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STRUCTURE AND ASSEMBLY

Common and Distinct Capsid and Surface Protein Requirements for Secretion of Complete and Genome-Free Hepatitis B Virions

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ABSTRACT During the morphogenesis of hepatitis B virus (HBV), an enveloped virus, two types of virions are secreted: (i) a minor population of complete virions containing a mature nucleocapsid with the characteristic, partially double-stranded, relaxed circular DNA genome and (ii) a major population containing an empty capsid with no DNA or RNA (empty virions). Secretion of both types of virions requires interactions between the HBV capsid or core protein (HBc) and the viral surface or envelope proteins. We have studied the requirements from both HBc and envelope proteins for empty virion secretion in comparison with those for secretion of complete virions. Substitutions within the N-terminal domain of HBc that block secretion of DNA-containing virions reduced but did not prevent secretion of empty virions. The HBc C-terminal domain was not essential for empty virion secretion. Among the three viral envelope proteins, the smallest, S, alone was sufficient for empty virion secretion at a basal level. The largest protein, L, essential for complete virion secretion, was not required but could stimulate empty virion secretion. Also, substitutions in L that eliminated secretion of complete virions reduced but did not eliminate empty virion secretion. S mutations that blocked secretion of the hepatitis D virus (HDV), an HBV satellite, did not block secretion of either empty or complete HBV virions. Together, these results indicate that both common and distinct signals on empty capsids and mature nucleocapsids interact with the S and L proteins during the formation of complete and empty virions.

IMPORTANCE Hepatitis B virus (HBV) is a major cause of severe liver diseases, including cirrhosis and cancer. In addition to the complete infectious virion particle, which contains an outer envelope layer and an interior capsid that, in turn, encloses a DNA genome, HBV-infected cells also secrete noninfectious, incomplete viral particles in large excess over the number of complete virions. In particular, the empty (or genome-free) virion shares with the complete virion the outer envelope and interior capsid but contains no genome. We have carried out a comparative study on the capsid and envelope requirements for the secretion of these two types of virion particles and uncovered both shared and distinct determinants on the capsid and envelope for their secretion. These results provide new information on HBV morphogenesis and have implications for efforts to develop empty HBV virions as novel biomarkers and a new generation of HBV vaccine.

KEYWORDS capsid, complete virion, empty virion, envelope, hepadnavirus, hepatitis B virus, virus secretion

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Hepatitis B virus (HBV) infects chronically ca. 300 million people worldwide and remains a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1, 2). HBV is a member of the *Hepadnaviridae*, a group of retroid viruses harboring a small (ca. 3.2-kbp), partially double-stranded (ds-), relaxed circular DNA (rcDNA) genome that is replicated via reverse transcription of an RNA intermediate called pregenomic RNA (pgRNA) (3, 4). The HBV DNA genome is enclosed within a protein capsid composed of 240 (for the major population) or 180 (for the minor population) copies of one viral protein, the core or C (HBc) protein (5–7), which is in turn enclosed by an outer envelope consisting of a host-derived lipid bilayer studded with three viral envelope or surface proteins, S, M, and L (8–10).

Upon entry into human hepatocytes, HBV delivers its rcDNA to the host cell nucleus, which is converted to a covalently closed circular DNA (cccDNA) that serves as the transcriptional template able to generate all viral RNA species required for productive viral replication (11–13), including the aforementioned pgRNA. In addition to serving as the template for viral reverse transcription (i.e., the precursor to rcDNA), pgRNA also serves as the mRNA for the viral C and polymerase (P) proteins. pgRNA is specifically packaged into an immature nucleocapsid (NC) in a process also dependent on the P protein and host factors (14–18). Viral reverse transcription within immature NCs, carried out by P, a specialized reverse transcriptase (RT), converts pgRNA to rcDNA found in mature NCs (3, 13, 19, 20). Mature NCs, but not immature ones that contain pgRNA or the single-stranded DNA (ssDNA) intermediate, are then selected for envelopment by the viral envelope proteins and secreted into the bloodstream as complete and infectious virions (3, 21–26).

In addition to the complete rcDNA-containing virions, HBV replication also leads to the production and release of two classes of incomplete or subviral particles, neither of which is infectious (27). The first is the classical Australian antigen, the HBV surface antigen (HBsAg) sphere or filament that is composed of only the viral envelope proteins, devoid of either the viral capsid or genome, and that is present in the blood of infected patients in 100- to 100,000-fold excess over virions (4, 25). The second class of subviral particles released into the blood is the more recently discovered and characterized empty virion (genome-free virion) that contains the capsid and envelope but no viral RNA or DNA (28, 29). Genome-free or empty virions are produced at ca. 100-fold excess or more relative to the number of DNA-containing virions by HBV in cell cultures, experimentally infected chimpanzees, and naturally infected humans (28–30).

It remains unknown how either rcDNA-containing mature NCs or empty capsids are selected for envelopment but immature, ssDNA- or pgRNA-containing NCs are excluded. In particular, the signal(s) on mature NCs or empty capsids that directs envelopment remains to be defined (23, 25, 27). When Summers and Mason discovered reverse transcription in hepadnaviruses using the duck hepatitis B virus (DHBV) as a model system over 3 decades ago, they proposed the now classical "maturation signal" hypothesis, which posits that a structural change is triggered in mature NCs, differentiating them from immature NCs and signaling for mature NCs to be enveloped (3). On the other hand, to explain the envelopment of empty capsids but not immature NCs, we have recently proposed that the presence of ssDNA or pgRNA in immature NCs triggers a single-strand blocking signal to actively prevent the envelopment of immature NCs (28). As genome maturation occurs within the capsid while envelopment occurs from without, the capsid shell would seem to be ideally situated to transmit the genome information (or the lack of any genome in empty capsids) from its interior to the exterior for recognition by the envelope proteins. The relationship, if any, between the maturation signal, which stimulates envelopment of mature NCs, and the single-strand blocking signal, which prevents envelopment of immature NCs, remains unclear, as does the relationship between these signals and the secretion signal on empty capsids.

The capsid does play an integral role in viral assembly and replication. The N-terminal two-thirds (the assembly domain or N-terminal domain [NTD]; amino acids 1 to 140) of the core protein (7, 31–33) provides the protective shell for the viral

genome, while its C-terminal domain (CTD; amino acids 150 to 183), connected to the NTD via a short linker peptide (amino acids 141 to 149), plays essential roles in pgRNA packaging and the ensuing reverse transcription (34–39). The highly basic CTD is shown to have nonspecific RNA and DNA binding activity (40, 41). While traditionally thought to be dispensable for capsid assembly, the CTD has also been shown to be important to facilitate capsid assembly under physiological (low) protein and salt concentrations *in vitro* and in human cells (42).

Subtle structural differences have been observed by cryo-electron microscopy (EM) between virion-derived HBV NCs, which were presumed at the time to be all mature and contain rcDNA, and recombinant HBV capsids derived from bacteria containing nonspecific RNA (43). It is now clear, however, that the majority of the virion-derived capsids used for that and other EM studies (9, 10) were almost certainly empty capsids and that only a small minority were in fact mature NCs. The structures observed therefore likely represent a mixture of empty capsids and mature NCs. More recent cryo-EM imaging and biophysical studies also revealed some structural differences among recombinant, *in vitro*-assembled capsids that were empty or packaged artificially with pgRNA or ss- or dsDNA (but not the P protein) (44, 45). The role, if any, of these putative structural differences in directing NC envelopment remains to be determined. In addition, NC maturation is associated with a dramatic dephosphorylation at the CTD of the capsid protein such that mature NCs are dephosphorylated whereas immature NCs are heavily phosphorylated (30, 46, 47). CTD phosphorylation is important for pgRNA packaging (48, 49) and DNA synthesis (39, 46, 50, 51). At least for DHBV, subsequent CTD dephosphorylation is shown to be required for second-strand DNA maturation and stability of mature NCs (39, 51). However, the CTD phosphorylation state, *per se*, does not play an essential role in directing envelopment of mature NCs or empty capsids during complete or empty virion formation (30). Whereas complete virions contain dephosphorylated mature NCs, empty virions contain phosphorylated empty capsids.

