplification was performed in a 10-μl mixture containing 2 μl of DpnI-treated DNA (about 1/10 of the extracted DNA), 1.6 μl of a 1.25 mM deoxynucleoside triphosphate mix, 1 μl of 10× PCR buffer, 125 ng of each primer, and 1.25 U of Taq polymerase (the conditions were 10 s at 94°C for denaturation, following by 30 cycles of amplification at 94°C for 10 s, 50°C for 10 s, and 72°C for 1 min). A second amplification was then performed with a nested primer set (1880S, 5'-TGTGGTTTACAGCCACAC-3'; and 570AS, 5'-AGGGTGTTTACAGCCACAC-3') in a 50-μl mixture containing 1 μl of the first PCR product (the quantity of each reagent was adjusted accordingly). The reaction mixtures were loaded into a 1.2% agarose gel. The PCR products obtained from mutant 164 were purified and cloned into the pGEM-T vector (Promega, Madison, Wis.). Sequencing was performed by use of a Sequenase kit (USB, Cleveland, Ohio).

**DNA agarose gel electrophoresis.** DNA samples were subjected to 1% agarose gel electrophoresis in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 7.8]) for 4 h at 100 V. Standard Southern blot procedures were followed (58). Lambda HindIII size markers were always included to identify the full-length relaxed circle form (4 kb), the full-length double-stranded linear form (3.2 kb), and the full-length single-stranded form (1.5 to 1.7 kb) of DNA.
Core-specific probe. The 0.6-kb DNA fragment from plasmid pSVC (58) was purified by HindIII and SacI digestion and by use of a QIAquick PCR purification kit (Qiagen Co., Valencia, Calif.). Approximately 25 ng of DNA was radio-labeled by use of a random-primed DNA labeling kit (Roche Co.).

Positive-strand-specific riboprobe. XhoI-linearized pGEM-4Z-HBV, which contains one copy of the HBV genome (58), was used to generate a 3.1-kb RNA fragment which specifically hybridizes to positive-strand HBV DNA. In vitro transcription to make the 32P-labeled RNA probe was performed by use of an AmpliScribe T7 transcription kit (Epiconcept Technologies, Madison, Wis.).

Western blot analysis. Cell lysates were prepared from cells at 4 days post-transfection with sodium dodecyl sulfate (SDS) lysis buffer (100 mM Tris-HCl [pH 8.0], 5% SDS, 0.2% bromphenol blue, 20% glycerol, 3% 2-mercaptoethanol) and then subjected to SDS-12% polyacrylamide gel electrophoresis, and core proteins were detected by standard Western blot procedures with rabbit anti-capsid antibodies (46).

Primer extension analysis. A primer extension protocol was adapted from the work of Roychoudhury et al. (39). Briefly, a 5’-end-labeled oligonucleotide (1930/AA, 5’-GAGGATCAACCTCAGATGTC-3’) was annealed at 65°C for 10 min to core-associated RNAs (extracted from one transfected 10-cm-diameter dish of cells) in a buffer containing 5% formamide, 1 mM EDTA (pH 7.8), 40 mM PIPES (piperazine-N,N-bis(2-ethanesulfonic acid), pH 6.2) and 400 mM NaCl. The mixture was then cooled down at room temperature. After the annealing step, 20 μl of 3 M sodium acetate, 150 μl of diethyl pyrocarbonate-treated water, and 400 μl of ethanol were added for precipitation. After centrifugation, the pellet was dissolved in 2 μl of a 10 mM deoxynucleoside triphosphate mixture, 2 μl of 0.1 M dithiothreitol, 10 μl of RNase inhibitor, and 1 μg of actinomycin D. The reaction was terminated by the addition of 1 μl of 0.5 M EDTA, pH 7.8, and 1 μl of RNase A for 30 min at 37°C. Two hundred microliters of Tris-EDTA-0.1 M NaCl was added to the reaction, followed by phenol-chloroform extraction and ethanol precipitation. After centrifugation, the pellet was dissolved in 3 μl of Tris-EDTA, and 3 μl of sequencing loading buffer was added. The mixture was then loaded into a 6% polyacrylamide sequencing gel.

Expression of HBV core proteins in E. coli. Constructs of pET-Blue1 containing truncated and full-length versions of HBV(20) core proteins were produced as described elsewhere (32). Briefly, the HBV core DNA fragments were amplified by PCRs with the common upstream primer 5’-ATGGACATTGACCCTGATTAA-3’. For the 1–154, 1–164, and 1–167 versions of the core protein, various downstream primers (for 1–154, 5’-TCTTCTTCTAGGTACCTGCCCGT-3’, for 1–164, 5’-TGTGACCTCTTGCAGCCGAGGG-3’, and for 1–167, 5’-ACGCGCCATTGTGACCTCGTCGCC-3’) containing stop codons at positions 155, 165, and 168, respectively, were used for PCR amplification of fragments from a pET-C183 DNA template which were then cloned into pET-Blue1 (32). Expression, purification, and characterization of the core particles were performed as detailed previously (32).

Nuclelease treatment of E. coli-derived capsids. Twenty micrograms of purified capsids was treated with 30 μl of micrococcal nuclease and 1 U of DNase I (Roche Co.) in the presence of 8 mM CaCl2 and 6 mM MgCl2 for 1 to 3 h at 37°C. Both nuclease-treated and untreated capsid preparations were run in 1% agarose gels containing ethidium bromide (EtBr). Gels were photographed under UV light and subsequently stained with Coomassie blue (32).

RESULTS

As reported previously, HBeAg mutant 164 is defective in positive-strand DNA synthesis yet seems to be able to secrete viral particles containing immature genomes (30). To compare the immature secretion phenotype of a naturally occurring HBeAg 197L mutant (58, 59) with the virion secretion behavior of the engineered HBeAg mutant 164, we reevaluated the phenotype and genomic structure of mutant 164.

Mutant 164 capsids contain an unusual DNA pattern. The intracellular replication of mutant 164 was first compared to that of the WT HBV genome by Southern blot analysis (Fig. 1A, left panel). To our surprise, the HBV DNA isolated from mutant 164 exhibited an unusual pattern, as follows. (i) A significant portion of its replicative intermediates displayed a higher molecular weight (MW) than the full-length ssDNA of the WT. (ii) There was no detectable accumulation of DNA signals which would comigrate with the full-length ssDNA of WT HBV. Instead, at least three strong bands appeared to migrate faster than the full-length ssDNA of the WT. (iii) The species with the highest MW was estimated to be about 2.2 kb.

This unusual DNA pattern for mutant 164 suggested that (i) mutant 164 probably can efficiently synthesize positive-strand DNA (i.e., dsDNA) in order to acquire a higher molecular weight than the full-length ssDNA of the WT and (ii) the smaller DNA genome of mutant 164 may result from the mutant encapsidating a subgenomic RNA species. The non-spliced 2.1- and 2.3-kb envelope protein-specific RNAs contain no RNA packaging signal (epsilon) and are not known to be encapsidated or reverse transcribed. We speculated that mutant 164 may encapsidate the 2.2-kb spliced viral RNA and reverse transcribe it to a spliced DNA genome (38, 43, 50). After all, important cis elements (DR1 and DR2) required for HBV DNA replication are located near the ends of the 3.5-kb pgRNA. A 5’ or 3’ deletion of the viral RNA template of 1.3 kb (from 3.5 to 2.2 kb) would have no DR1 or DR2 for DNA synthesis.

Mutant 164 does not have an immature secretion phenotype. As shown in Fig. 1A (middle panel), the majority of the secreted viral genome of mutant 164 had a higher molecular weight than the ssDNA of the WT. Therefore, the vast majority of the viral microRNAs associated with mutant 164 virions does not appear to be immature genomes (30). Consistent with the intracellular RNA data (Fig. 1A, left panel), mutant 164 exhibited a reduced molecular weight for its secreted DNA genome. Furthermore, the DNA pattern of mutant 164 virions was also very different from that of the F97L mutant, which exhibited a so-called immature secretion profile (Fig. 1A, right panel) (58, 59).

Comparison between two different methods of capsid preparation. The capsid particles used to generate the data shown in Fig. 1A were prepared by the PEG precipitation method (39). This method involves the use of a nuclease treatment to remove contamination of the input plasmid DNA from capsule preparations. As mentioned briefly in the introduction, the genomic DNAs of class II capsid mutants of DHBV were shown to be susceptible to nuclease digestion due to capsid instability or disassembly occurring during genome maturation (22). The reduction in the genomic DNA size of mutant 164 may have been caused by the in vitro exposure to nuclease during the PEG procedure. To address this issue, we compared the Southern blot patterns of the core particle-associated DNA of mutant 164 by performing the PEG method with or without the nuclease treatment. As shown in Fig. 1B, we found no difference in the Southern blot patterns of mutant 164 with or without micrococcal nuclease. Similarly, when capsids were prepared by anticoag antibody-mediated immunoprecipitation, we also found no difference in the Southern blot patterns with or without nuclease treatment. Taken together, these results (Fig. 1B and C) indicate that the smaller genome of mutant 164 was not due to in vitro digestion by the micrococcal nuclease or DpnI. These two methods of capsid preparation can be used interchangeably for mutant 164.

Mutant 164 capsids contain double-stranded DNA which can be heat denatured into single-stranded DNA. The results
shown in Fig. 1 suggested that mutant 164 most likely contains a dsDNA genome with a reduced molecular weight. To determine the molecular structure of the DNA species present in mutant 164 core particles, we heat denatured the DNA samples before gel electrophoresis. After heat denaturation (100°C for 5 min), all dsDNA species should convert to ssDNA. As shown in Fig. 2, both the WT and mutant 164 appeared to contain dsDNA, which converted to ssDNA upon heat denaturation. We also noted that the resulting ssDNA species of mutant 164 migrated faster than the full-length WT ssDNA, again suggesting that the DNA isolated from mutant 164 core particles was indeed smaller. Furthermore, we observed no full-length ssDNA of the WT size in the heat-denatured mutant 164 DNA sample.

**Mutant 164 contains positive-strand DNA.** The strong signal intensity of the dsDNA genome of mutant 164 was inconsistent
FIG. 5. Functional analysis of the C-terminal domain of the core protein by restoring amino acids to mutant 164. (A) C-terminal amino acid sequences of the various truncated core proteins used for this study. The four arginine (R)-rich domains (I to IV) as well as the phosphorylation sites (*) at serine residues 155, 162, and 170 are indicated. Missense mutations are underlined. (B) Western blot analysis of truncated core proteins by use of a rabbit anticore antibody. The weaker signal obtained for SVC169 was not representative. (C) Wild-type and mutant core proteins were supplied in trans to core-defective HBV replicon 1903 (58). Their respective complementation effects on intracellular HBV DNA replication were analyzed by Southern blotting. The dot represents incomplete nuclease digestion of the input plasmid during the HBV DNA extraction from PEG-precipitated capsids. All samples were run in the same gel, but the exposure time for autoradiography was shorter for the first two lanes. (D) Northern blot analysis of immunoprecipitated core-associated RNAs (see the legend to Fig. 4) of the various mutants shown in panel A. trans-Complementation experiments were conducted as described for panel C. Top, no nuclease treatment; bottom, nuclease treatment. (E) Primer extension analysis of 5′ ends of core-associated RNAs, including both 3.5- and 2.2-kb RNA species. No precore RNA was encapsidated. As a control, the cytoplasmic total RNA was used in the primer extension assay. The identities of the bands corresponding to precore and core RNAs were based on an adjacent DNA sequencing ladder (data not shown).
with a previous report that mutant 164 can synthesize positive-strand DNA at only 10 to 20% of the level of WT HBV (30).