On the other hand, there is strong and accumulating genetic evidence that implicates the HBc NTD in interaction with the viral envelope protein(s) to facilitate secretion of the DNA-containing virions. HBc NTD mutants, particularly those on the capsid surface, have been identified that remain competent for rcDNA synthesis but that are selectively defective in complete virion formation (25, 52–55). NTD variants that apparently relieve the block to envelopment of immature NCs (the so-called immature secretion mutants) have also been isolated (56). Furthermore, the snow goose hepatitis B virus is found to secrete ssDNA in their virions naturally (57), and elegant analyses using chimeric constructs have mapped the determinants responsible for this remarkable phenotype to just two residues in the NTD of the core protein (58). The role, if any, of the NTD in the secretion of empty virions, however, is not yet known.

Regarding the envelope proteins, both L and S, but not M, are required for secretion of DNA-containing HBV virions (8). L and M share the same sequences of the entire S protein in their C-terminal parts, with the N-terminal extension of M (pre-S2) being also entirely contained in L, which has an additional N-terminal extension (pre-S1). Specifically, the so-called matrix domain (MD) in L, a short linear sequence in the C-terminal part of pre-S1 and N-terminal part of pre-S2, is essential for complete virion formation (25, 59–64). The NTD residues on the capsid surface, the substitution of which prevents complete virion formation as described above, are thought to interact with the MD of L during complete virion formation and are thus called the matrix binding domain (MBD) (65).

Interestingly, HBV envelope proteins are also required for the envelopment and secretion of the HBV satellite, hepatitis D virus (HDV or delta agent) (66, 67). An important human pathogen itself, HDV virion contains a genomic RNA in complex with two HDV proteins, the small and large delta antigens enclosed within the HBV envelope proteins. The HDV proteins bear no resemblance to the HBV capsid protein. In contrast to formation of complete HBV virions, HDV virion formation requires only the HBV S, but not M or L, protein (68, 69). Evidently, sufficient levels of L must also be incorporated

into the HDV envelope (66, 70), presumably via interaction with S, since HDV infects the same cells as HBV in a process that is dependent on HBV L and uses the same cell surface receptor as HBV (70, 71).

To understand better the determinants involved in HBV virion assembly, we have now compared the core and envelope requirements for the secretion of empty HBV virions with those for complete virions. Our results showed that mutations in HBc or L that completely blocked the formation of complete virions still allowed secretion of empty virions. Furthermore, S alone was sufficient to allow empty HBV virion secretion at a low level, which was stimulated by L, even though L is absolutely required for complete virion secretion. In addition, S mutations that blocked HDV secretion did not inhibit secretion of either complete or empty HBV virions. These results thus demonstrated that morphogenesis of empty and complete HBV virions involved both common and distinct signals on both HBc and envelope proteins, and the S requirement for HDV secretion was also different from that of HBV empty virions.

RESULTS

HBc NTD mutations that block complete virion secretion did not block empty virion secretion. A number of HBc NTD residues located on the surface of the capsid shell have been identified as critical for secretion of complete virions (54). In particular, alanine substitutions at L60, L95, K96, and I126 were shown to block secretion of complete virions. To determine if these same mutations could differentially affect the secretion of empty virions, they were introduced into an HBV genomic (replicon) construct able to engender viral replication and secretion. To allow flexibility of the envelope proteins used to support virion secretion, the replicon construct used in some experiments (as indicated in the figures) was defective for expressing any envelope proteins. In that case, a second plasmid expressing envelope proteins was cotransfected to allow virion secretion. As indicated in the appropriate figure legends, an N-terminally truncated L protein, missing N-terminal residues 2 to 30 and thus lacking the myristylation and intracellular retention signal of L but remaining fully competent in supporting complete virion formation (61), was used as the wild-type (WT) reference. This construct has the advantage that the truncated L protein is readily released into the culture supernatant to allow easy monitoring of envelope expression and secretion (61), and it served as the reference here for testing the effects of all of the L protein mutants on virion secretion. Due to this N-terminal truncation, virion secretion supported by this construct may not be compared directly to that supported by the full-length L construct.

Culture supernatant from transfected human hepatoma cells able to support HBV replication and secretion (Huh7 or HepG2 cells, as indicated in the figures) was collected and concentrated. Viral particles, including (enveloped) virions as well as naked capsids (nonenveloped) were then resolved by native agarose gel electrophoresis. Virion-associated DNA was detected by Southern blotting to determine the levels of complete virions, and virion-associated HBc protein was detected by Western blotting to determine the levels of empty virions. As we reported previously, complete virions account for only a small proportion (1% or less) of all virions secreted by HBV *in vitro* and *in vivo* (28, 29); the levels of virion-associated HBc protein essentially reflect the levels of empty virions (with contribution from the HBc associated with complete virions to the total virion HBc signal being negligible). We could confirm that, in contrast to WT HBc that supported secretion of complete (DNA-containing) and empty virions (Fig. 1A, lanes 1 and 5), no DNA-containing virions were detectable by the L60A, L95A, K96A, or I126A HBc NTD mutant (Fig. 1B and C, lanes 2 and 6). In contrast, secretion of empty virions was readily detectable by all of these NTD mutants (Fig. 1B and C, lanes 10 and 14). To further confirm the authenticity of secreted HBV virions, viral particles released into the culture supernatant of transfected cells were analyzed by CsCl gradient centrifugation. Peak fractions containing complete and empty virions were resolved by native agarose gel electrophoresis. Again, it was clear that no DNA-containing virions were detectable by the L60A, L95A, K96A, or I126A HBc NTD

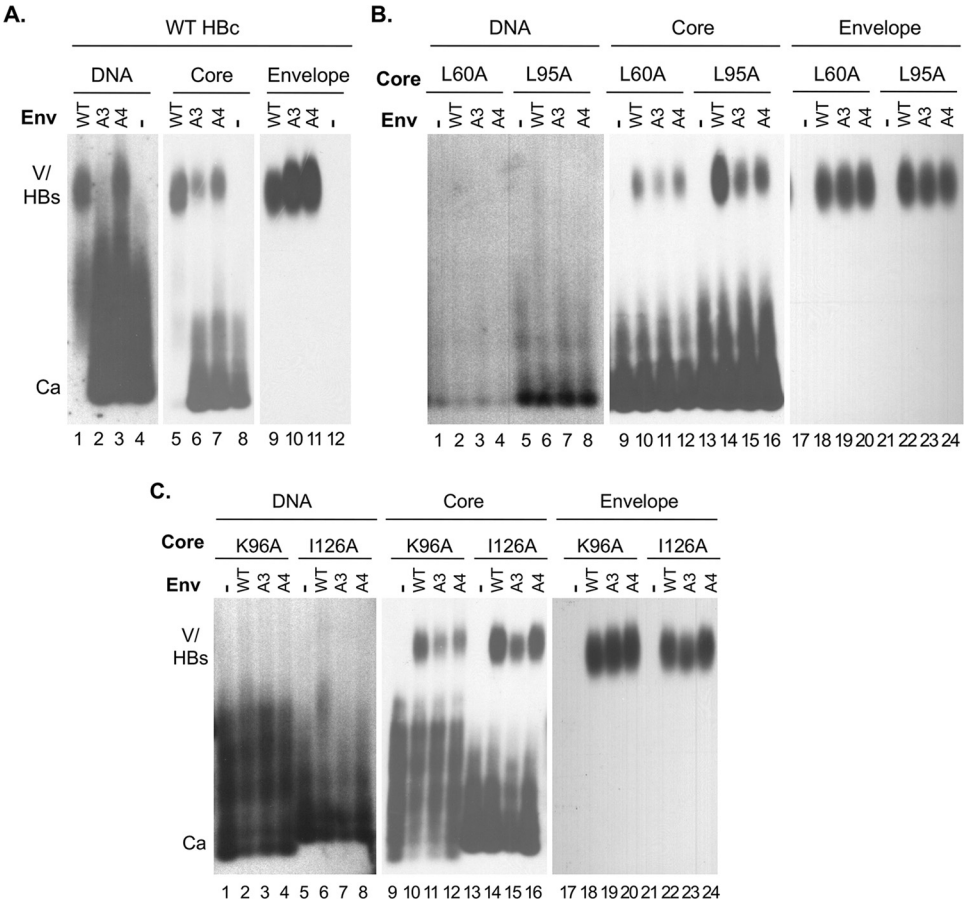

FIG 1 Analysis of complete and empty virion secretion by HBc NTD mutants in combination with pre-S1 mutants. Huh7 cells were transfected with an HBV genomic construct defective in envelope protein expression (pCMV-HBV/Env$^-$) expressing either the WT (A) or the indicated HBc NTD mutants (B and C), complemented with a second plasmid expressing all three envelope proteins with WT or mutant pre-S1 (A3 and A4) sequences (pSV45-31L). Seven days later, the concentrated culture supernatant containing both secreted virions and naked NCs was analyzed for virion secretion by native agarose gel electrophoresis. Following transfer to nitrocellulose membrane, viral DNA was detected by ^{32}P-labeled HBV DNA, followed by detection of HBc (core) protein using an HBc-specific polyclonal antibody and envelope proteins using an anti-HBs polyclonal antibody, as indicated above the panels. HBV virions (V), HBsAg particles (HBs), and capsids (Ca) are indicated. Lane −, no Env.

mutant (Fig. 2B to E, lanes 1 to 3). In contrast, secretion of empty virions was readily detectable by all of these NTD mutants, as evidenced by the presence of HBc protein in the virion fractions (Fig. 2B to E, lanes 5 to 7).

Neither the N-terminal nor C-terminal part of HBc CTD was required for empty virion secretion. The lack of effect on secretion of empty virions of the HBc NTD mutants prompted us to determine a potential role of the HBc CTD in their secretion. However, as we reported recently (42), a CTD truncation construct (C149) with the entire CTD (amino acids 150 to 183) deleted failed to accumulate to any significant level in human hepatoma cells due to the apparent need for CTD to stimulate capsid assembly in human cells, rendering it difficult to determine if C149 (i.e., with the entire CTD deleted) is competent in virion secretion or not. On the other hand, it was reported that HBc truncated at residue 164 (C164; i.e., deleting the C-terminal part of CTD) remains competent for secretion of DNA-containing virions (34, 72). Thus, we were interested in determining whether C164 could support secretion of empty virions. In addition, since the C-terminal Cys (i.e., Cys183) is involved in cross-linking HBc dimers in the capsids and can stabilize the capsids (32, 73, 74), we constructed another mutant, C164Cys, which expresses C164 plus a terminal Cys residue. An expression construct for the WT HBc, C164, or C164Cys was cotransfected with a second construct expressing all

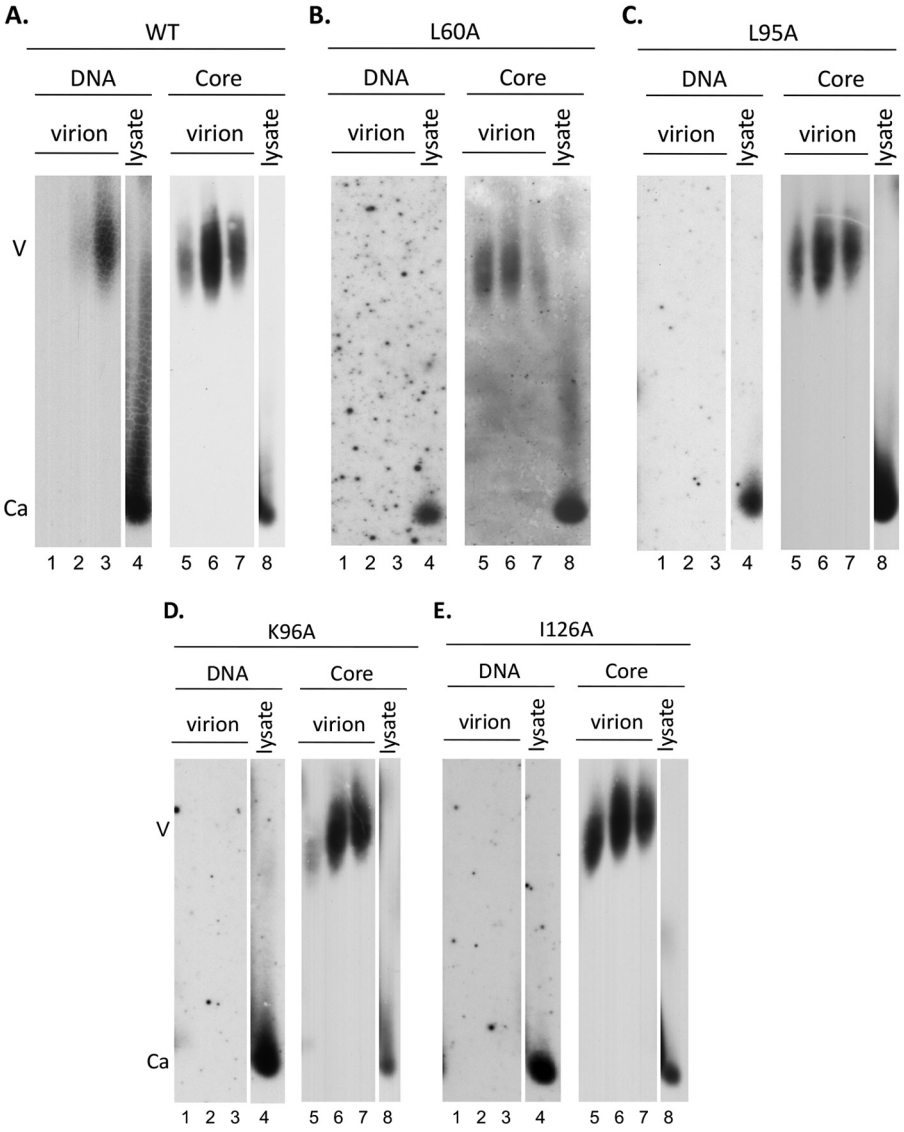

FIG 2 CsCl density gradient analysis of virion secretion by HBc NTD mutants. HepG2 cells were transfected with HBV genomic constructs (pCIdA-HBV/pgRNA) containing the WT HBc or HBc mutants as indicated. Seven days later, the concentrated culture supernatant was analyzed for virion secretion by CsCl gradient ultracentrifugation. Fractions containing HBV virions as well as cytoplasmic lysate (lysate) containing intracellular NCs were resolved by native agarose gel electrophoresis. Following transfer to nitrocellulose membrane, viral DNA was detected by ^{32}P-labeled HBV DNA, followed by detection of HBc (Core) protein using an HBc-specific polyclonal antibody, as indicated above the panels. HBV virions (V) and capsids (Ca) are indicated.