To further address this issue, we reprobed the same filter used to generate Fig. 2 (left panels) with a positive-strand-specific riboprobe. As shown in the right panels, positive-strand DNA was present at similar levels in both the WT and mutant 164. We noted that there were some mysterious but reproducible positive-strand signals migrating below the full-length ssDNA position of mutant 164 when there was no heat denaturation. It is our interpretation that mutant 164 encapsidates multiple species of spliced RNAs and that these mysterious signals represent low-MW species of spliced dsDNA (Fig. 3).

The shorter DNA species in mutant 164 capsids are derived from the 2.2-kb spliced RNA. Although we hypothesized that the shorter dsDNA genome of mutant 164 may be a product of reverse transcription from the encapsidated spliced RNA, we had no direct experimental evidence for this hypothesis. To this end, we performed nested PCRs using primers spanning the known predominant splice sites (Fig. 3A), with virion-associated DNAs isolated from the WT and mutant 164 as templates (see Materials and Methods).

As shown in Fig. 3B, only shorter fragments, designated “a” and “b,” were obtained from mutant 164 by nested PCR. Unlike the case for the WT control, we were not able to detect a 1.9-kb DNA product from the mutant 164 DNA template. Amplified products from mutant 164 ranging from 0.2 to 0.4 kb and from 0.5 to 1.3 kb were cloned into a TA cloning vector. Sequencing was performed with a primer located outside the putative introns. Spliced junction sequences (GT-AG) were identified in each clone. All clones analyzed from the 0.2- to 0.4-kb range contained the same spliced DNA (2067/489). For the 0.5- to 1.3-kb range, a more heterogeneous population of spliced DNA species was identified. For example, the 2447/489 spliced DNA species was identified in about 70% of the sequenced clones (17 of 25). The 2471/489 species was identified in four clones, and the 2471/282 spliced DNA was identified in two clones. One clone of each doubly spliced DNA species (2471/2902–2985/489) and (2447/2902–2985/489) was identified (Fig. 3C) (7). Consistent with the Southern blot data (Fig. 1 and 2), no full-length nonspliced DNA genome was detected in mutant 164 by PCR analysis (Fig. 3B).

A triple mutant encapsidates alternatively spliced RNA species. A missense mutation which abolished the most predominant splice acceptor site at nucleotide (nt) 489 (43, 45, 50, 55)
was introduced into the mutant 164 genome. As shown in Fig. 3D, the two strongest signals (\(*\)), presumably corresponding to the single-stranded and double-stranded forms of spliced RNAs, were lost in this splice mutant, designated 164/487 KO. When an additional mutation was introduced into the predominant splice donor site at nt 2449 (Fig. 3D), the triple mutant 164/487–2449 KO continued to contain shorter DNA genomes. Most interestingly, this triple mutant contained increased signal intensity at a position (marked “\(\ddagger\)”) in Fig. 3D) that exactly comigrated with the full-length ssDNA of WT HBV. To clarify if this was indeed full-length ssDNA or fortuitously comigrating spliced dsDNA, we again performed the heat denaturation experiment. As shown in Fig. 3E, no full-length ssDNA-like species were found in the 164/487–2449 KO mutant after heat denaturation. Therefore, RNA species generated via alternative or cryptic splice sites must have been encapsidated by this triple mutant. Indeed, PCR cloning and sequencing confirmed that the predominant DNA species in the triple mutant was reverse transcribed from the RNA template spliced at nt 2067 and 282 (data not shown).

In summary, we demonstrated by PCR cloning, sequencing, mutagenesis, and heat denaturation experiments that the majority of the shorter DNA genome of mutant 164 originated from encapsidated spliced RNAs. No reversion to the WT phenotype containing the nonspliced DNA genome was ever observed when the predominant splice sites were abolished in the 164/487–2449 KO mutant.

Similar level of 3.5-kb full-length pgRNA and higher level of 2.2-kb RNA in mutant 164 capsids compared to WT HBV. To confirm that the origin of the shorter dsDNA genome of mutant 164 was indeed encapsidated spliced RNAs, we analyzed the core particle-associated RNAs of mutant 164 and WT HBV by Northern blot analysis. As shown by the results of a duplicate experiment in Fig. 4A, when core particle-associated RNAs were isolated by immunoprecipitation without nuclease treatment, mutant 164 appeared to have encapsidated a similar or slightly weaker level of full-length 3.5-kb pgRNA than the WT, despite the fact that we were unable to detect any full-length ssDNA in mutant 164 capsids (Fig. 1 to 3). Furthermore, we noted that mutant 164 reproducibly packaged more 2.2-kb RNA species than WT HBV (bracketed regions; compare lanes 2 and 1 and lanes 6 and 5).

Reverse-transcribing RNA templates between 2.2 and 3.5 kb from mutant 164 are more susceptible to nuclease digestion. When immunoprecipitated core particles were not digested with nuclease, the putative full-length 3.5-kb pgRNA (\(*\)) was often present in both the WT and mutant 164, even after extensive washing of the precipitated capsids (Fig. 4A). To see if this 3.5-kb RNA signal was derived from encapsidated pgRNA or from nonencapsidated viral RNAs which were non-specifically bound to the capsid surface, we included the polymerase-deficient 2310 mutant (39). It is well known that polymerase is required for the packaging of pgRNA. Since mutant 2310 is deficient in the synthesis of polymerase (39), it is defective in RNA encapsidation. However, after a long exposure, we could detect weak signals from the capsids of mutant 2310. Therefore, there is no doubt that pgRNA can bind non-specifically to the surfaces of capsids. However, the trace amount of RNA which non-specifically bound to the capsid exterior was very small and insignificant (Fig. 4A).

Upon nuclease treatment, mutant 164 lost all continuously smearing signals between 2.2 and 3.5 kb (Fig. 4A, dotted areas), and only the shorter RNA species around 2.2 kb and below were spared (regions below the dotted areas). Since these nuclease-sensitive signals were not discrete in size and since there is no known spliced RNA within the range of 2.6 to 3.5 kb, we interpreted these nuclease-sensitive continuously smearing signals (dotted areas) as reverse-transcribing RNA templates that were concomitantly degraded by RNase H during minus-strand DNA synthesis. In contrast, wild-type HBV capsids that underwent nuclease treatment showed signals between 2.2 and 3.5 kb that were well protected from nuclease (Fig. 4A, lanes 3 and 7). Unlike the DHBV system with a more sensitive RNase protection assay (33), our Northern blot analysis of WT HBV core particle-associated RNA did not seem to reveal any apparent 3′-end nuclease sensitivity of WT HBV.

These results suggest that although mutant 164 could still encapsidate the 3.5-kb pgRNA, somehow its capsid-associated RNA species between 2.2 and 3.5 kb were more exposed and susceptible to micrococcal nuclease digestion in vitro. Unlike these higher MW RNA species, at least a substantial fraction of the viral RNAs around and below 2.2 kb in mutant 164 capsids appeared to remain resistant to nuclease treatment (Fig. 4A, lanes 4 and 8). At present, we cannot exclude the possibility that some of the 2.2-kb and lower MW RNAs (Fig. 4A, lanes 2 and 6, bracketed areas) were also sensitive to nuclease treatment. As shown in Fig. 4A, after nuclease digestion, the signal intensity around the 2.2-kb region for mutant 164 (lanes 4 and 8, signals below the dotted areas) was no longer significantly stronger than the corresponding region for WT HBV (lanes 3 and 7, respectively), as observed before nuclease treatment (bracketed areas; compare lanes 2 and 1 and lanes 6 and 5) (see Discussion).

To make sure that the results shown in Fig. 4A were independent of the methods used for capsid preparations, we compared the nuclease sensitivities of mutant 164 capsids isolated by either the PEG method or the anticore antibody-mediated immunoprecipitation method. As shown in Fig. 4B, we observed no difference in the results from both methods. In general, we tended to have a larger yield of recovered capsids by the PEG method.

Preferential encapsidation rather than preferential splicing of the 2.2-kb RNA by mutant 164. The predominant 2.2-kb RNA in mutant 164 capsids may result from increased production of the 2.2-kb RNA by mutant 164. Alternatively, it may result from preferential or more productive encapsidation of the 2.2-kb RNA than of the 3.5-kb RNA by mutant 164. As shown in Fig. 4C, left panel, when the total intracellular viral RNAs were compared between the wild type and mutant 164 by Northern blot analysis with a full-length HBV DNA probe, the 2.1- to 2.3-kb RNA species of mutant 164 were reproducibly reduced in intensity relative to those of wild-type HBV. These 2.1- to 2.3-kb RNA species include the 2.1-kb nonspliced RNA for the small envelope protein, the 2.3-kb nonspliced RNA for the large envelope protein, and the spliced 2.2-kb RNA, which is unrelated to the envelope proteins. To improve the resolution of these RNA species with very similar MWs, we used a core-specific probe specific for the spliced 2.2-kb RNA that did not cross-hybridize with RNA species coding for the envelope proteins. Again, the 2.2-kb RNA signal was reduced
by about 70% based on a quantitative image analysis (Fig. 4C, right panel). These results argue against the possibility of enhanced splicing. Instead, they favor the possibility of preferential encapsidation of the 2.2-kb RNA by mutant 164 capsids.

**Correlations between numbers of C-terminal arginine residues, viral DNA signal intensities, and genomic sizes.** The core protein of mutant 164 contains only the first two arginine-rich motifs and one arginine residue from the third motif (Fig. 5A; also see Fig. 7). The apparent preference for encapsidating the 2.2-kb RNA of mutant 164 (Fig. 4) suggests the important influence of the C-terminal arginine (R)-rich domains I to IV (Fig. 5A; also see Fig. 7) of HBcAg on the specificity and/or efficiency of productive viral RNA encapsidation. In addition, since no full-length DNA was found in mutant 164, despite the presence of a full-length 3.5-kb RNA, it was logical to ask if reverse transcription of the 3.5-kb pgRNA and/or capsid stability could also be affected by the C-terminal truncation of mutant HBcAg 1–164. A previous study by Nassal (30) has shown that the mutant HBcAg 1–173 can replicate like wild-type HBV. Therefore, the absence of amino acids 165 to 173 is entirely responsible for all of the phenotypes of mutant 164 described so far (Fig. 1 to 4). To map this critical domain in further detail, we constructed expression vectors for serially truncated core proteins (Fig. 5A). Western blot analyses demonstrated that all of the truncated core proteins were expressed at the expected sizes and at similar levels (Fig. 5B).

These serially truncated core proteins were then provided in trans by cotransfection with a core-deficient HBV genome (carrying plasmid 1903, which is a tandem dimer replicon lacking the initiation codon for HBcAg) (58). As shown in Fig. 5C, trans-complementation of plasmid 1903 with the truncated core protein of mutant 164 in a simian virus 40 expression vector reproduced the phenotype that was previously observed for mutant 164. However, weaker signals of DNA replication from plasmid pSVC164 than from mutant 164 under the control of the highly potent cytomegalovirus promoter were noted. Interestingly, we also noted that there appeared to be a general correlation between the increase in signal intensity and the size of core particle-associated DNAs when the C terminus of HBcAg was gradually extended from amino acids 164 to 173 (Fig. 5C). Such a correlation suggests that a common mechanism is likely involved in both replication efficiency and genomic size (see Discussion).