HBV proteins except HBc into HepG2 and Huh7 cells in the aforementioned transcomplementation assay. Both naked capsids and virions were released by the transfected HepG2 cells (Fig. 3A, lanes 1 to 3) or Huh7 cells (Fig. 3A, lanes 5 to 7) under all three cotransfection conditions, indicating that deletion of the C-terminal part of HBc CTD (i.e., amino acids 165 to 183) did not block secretion of empty virions.

It was noticeable that the levels of virions (or naked capsids) containing C164 were lower than those containing the WT HBc in both cell lines, and addition of the C-terminal Cys residue in C164Cys enhanced the levels of both virions and naked capsids (Fig. 3A, top). These results suggested that the deletion of amino acids 165 to 183 from the HBc CTD may partially impair HBc expression and/or assembly in hepatoma cells. We thus measured the amount of intracellular HBc in the lysate of transfected cells by both agarose gel electrophoresis to detect assembled capsids (Fig. 3B,

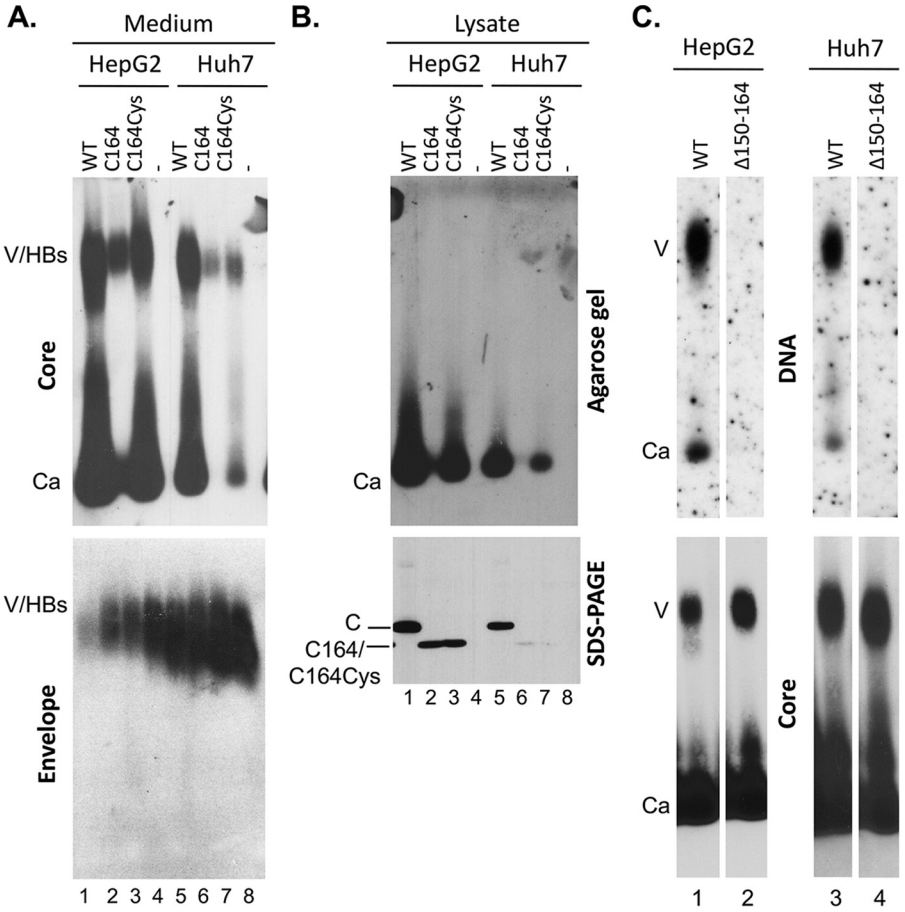

FIG 3 Analysis of empty virion secretion by HBc CTD deletion mutants. HepG2 and Huh7 cells were transfected with a plasmid expressing the WT HBc (pCI-HBc), the CTD deletion mutant C164 (pCI-HBc-C164), C164Cys (pCI-HBc-C164Cys), or HBcΔ150–164 (pCI-HBcΔ150–164), as indicated, together with an HBV genomic construct expressing all viral proteins except HBc (pSVHBV1.5C$^-$). The samples represented in lanes 4 and 8 of panels A and B were from cells transfected with the HBc-defective genomic construct alone. Seven days later, the concentrated culture supernatant was analyzed for virion secretion by native agarose gel electrophoresis (A and C). The cytoplasmic lysate containing intracellular NCs was also resolved by native agarose gel electrophoresis (B, top) or SDS-PAGE (B, bottom). Following transfer to nitrocellulose (A; B, top; C) or polyvinylidene difluoride (B, bottom) membrane, HBc (core) was detected using an HBc-specific polyclonal antibody (A, top; B, top; C, bottom) or monoclonal antibody (B, bottom). The viral envelope proteins were detected using an anti-HBs polyclonal antibody (A, bottom). Viral DNA was detected by ^{32}P-labeled HBV DNA probe (C, top). HBV virions (V), HBsAg particles (HBs), and capsids (Ca) are indicated, as are the full-length HBc (C) and truncated C164 and C164Cys proteins.

top) and SDS-PAGE to detect total HBc proteins (assembled and nonassembled) (Fig. 3B, bottom) expressed in the cell. Indeed, the levels of intracellular capsids matched those of capsids released into the culture supernatant (compare the top panels of Fig. 3A and B). The total intracellular C164 or C164Cys level was also lower than the level of WT HBc, especially in Huh7 cells (Fig. 3B, bottom). Thus, there was a partial defect of C164 in either expression and/or assembly in human hepatoma cells. The addition of a C-terminal Cys residue, as normally found in WT HBc, was able to enhance capsid assembly and/or stability. Thus, while the total core protein levels of C164 and C164Cys were similar (Fig. 3B, bottom), C164Cys had much higher levels of intracellular capsid levels than C164 (Fig. 3B, top) and, correspondingly, higher levels of virions and naked capsids released into the supernatant (Fig. 3A, top).

As the C-terminal portion of CTD (i.e., residues 165 to 183) was not required for secretion of DNA-containing (34, 72) or empty virions (Fig. 3), we were interested in determining if the N-terminal portion of CTD (i.e., residues 150 to 164) was required. Therefore, we constructed an HBc mutant with deletion of residues 150 to 164

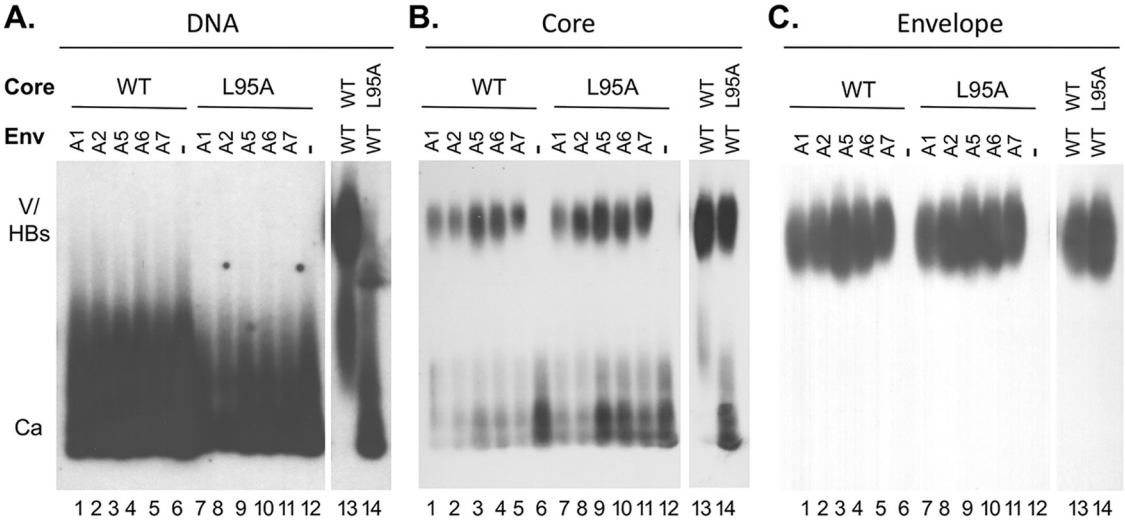

FIG 4 Analysis of complete and empty virion secretion by WT HBc and the NTD L95A mutant in combination with pre-S1 and pre-S2 mutants. Huh7 cells were transfected with an HBV genomic construct defective in envelope protein expression (pCMV-HBV-Env$^-$) expressing either WT or the L95A HBc NTD mutation, complemented with a second plasmid (pSV45-31L) expressing all three envelope proteins with WT or mutant pre-S1 (A1, A2, and A5 to A7) sequences. Seven days later, the concentrated culture supernatant was analyzed for virion secretion by native agarose gel electrophoresis. Following transfer to nitrocellulose membrane, viral DNA was detected by ^{32}P-labeled HBV DNA (A), followed by detection of HBc (core) protein using an HBc-specific polyclonal antibody (B) and envelope proteins using an anti-HBs polyclonal antibody (C). HBV virions (V), HBsAg particles (HBs), and capsids (Ca) are indicated.

(HBcΔ150–164), the reciprocal of HBc164, and tested its ability to support virion secretion in the transcomplementation assay. As shown in Fig. 3C, HBcΔ150–164 was competent in secretion of empty virions. Not surprisingly, HBcΔ150–164 failed to support any viral DNA synthesis, given the essential role of CTD residues 150 to 164 in supporting pgRNA packaging (34). The failure of HBcΔ150–164 to support viral DNA synthesis, however, precluded the testing of its ability to support secretion of complete virions. Together, these results thus indicated that neither the C-terminal (residues 165 to 183) nor N-terminal (i.e., residues 149 to 164) part of CTD of HBc was essential for secretion of empty virions.

L mutants defective in complete virion secretion remained competent for empty virion secretion. To ascertain the requirements from the envelope proteins for empty virion secretion compared to those for secretion of complete virions, we tested the effects of mutations in the MD of L that have been shown to block secretion of complete virions. WT HBc and all mutants were expressed from the HBV replicon defective in envelope protein expression (Fig. 1). They were complemented with another construct expressing the WT Env or A3 or A4 L mutant to determine their virion secretion capacity. As reported earlier (61), the A3 and A4 L mutants were defective and competent, respectively, in the secretion of complete virions (Fig. 1A, lanes 2 and 3). These L proteins were tested in combination with all of the HBc mutants tested above. The results showed that both the A3 and A4 L mutations allowed secretion of the WT and mutant HBc capsids as empty virions (Fig. 1A, lanes 5 to 8, and B and C, lanes 9 to 16). We then decided to test a number of additional L mutants, which were defective in secretion of complete virions, in combination with WT HBc and the L95A HBc mutant. As reported before (61), all of the L mutations (A1, A2, and A5 to A7) eliminated secretion of complete virions that was supported by the WT L (Fig. 4A, compare lanes 1 to 12 and lane 13); in contrast, they all allowed secretion of the WT or the L95A mutant capsids as empty virions (Fig. 4B, lanes 1 to 12). The release of HBsAg particles (with envelope proteins alone, without any capsids) was not affected significantly by any of the L mutations (Fig. 1A, lanes 9 to 12, and B and C, lanes 17 to 24, and 4C).

The small surface protein was sufficient for basal level secretion of empty HBV virions. Since none of the L mutants tested, which abolished secretion of complete virions, blocked empty virion secretion significantly, we were led to the possibility that