**Importance of arginine-172 and arginine-173 for the full-length HBV DNA phenotype.** When the entire arginine-rich domain III was gradually restored up to HBcAg 1–169 and 1–171, there was still no full-length HBV DNA at the WT level, although the size of the viral DNA was clearly increased relative to that of mutant 164 (Fig. 5C). However, when the truncated protein HBcAg 1–173 (plasmid pSVC173RR) was used in the complementation assay, we observed a complete restoration of the WT-like phenotype with a full-length HBV DNA (Fig. 5C) (30). Therefore, arginine-172 and arginine-173 are critical for the full-length HBV DNA phenotype.

**Importance of positive charge at amino acids 172 and 173 for the full-length HBV DNA phenotype.** To distinguish the size effects of Arg-172 and Arg-173 from their charge effects, we replaced these two arginine residues with two lysine (K) residues (also positively charged amino acids) or two glycine (G) residues (no charge) (Fig. 5A). While the HBcAg173KK protein was able to exhibit the WT phenotype with a full-length HBV DNA, HBcAg173GG had the same phenotype as HBcAg 1–169 or HBcAg 1–171. The phenotype of HBcAg 1–179, which carries all four R-rich domains, was similar to that of HBcAg 1–173, suggesting that Arg-174, Arg-175, and Arg-179 are not required for viral DNA replication in this tissue culture system. In conclusion, positive charges at positions 172 and 173 are important for the full-length HBV DNA phenotype.

**Full protection of encapsidated viral RNAs in HBcAg 1–171.** To further investigate the DNA phenotypes of mutants in Fig. 5C, we performed Northern blot analyses to examine their respective encapsidated RNA species. A rabbit anti-core polyclonal antibody was used to immunoprecipitate the capsid particles without nuclease treatment. The core particle-associated RNAs were prepared by trans-complementation of the core-deficient HBV genome (plasmid 1903) with different plasmids.
i.e., pSVC169, pSVC171, pSVC173GG, pSVC173RR, and pSVC173KK. As shown by Northern blot analysis (Fig. 5D, top panel), core-associated RNAs from all mutants migrated at about the same position as that of WT HBV, suggesting that they all can package the full-length 3.5-kb pgRNA. We also observed a higher level of encapsidated RNA for mutants 171, 173KK, and 173GG by either Northern blotting (Fig. 5D, top panel) or primer extension analysis (Fig. 5E). As expected, no precore RNAs were encapsidated by the WT or the mutants (Fig. 5E). However, when capsids were treated with nuclease after immunoprecipitation, encapsidated RNAs of mutant 164 and 169 were not well protected (Fig. 5D, bottom panel). Although mutant 169 was better protected than mutant 164, its 3.5-kb RNA was not as fully protected as that of mutant 171. Therefore, in addition to the three arginines at positions 165 to 167, nonarginine residues such as serine-170 and proline-171 also contribute to the full protection of the encapsidated viral RNAs. For reasons that are unclear, the 173RR mutant reproducibly displayed a weaker signal intensity than the 171, 173GG, and 173KK mutants.

Phosphorylation at serine-170 of HBcAg is important for a normal DNA phenotype. Because serines and arginines are closely clustered together at the C terminus of HBcAg (Fig. 5A; also see Fig. 7), the aforementioned phenotypes of mutant 164 and other C-terminal mutants may have been caused in part by the lack of serines rather than entirely by the lack of arginines. There are a total of seven serine residues (ayw subtype) located between HBcAg positions 155 and 181. Thus far, the three serines located in the SPRRR motifs have been studied, and it has been shown that phosphorylation and dephosphorylation at positions 155, 162, and 170 (ayw subtype numbering system) play an important role in pgRNA encapsidation and DNA replication (14, 23). In this study, we attempted to improve our resolution by dissociating the role of arginines from that of their adjacent serines.

We changed serine-170 into alanine (A) (to prevent phosphorylation), aspartic acid (D), glutamic acid (E) (to mimic phosphorylation), or threonine (T) (to allow phosphorylation) in various HBcAg contexts (Fig. 6A). As shown in Fig. 6B, all of the proteins produced from these serine-170 mutants in various contexts were expressed at similar levels. A trans-complementation experiment using the core-deficient replicon 1903 was performed by cotransfection. Consistent with the results shown in Fig. 5C, the SVC173RR/S170S and SVC173RR/S170T mutants exhibited a WT-like full-length DNA phenotype (Fig. 6C). However, except with the SVC171/S170A and SVC173RR/S170A mutants, we did not observe any striking effect on the DNA pattern when serine-170 was changed to an aspartic acid or glutamic acid in the context of HBcAg 1-171 or HBcAg 1-173RR. Therefore, phosphorylation at serine-170 is likely important for a normal level of HBV DNA, particularly for positive-strand DNA in the context of the 173RR mutation.

Serine-170 of HBcAg and viral RNA phenotype. To distinguish the effects of Ser-170 on RNA encapsidation from those on reverse transcription, we measured the total RNA (3.5-kb RNA plus 2.2-kb RNA plus smaller RNAs) encapsidation efficiencies of serine mutants by performing primer extension assays (Fig. 6D). Consistent with the results shown in Fig. 5E, we observed stronger signals from the SVC171/S170S and SVC173GG mutants. In addition, the SVC171/S170D and SVC173KK mutants also exhibited higher levels of core particle-associated RNAs. Since the RNA signal of the S170A mutant was weaker than those of the SVC171/S170S and SVC171/S170D mutants (Fig. 6D), while the DNA signal of the S170A mutant was also weaker than those of the WT and the rest of the serine mutants (Fig. 6C), dephosphorylation at serine-170 may have a negative effect on RNA encapsidation.

DISCUSSION

We have characterized the genomic structure and function of mutant 164 and have begun to extend that study to other arginine-deficient mutants of HBcAg (Fig. 7). We also have confirmed a previous report that the C-terminal 10 amino acids of HBcAg are dispensable for HBV DNA replication (30). Furthermore, our Northern blot analysis of the encapsidated RNA of mutant 164 lends support to the conclusion that the last 19 amino acids of HBcAg are not required for encapsidation of the full-length pgRNA (Fig. 4A and B).