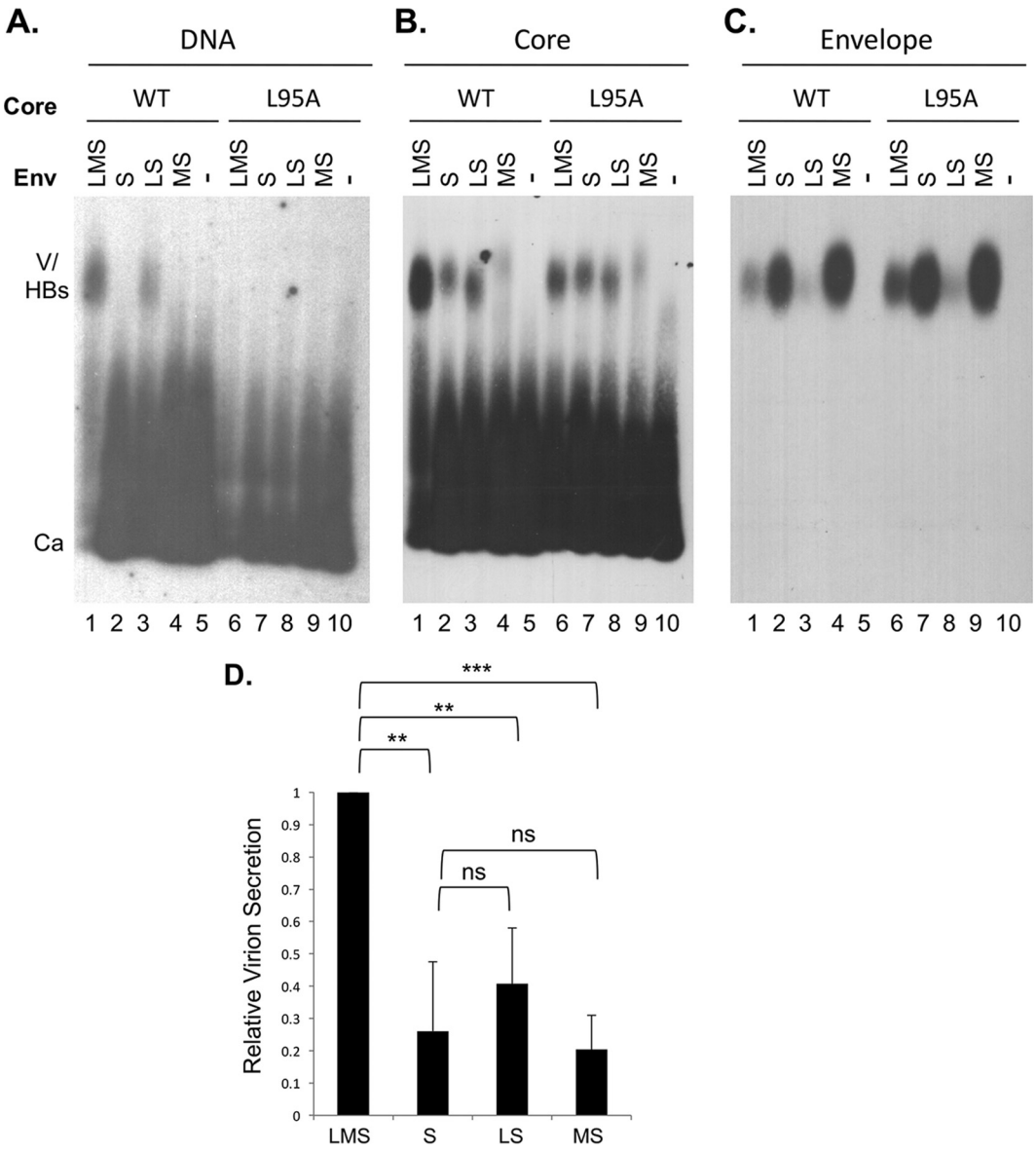

FIG 5 Analysis of complete and empty virion secretion by WT HBc and the L95A mutant supported by different combinations of the three envelope proteins. (A to C) Huh7 cells were transfected with an HBV genomic construct defective in envelope protein expression (pCMV-HBV-Env$^-$) expressing either WT or the L95A NTD mutant, complemented with a second plasmid expressing all three envelope proteins (LMS) (pSVB45H), S alone (pSVBX24H), L and S (LS) (pSVLS), or M and S (MS) (pSV33H). Seven days later, the concentrated culture supernatant was analyzed for virion secretion by native agarose gel electrophoresis. Following transfer to nitrocellulose membrane, viral DNA was detected by ^{32}P-labeled HBV DNA (A), followed by detection of HBc (core) protein using an HBc-specific polyclonal antibody (B) and envelope proteins using an anti-HBs polyclonal antibody (C). HBV virions (V), HBsAg particles (HBs), and capsids (Ca) are indicated. (D) The HBc protein signals in virions (relative virion secretion) were quantified and compared to signals with L plus M plus S, which was set to 1.0. Statistical analysis was performed using a Student *t* test, two-tailed and unpaired. ns, not significant ($P > 0.05$); **, $P < 0.01$; ***, $P < 0.001$.

L may not be needed for empty HBV virion secretion, in contrast to requirements for the secretion of complete HBV virions but similar to those for the secretion of HDV virions. To test this possibility, the WT or L95A HBc mutant with different combinations of the envelope proteins was used for determining complete and empty HBV virion secretion. As expected, secretion of complete virions required L (Fig. 5A, lanes 1 to 5), and the L95A HBc mutation abolished secretion of complete virions (Fig. 5A, lanes 6 to 10). In contrast, S alone, in the absence of either L or M, was able to support secretion of empty virions with the WT or L95A mutant HBc (Fig. 5B, lanes 2 and 7). It was noticeable that the secretion of total HBs (with or without the capsid or viral DNA) was higher

when S was expressed alone or in combination with M but was decreased when S was coexpressed with L (Fig. 5C), consistent with the well-known effect of intracellular S retention by L (75).

WT but not mutant L could stimulate secretion of empty virions and suppress release of naked NCs when combined with WT but not mutant HBc. Close inspection of the empty virion secretion results showed that L plus M and, to a lesser extent, L alone could enhance empty virion secretion that was supported by S alone at a lower level. Thus, although the L plus S and L plus M plus S combinations had less overall HBsAg secretion (due to the retention property of L) than S or M plus S (Fig. 5C, lanes 1, 3, 6, and 8 compared to lanes 2, 4, 7, and 9), they actually showed more empty virion secretion when combined with the WT HBc (Fig. 5B, compare lanes 1 and 3 to lanes 2 and 4, and D). Furthermore, L mutations (A1 to A3 and A5 to A7) that blocked complete virion secretion mostly eliminated this enhancement of virion secretion with WT HBc although they still allowed empty virion secretion with WT HBc at a reduced level (Fig. 1A, lane 5 versus lane 6, and 4B, lane 13 versus lanes 1 to 5). Interestingly, the stimulatory effect of WT L on empty virion secretion was also mostly lost when the mutant, instead of WT, HBc proteins were used. Thus, levels of empty virion secretion by the L60A, K96A, and I126A HBc mutants were not stimulated or were stimulated only weakly by the WT L compared to stimulation with the mutant L proteins (Fig. 1B, lanes 10 to 12, and C, lanes 10 to 12 and 14 to 16). The effect of L95A on empty virion secretion seemed to be somewhat variable. Empty virion secretion by this mutant was still stimulated by the WT (but not mutant) L protein (Fig. 1B, lanes 14 to 16, and 4B, lanes 7 to 11 and 14), and its secretion with WT L was only slightly (within 2-fold) less than that with the WT HBc (Fig. 4B, lanes 13 and 14). On the other hand, in other experiments, levels of empty virions formed by L95A were clearly less than levels with WT HBc and were stimulated little by the presence of WT L compared to the level with S alone (Fig. 5B, lanes 6 to 9). Interestingly, the combination of the M and S envelope proteins appeared to support somewhat less efficient secretion of empty virions than S alone (Fig. 5D) although the difference did not reach statistical significance. On the other hand, in the absence of M, L plus S supported less secretion of empty virions than L plus M plus S (Fig. 5D) (see more on the role of M in the Discussion).

It was also notable that L plus M plus S (and to a lesser degree, L plus S), but not S alone or M plus S, dramatically reduced the naked capsid signals in the culture supernatant (Fig. 1A, lanes 1 and 5, 4A and B, lanes 13, and 5A and B, lanes 1 and 3). Mutations in either L (A1 to A3 and A5 to A7) or HBc (L60A, L95A, K96A, and I126A) that prevented secretion of complete virions or eliminated the enhancing effect on empty virion secretion also eliminated this suppressive effect on the release of naked capsids (Fig. 1A, lanes 2, 6B and C, lanes 2, 3, 6, 7, 10, 11, 14, and 15, and 4A and B, lanes 1 to 5, 7 to 11, and 14). Thus, L apparently interacted with capsids to suppress naked capsid release in a process requiring the same pre-S1 (MD) and HBc NTD residues as those that play an essential role in complete virion secretion and a stimulatory role in empty virion secretion. Even the A4 L mutant, which remained efficient for complete virion formation, was less efficient than the WT L in stimulating empty virion secretion (Fig. 1A, lanes 3 and 7 versus lanes 1 and 5) and also failed to suppress the release of naked capsids (Fig. 1A, lanes 3 and 7, B and C, lanes 4, 8, 12, and 16), similar to all the other L mutants tested here. This result indicated that even L residues that were not essential for complete virion secretion could also stimulate empty virion secretion and suppress release of naked capsids. The suppressive effect on the release of naked capsids by the envelope proteins seemed to be dependent on how they were expressed. Thus, this effect was not so obvious in other experiments when the envelope proteins were expressed from the genomic (replicon) construct (pSVHBV1.5C^{-}) (Fig. 3A, lanes 1 and 5, and C, lanes 1 and 3, and 6, lane 1 versus 2 and lane 5 versus 6, where the envelope proteins were expressed from constructs that differed from the ones used in the experiments shown in Fig. 1, 4, and 5) (see also Discussion below).

S mutations defective in HDV secretion did not block complete or empty HBV virion secretion. Since S alone allowed secretion of empty HBV virions, similar to HDV,

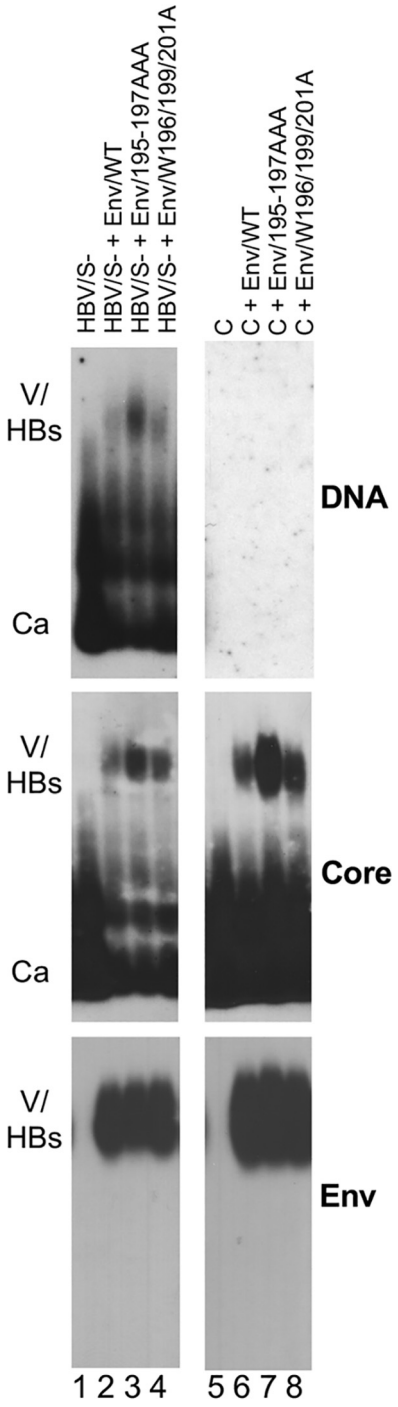

FIG 6 Analysis of complete and empty virion secretion by envelope mutants defective in HDV secretion. Huh7 cells were transfected with an HBV genomic construct defective in expressing the envelope proteins (pCMV-HBV-Env$^-$) or a plasmid expressing HBc alone (pCI-HBc) (C), as indicated, together with a second plasmid expressing all three envelope proteins with the WT or the indicated mutant sequences (pT7HB2.7). Seven days later, the concentrated culture supernatant was analyzed for virion secretion by native agarose gel electrophoresis. Following transfer to nitrocellulose membrane, viral DNA was detected by ^{32}P-labeled HBV DNA, followed by detection of HBc (core) protein using an HBc-specific polyclonal antibody, and envelope proteins using an anti-HBs polyclonal antibody, as indicated. HBV virions (V), HBsAg particles (HBs), and capsids (Ca) are indicated.

we were interested in determining whether the S requirements for secretion of HDV and empty HBV virions are the same or different. To this end, we tested two different S mutants in the cytosolic loop II (CYLII) of S, W196/199/201A (W196–201A) (76, 77) and residues 195 to 197 with alanine substitutions (S195–197AAA) (78), which are known to

eliminate HDV secretion but remain competent for subviral HBsAg secretion, for their ability to support empty HBV virion secretion. In these experiments, HBc was provided either from the HBV Env-defective replicon plasmid (expressing all other HBV proteins and competent for intracellular replication) (Fig. 6, lanes 1 to 4) or from an HBc-expressing plasmid that expressed only the HBc protein (Fig. 6, lanes 5 to 8). In neither case was the secretion of empty HBV virions affected by the S CYLII mutations. The S mutations also did not affect secretion of complete virions (Fig. 6, lanes 2 to 4). These results indicated that the S requirements for secretion of empty (or complete) HBV virions were distinct from those for HDV virion secretion.

DISCUSSION

Secretion of both empty and complete HBV virions requires interactions between the viral capsid and one or more viral envelope proteins. On the capsid side, we have shown here that substitutions of the HBc NTD residues on the surface of the capsids in the previously defined MBD (Fig. 7A) that block the secretion of DNA-containing virions did not prevent the secretion of empty virions. Furthermore, the HBc CTD was also not essential for empty virion secretion. On the envelope side, substitutions in the MD (Fig. 7B) in the pre-S1 domain of L that block secretion of complete virions also did not block empty virion secretion. Furthermore, among the three viral envelope proteins, the smallest, S, alone was sufficient for empty virion secretion at a basal level, and the L (and possibly M) protein could enhance empty virion secretion in contrast to the secretion of complete virions, which absolutely requires both S and L. Thus, the secretion requirements of empty HBV virions are distinct from those of complete virions and share some similarity to the requirements of the satellite virus, HDV, whose secretion is also supported by S alone. However, S mutations that block HDV secretion (Fig. 7B, CYLII) had no effect on secretion of either empty or complete HBV virions. In addition, residues in the MBD on the capsid surface as well as those in MD of pre-S1, while not essential for secretion of empty virions, could enhance empty virion secretion and could also suppress the release of naked (nonenveloped) capsids from hepatoma cell cultures.

Our results indicate that the secretion of complete and empty HBV virions is mediated via distinct, as well as shared, signals. These results, together with the information available in the literature, allow us to propose a two-signal model for HBV virion secretion (Fig. 7C). While additional biochemical and structural studies will be required to confirm directly the interactions predicted here, the genetic evidence provided by our mutagenesis experiments strongly supports this model. First, empty capsids display a basal and constitutive positive signal (secretion signal I) that induces their envelopment, which is mediated, at a basal level, via interactions with S (Fig. 7C, top). Although the structural basis of signal I remains to be defined, we have recently obtained evidence supporting a critical role of the HBc linker in empty virion secretion (95). Thus, the linker peptide may harbor signal I or is involved in generating the signal. In support of the proposed capsid-S interactions, peptides derived from S were shown to interact with capsids *in vitro* (79, 80). However, the specific site on S that interacts with empty capsids to direct empty virion secretion remains to be defined. Our results here and previous findings (76, 77) do indicate that the S requirements for interacting with HBV capsids to secrete empty (or complete) HBV virions are distinct from those for interacting with the HDV ribonucleoprotein (RNP) to secrete HDV virions. Additional comparative studies to define the HBV S requirements for empty and complete HBV virion formation and those for HDV virion formation should provide important insights into the molecular details driving HBV and HDV morphogenesis, in particular, how HBV S recognizes two seemingly rather different substrates (the HBV capsid and the HDV RNP).

Second, a positive signal distinct from signal I, which we call secretion signal II and which likely consists of the previously defined MBD within the HBc NTD and is located spatially on the capsid surface, emerges on mature, rcDNA-containing NCs, which primarily interact with the MD of L to mediate secretion of DNA-containing or complete