Interestingly, we have identified several novel phenotypes of mutant 164, including (i) a lack of detectable full-length minus-strand DNA (Fig. 1 to 3), (ii) the predominant presence of spliced dsDNA (Fig. 1 to 3), (iii) secreted virions containing mature genomes of spliced dsDNA (Fig. 1), (iv) a reproducibly reduced level of the 2.2-kb spliced RNA (Fig. 4C), (v) preferential encapsidation of the 2.2-kb spliced RNA (Fig. 4A and C), and (vi) the nuclease sensitivity of the putative reverse-transcribing RNA templates (Fig. 4A and B).

As summarized in Fig. 7, further investigations of HBcAg mutants revealed the following: (i) when arginines and serines were systematically restored at the truncated C terminus, the core-associated DNA and nuclease-resistant RNA gradually increased in both size and signal intensity (Fig. 5C and D); (ii) positive charges at amino acids 172 and 173 are important for the formation of the full-length relaxed circle (RC) form of DNA (Fig. 5C); (iii) phosphorylation at serine-170 is required for efficient RNA encapsidation and positive-strand DNA synthesis (Fig. 6C and D); and (iv) HBcAg 1–171 is sufficient to confer full protection from nuclease digestion on encapsidated pgRNA (Fig. 5D).

Mutant 164 is not a so-called class II mutant or an immature secretion mutant. As mentioned in the introduction, despite a severe deficiency in positive-strand DNA, mutant 164 was able to secrete an immature genome (30). This discrepancy with the genome maturation signal hypothesis can now be explained by the finding here that mutant 164 is not a bona fide class II mutant (Fig. 1 and 2) (48, 57). At present, we do not have direct evidence that mutant 164 can produce the spliced dsDNA in its full-length size, nor do we know if the spliced dsDNA genome of mutant 164 is in a relaxed circle or double-stranded linear configuration. However, according to a primer extension analysis of the 5′ end of the positive-strand DNA in mutant 164, almost all of the ends are primed in situ (30, 44). Therefore, the RNA primer translocation to DR2 may have been very inefficient in mutant 164 (28, 40). Based on these studies (Fig. 1), mutant 164 does not secrete immature genomes in a nonspecific manner as the naturally occurring HBcAg 97L variants do (8, 26, 58, 59, 60).

Moderate degree of selectivity in viral RNA encapsidation by mutant 164. The packaging signal of hepadnaviruses is recognized by polymerase (1, 19) rather than by the nucleo-
capsid-like retroviruses (3, 25, 61). For HBV, this packaging signal (ε) is necessary and sufficient for pgRNA encapsidation (21). Both spliced subgenomic RNAs and nonspliced pgRNA contain the ε signal and can be encapsidated, reverse transcribed, and secreted (17, 43, 50). Mutant 164 predominantly, if not exclusively, contains the spliced DNA genome (Fig. 1, 2, and 3). This may be caused, in theory, by (i) increased splicing to generate the 2.2-kb RNA species, (ii) an intrinsic preference for the spliced RNA by mutant HBcAg, or (iii) selectivity against the nonspliced 3.5-kb pgRNA. As shown in Fig. 4A, both the wild type and mutant 164 were capable of encapsidating the full-length 3.5-kb pgRNA. Furthermore, the RNA species corresponding to the 2.2-kb region (Fig. 4A, bracketed areas) were more enriched in mutant 164 than in WT HBV, suggesting a small degree of preferential encapsidation of smaller RNAs by mutant 164. The possibility of increased splicing of the 2.2-kb RNA is discussed below.

**Mutant 164 exhibited a reduction of less than twofold in 2.2-kb RNA splicing.** In contrast to our prediction, the amount of 2.2-kb spliced RNA in the cytoplasm produced by mutant 164 was about 70% of that produced by WT HBV (Fig. 4C, right panel). It remains unclear why truncated HBcAg 164 resulted in a reduced level of cytoplasmic 2.2-kb spliced RNA, even though the effect was small. In the case of human immunodeficiency virus type 1, the RNA-binding protein Rev has been shown to mediate the export of intron-containing viral RNAs and thus can influence the proportion of viral RNA species (9). It has recently been reported that the DHBV core antigen is colocalized with SC-35, a host splicing factor present in the spliceosome (29). Indeed, the arginine-rich domains of HBcAg resemble the sequences of the well-known serine/arginine (SR)-rich splicing factors (11). It will be interesting to see if HBcAg does indeed play a role in HBV RNA splicing.

**Nuclease-sensitive capsids packaged nonspliced 3.5-kb RNA, and nuclease-resistant capsids packaged spliced 2.2-kb RNA.** At present, we cannot exclude the possibility that in addition to the RNA species between 2.2 and 3.5 kb, some of the 2.2-kb RNAs of mutant 164 may also be sensitive to nuclease. As shown in Fig. 4A, before nuclease treatment the 2.2-kb signal in mutant 164 was stronger than that of WT HBV, probably due to preferential encapsidation of the spliced 2.2-kb RNA. However, after nuclease treatment the 2.2-kb signal in mutant 164 became more or less equalized to that of WT HBV (Fig. 4A). We hypothesize here that there were two overlapping subpopulations of RNA-encapsidated capsids of mutant 164. One subpopulation initially encapsidated the 3.5-kb pgRNA, which was being reduced in size by the endogenous RNase H activity. Capsids from this subpopulation were less stable and were susceptible to nuclease digestion due to the excessive amount of negative charge inside the capsid. The other subpopulation encapsidated only the spliced 2.2-kb and shorter RNA species, but no 3.5-kb RNA. This subpopulation was more stable and was resistant to nuclease digestion due to its more balanced charge density (see below).

**Which portion of the reverse-transcribing RNA templates of mutant 164 is exposed?** Previously, a 1.7-kb viral RNA associated with HBcAg mutant cd163, which has a truncation of 20 amino acids at the C terminus, lacked sequences corresponding to the 3′ half of the pgRNA (2). We noted that the capsids of mutant cd163 were prepared by immunoprecipitation with nuclease treatment (2). We speculated that cd163 probably exposed its pgRNA to the capsid exterior, like mutant 164, and became susceptible to nuclease digestion during capsid preparation (Fig. 4A). Recently, the 3′ end of the wild-type DHBV pgRNA was found to be heterogeneous and underrepresented by use of an RNase protection assay and an RNase H mapping assay (33). We did not detect any apparent nuclease sensitivity of the core particle-associated RNA of WT HBV, as shown in Fig. 4A. This was likely due to the difference in detection sensitivity between Northern blotting and RNase protection assays (33). Alternatively, it may have been related to the species difference between HBV and DHBV.

While previous reports pointed to the possibility of 3′ end exposure, the data shown in Fig. 4A suggest that it is equally possible that the entire molecule (or a very large portion) was exposed. If only a 1.3-kb portion were exposed and susceptible to nuclease digestion, one would expect to see the downshift in the RNA signal from the 3.5- and 2.2-kb regions to RNAs of 2.2 kb and less. As such, the signal intensity around the 2.2-kb region should have been increased, instead of decreased, after nuclease treatment. Further investigation is needed to determine if it is the 5′ end, the 3′ end, or indeed the entire molecule of the 3.5-kb RNA template of mutant 164 that is exposed.

**Correlation between nuclease sensitivity and capsid instability.** There are at least two different models to interpret the relationship between nuclease sensitivity and capsid stability from our study of different C-terminal truncation mutants. In the first model, the nuclease sensitivity of mutant 164 would be an all-or-nothing phenomenon (Fig. 4A). Capsids which encapsidated the 3.5-kb RNA appeared to be less stable, and the entire RNA molecule (or a very large portion of it) was susceptible to nuclease digestion. On the other hand, this kind of capsid was still stable enough to allow RNA encapsidation and reverse transcription of the 3.5-kb RNA template. In the second model, the nuclease sensitivities of the WT and mutants 169, 171, and 173 did not seem to be an all-or-nothing phenomenon. Different degrees of nuclease sensitivity could be correlated with different degrees of exposure, and only a certain portion of the viral genome was exposed to the exterior of a stable capsid. Recently, the 3′ region of the pgRNA of WT DHBV was found to be susceptible to nuclease digestion (33). Therefore, nuclease sensitivity may not be a property that is necessarily associated with capsid instability; for example, it may be related to a “predisassembly” status of a stable and flexible WT capsid.

**Correlation between capsid stability and encapsidation or replication efficiency.** As shown in Fig. 5C, there was a general correlation between increases in signal intensity and increases in the sizes of DNA products when the C terminus of HBcAg was gradually extended from amino acids 164 to 173. A similar correlation is shown in Fig. 5D. Nuclease-resistant RNAs increased in size and intensity from mutants 164 to 169 and 171. These results suggest that the efficiencies of RNA encapsidation and DNA synthesis are causally associated with nuclease sensitivity and capsid stability. In a previous study, DHBV class II mutants were shown to be fully replication competent, and reductions in genomic DNA size were mainly caused by capsid instability and exposure to nuclease treatment during capsid preparation (22). If capsid instability was indeed the only fac-
tor and replication efficiency was not a contributing factor to the results shown in Fig. 5C and D, one would have expected to see equal intensities between differently sized products from different mutants (Fig. 5C). We are in the process of testing the nuclease sensitivities of the genomic DNAs of mutants 169, 171, and 173.

Arginine-mediated nucleic acid binding and intracapsid organization of viral genomes. To explain the lower replication efficiencies of the nuclease-sensitive mutants 164 and 169, we further hypothesize here that the arginine residues between amino acids 165 and 173 are important for causing the reverse-transcribed replicative intermediates to stay within the capsids. Presumably, exposed RNA templates can no longer serve as active substrates to continue DNA synthesis. The polymerase inside the capsids cannot reach the substrate once part of the replicating RNA or DNA template is exposed irreversibly to the capsid exterior and becomes nuclease sensitive. It has also been reported that nonencapsidated DHBV polymerase is enzymatically inactive (56).

Phosphorylation at Ser-170 of HBcAg is required for efficient RNA encapsidation and a full-length positive-strand DNA phenotype. HBcAg contains three major serine phosphorylation sites, at Ser-155, Ser-162, and Ser-170 (27). Our analysis of Ser-170 in the contexts of HBcAg 1–171 and 1–173 indicated that phosphorylation at this position is required for optimal RNA encapsidation (Fig. 6D) and a full-length positive-strand DNA phenotype (Fig. 6C). Although our results for the S170A mutant are in agreement with previous reports (14, 23), there is a minor discrepancy. Previously, the CM6 mutant containing an S170A mutation in the full-length HBcAg 1–183 context encapsidated barely detectable pgRNA, and no HBV DNA replication was observed (23). In our study, although the overall DNA signal of mutant S170A was significantly reduced, it remained detectable by Southern blot analysis (Fig. 6C and D). This minor discrepancy is probably more related to the detection sensitivity than to the sequence context. Finally, our results. (i) HBcAg mutant 164, which lacked eight arginines from the C terminus, appeared to preferentially package the 2.2-kb spliced subgenomic RNA (Fig. 4A). Mutant 164 capsids containing the 3.5-kb RNA appeared to be less stable and more nuclease sensitive than those containing the 2.2-kb RNA (Fig. 4A and B). (ii) When downstream arginine residues were added back to mutant 164 gradually, core particle-associated DNA and RNAs correspondingly increased in size and intensity (Fig. 5C and D). (iii) The amount of encapsidated RNA in E. coli-expressed HBV full-length capsids was estimated to be approximately 3,400 nt (62). This number coincides with the size of the pgRNA, 3.5 kb. (iv) When negatively charged residues were introduced into the N terminus of the DHBV core protein, a reduced size of the capsid-associated DNA was observed (22). The size reduction of the genomic DNA appeared to be caused by nuclease digestion. Again, exposure of the excessive negative charge of viral DNA in these mutants may be a way for capsids to manage an unbalanced charge density. (v) Most recently, it has also been reported that a retroviral nucleocapsid-RNA interaction is essential for structural stability but not for particle assembly and release (52). While we emphasized here the balance of the charge density between arginines and nucleic acids, we should point out that the interior, serine dephosphorylation may help to reduce the total charges are being accumulated by the DNA in the capsid interior, leading to the exposure of nuclear localization signals and the nuclear import of capsids.

This charge balance and capsid stability hypothesis is consistent with or supported by several independent experimental results. (i) HBcAg mutant 164, which lacked eight arginines from the C terminus, appeared to preferentially package the 2.2-kb spliced subgenomic RNA (Fig. 4A). Mutant 164 capsids containing the 3.5-kb RNA appeared to be less stable and more nuclease sensitive than those containing the 2.2-kb RNA (Fig. 4A and B). (ii) When downstream arginine residues were added back to mutant 164 gradually, core particle-associated DNA and RNAs correspondingly increased in size and intensity (Fig. 5C and D). (iii) The amount of encapsidated RNA in E. coli-expressed HBV full-length capsids was estimated to be approximately 3,400 nt (62). This number coincides with the size of the pgRNA, 3.5 kb. (iv) When negatively charged residues were introduced into the N terminus of the DHBV core protein, a reduced size of the capsid-associated DNA was observed (22). The size reduction of the genomic DNA appeared to be caused by nuclease digestion. Again, exposure of the excessive negative charge of viral DNA in these mutants may be a way for capsids to manage an unbalanced charge density. (v) Most recently, it has also been reported that a retroviral nucleocapsid-RNA interaction is essential for structural stability but not for particle assembly and release (52). While we emphasized here the balance of the charge density between arginines and nucleic acids, we should point out that the interior, serine dephosphorylation may help to reduce the total charges are being accumulated by the DNA in the capsid interior, leading to the exposure of nuclear localization signals and the nuclear import of capsids.

Charge balance and capsid stability hypothesis. Based on our studies reported here and previous reports by others, we propose a charge balance and capsid stability hypothesis. In this working model, we assume that the most active replication complex and the most stable capsid structure can be achieved by an adequate balance between positive and negative charge densities of the nucleic acid and protein complex in the capsid interior. The negative charges are mainly from encapsidated RNAs and reverse-transcribed DNA. The positive charges are mainly from the basic amino acids of HBcAg. This hypothesis further assumes that the HBV polymerase is not a very processive enzyme and that dissociation between the polymerase and DNA or RNA substrates can occur frequently during the process of DNA synthesis. Although the polymerase and minus-strand viral DNA are covalently linked to each other initially (53, 63), such a linkage probably cannot prevent exposure of the RNA templates of arginine-deficient mutants, which are hybridized only transiently with the nascent minus-strand DNA owing to the ongoing RNase H activity. In this model, arginines at the C terminus of HBcAg, particularly HBcAg 165–173, are important for nucleic acid binding and intracapsid organization of larger viral genomes.

In addition to arginines, serine phosphorylation and dephosphorylation probably play an important role in modulating the dynamic balance of charge density. During minus-strand DNA synthesis, the charge density more or less remains constant, since while minus-strand DNA is being synthesized, RNA is being degraded by RNase H. However, when the nascent positive-strand DNA is being elongated and more negative charges are being accumulated by the DNA in the capsid interior, serine dephosphorylation may help to reduce the total load of negative charges. This hypothesis is consistent with a previous report that mature genomes in secreted virions are hypophosphorylated (36). For those maturing capsids whose serines are not dephosphorylated, capsid instability will occur due to an excessive amount of negative charge in the capsid interior, leading to the exposure of nucleic acid sequences and the nuclear import of capsids.
duced by micrococcal nuclease in capsids 154, 164, and 167, but not in the full-length capsid 183 (Fig. 8B). We also noted a gradual increase in EtBr staining for capsids 154 to 183 before the nuclease treatment. However, the gradual incremental increase in the EtBr staining pattern became more obvious after the nuclease treatment (Fig. 8B). These results appeared to be reminiscent of the increased degree of protection from nuclease when more and more arginines were added back to the C terminus of mutant 164 (Fig. 5D). Most surprisingly, when the EtBr-stained agarose gel (Fig. 8B, left panel) was subsequently restained with Coomassie blue (Fig. 8B, right panel), the banding intensities of capsid particles of mutants 164 and 167 were found to be significantly diffused or reduced, while capsids 154 and 183 remained unchanged before and after nuclease treatment. Analyses of the nuclease-digested capsids of mutants 164 and 167 by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining indicated that the core protein monomers of these two samples remained intact after nuclease treatment.
HBcAg 149 expressed in stability between full-length wild-type HBcAg 183 and mutant capsid instability after nuclease treatment remains to be investigated. Mutant 154 exhibited nuclease sensitivity yet had no apparent densities leading to capsid instability or disintegration. Why of mutant 164 and 167 capsids resulted in unbalanced charge mutants 154 and 183 (WT). It is tempting to speculate here change their Coomassie blue staining patterns, but not those of RNAs from the surfaces of mutant 164 and 167 capsids would these nuclease-sensitive nucleic acids were largely nonencapsidated (data not shown). We believe that it is highly unlikely that interior. If this charge balance model is true, it will be interesting to see if it is also applicable to retroviruses and retrotransposons.

ACKNOWLEDGMENTS

This work was funded by NIH grants R01 CA 70336 and CA 84217 to C.S. S.L.P. and P.K.C. were supported by a James McLaughlin postdoctoral fellowship. We are grateful to Michael Nassal for plasmid 164.

REFERENCES