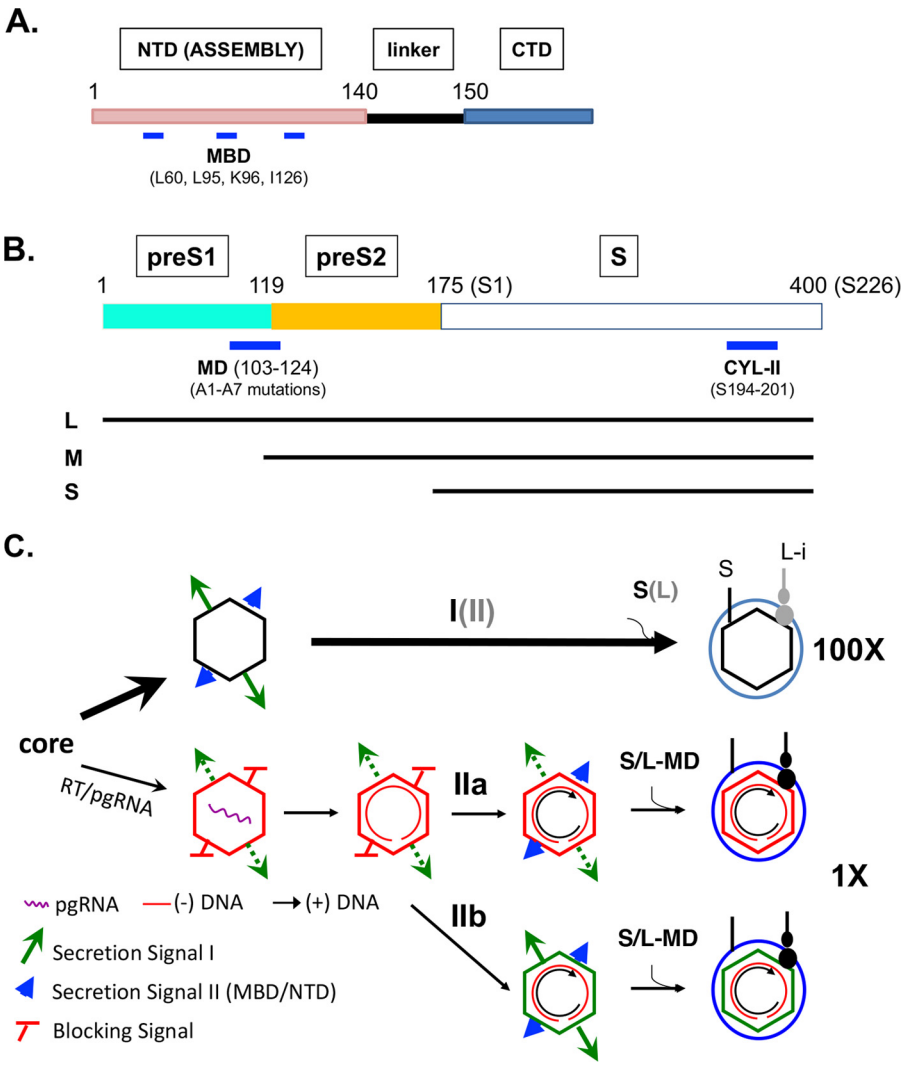

FIG 7 Schematics of HBc and HBV envelope protein domain structure and a working model for HBV virion secretion. (A) HBc domain structure. The boundaries of the HBc NTD, linker, and CTD domains are indicated on the top. The MBD (scattered within the NTD on the linear sequence but located on the capsid surface spatially) that is involved in interactions with the L envelope protein for secretion of complete virions is indicated at the bottom. The positions of HBc mutations used in this study are also indicated. (B) Domain structure of the HBV envelope proteins. The boundaries of the pre-S1, pre-S2, and S domains are indicated on the top, with the positions within the S domain also indicated (i.e., position 175 as 1 or S1 within the S domain). The MD at the junction between pre-S1 and pre-S2 (mostly within pre-S1), which is involved in interactions with the HBc MBD for secretion of complete virions, is indicated in the middle (A1 to A7, denoting mutants within the MD that were used in this study). Also indicated is the CYLII involved in secretion of HDV. The bottom three lines denote the viral L, M, and S envelope proteins, with L containing all three (pre-S1, pre-S2, and S) domains, M containing the pre-S2 and S domains, and S containing the S domain only. (C) Model for two distinct signals in the secretion of empty versus complete HBV virions. The hexagons denote the capsids, and the outer circle indicates the viral membrane, with the S, pre-S2, and pre-S1 domains of the envelope protein denoted by the vertical bar, the small filled ball, and large filled ball, respectively. Secretion signal I or II is proposed to exist on the surface of empty capsids or mature NCs to direct their interactions with the S or L envelope protein, respectively. Immature NCs display a blocking signal that negatively regulates NC envelopment and virion formation, which can be overwhelmed by secretion signal II in pathway IIa but has to be eliminated (resulting in reexposure of secretion signal I) also in pathway IIb for secretion of complete (i.e., rcDNA-containing) virions. Gray lettering in parentheses denotes the proposition that signal II stimulates but is not essential for empty virion formation, and L stimulates but is not essential for empty virion formation. Dashed green arrows denote the sequestered or suppressed secretion signal I. Loss of the blocking signal upon NC maturation is denoted by the change in capsid shell coloring (from red to green). L-i refers to one of the two different topologies of L, with an internal (i) localization of the pre-S domain (90, 94). The pre-S1/pre-S2 MD in L can only interact with the capsid in the L-i topology. The alternative topology, L-e, with the pre-S region located externally and involved in virus entry into cells instead of release, is not depicted for clarity. The M envelope protein is omitted as its role in virion secretion remains to be more clearly defined. See the text for details.

virions. Assuming that the constitutive secretion signal I remains on immature NCs, we hypothesized that immature NCs acquire a negative (retention) signal that is dominant over secretion signal I so as to inhibit their envelopment (28). The emergence of signal II on mature NCs could, by itself, overcome the negative effect of the inhibitory signal on envelopment (Fig. 7C, IIa). For example, the pre-S1 (MD)-mature NC interactions alone, without any contribution of S, may be sufficient to direct secretion of complete virions. If the negative signal of envelopment on immature NCs indeed represents the sequestration of a positive secretion signal (which could be the secretion signal I suggested here), as we proposed earlier (28), the emergence of a different positive signal (represented by signal II) could be sufficient to trigger secretion of complete virions. Alternatively, additional structural changes of mature NCs, distinct from the emergence of signal II, may be required to remove the blocking signal, e.g., by desequestration (exposing) of the secretion signal (signal I) previously hidden in immature NCs upon NC maturation, which, together with signal II, facilitates the envelopment and secretion of mature NCs (Fig. 7C, IIb).

We have also shown here that the same interactions between the MBD on capsids (signal II) and the MD on L, though not absolutely required for secretion of empty virions, could stimulate empty virion secretion (Fig. 7C, top). HBc MBD mutants were less competent for empty virion secretion than WT HBc when they were complemented with WT envelope proteins. Also, MD mutations in L mostly eliminated the stimulatory effect of L on empty virion secretion with WT HBc. Indeed, peptides related to the MD in pre-S1 were shown to bind mostly empty capsids isolated from HBV-infected human liver (79, 80) and CTD-deleted capsids (C144) from insect cells (80). Thus, it appears that signal II, postulated to emerge (inducibly) on mature NCs, may also be present on empty capsids (constitutively), and, like signal I, would also have to be suppressed on immature NCs by the presence of pgRNA or ssDNA or be overwhelmed by the secretion-inhibitory signal to prevent empty capsid secretion. We emphasize that the structural basis for the emergence or sequestration of either signal I or II on empty capsids and mature NCs remains to be elucidated. As discussed in the introduction, although CTD dephosphorylation is correlated with NC maturation, it is not necessary for secretion of either complete or empty virions (30). A possible structural correlate of the secretion signals is the dynamic stability of the capsids: both mature NCs and empty capsids, which are competent for virion formation, are less stable than immature NCs, which are incompetent for virion formation (81).

The role of the M envelope protein in the secretion of virions appears to be complex. On one hand, M plus S was not better (and may even be worse) than S alone in supporting secretion of empty virions (Fig. 5), suggesting that the pre-S2 domain of M, in an N-terminal open configuration, and perhaps its pre-S2-linked N/O glycosylation (1) do not stimulate, and may even interfere with, empty virion secretion. On the other hand, the less efficient secretion of empty virions by L plus S than of L plus M plus S suggests that M is needed to enable L to stimulate secretion of empty virions. We note that the absence of M (i.e., comparing L plus M plus S and L plus S) also led to somewhat reduced secretion of complete virions (Fig. 5A), consistent with a previous report (82), suggesting that M might also play an auxiliary role in the secretion of complete virions. It is also possible that the absence of M indirectly affected virion secretion by increasing the ratio of L to S (or S plus M), which would decrease secretion of virions as well as HBsAg particles due to the intracellular retention function of L. The somewhat lower levels of HBsAg in the culture supernatant secreted by L plus S than by L plus M plus S (Fig. 5C) would be consistent with this possibility. Future studies will be required to resolve these possibilities.

The effect of the L95A mutation on the secretion of empty virions appeared to be weaker (and more variable) than that with the other HBc MBD mutants. This may suggest that the mutant could still interact with L (MD) to stimulate empty virion secretion though it would clearly be defective in secretion of complete virions. So, this mutant may impair secretion of complete virions in a way that is somewhat different from that of the other HBc MBD mutants. Interestingly, we showed recently that this

mutant also does not cause hyperdestabilization of mature NCs to increase cccDNA formation, in contrast to the other MBD mutants (83). Also, since other MBD mutants of HBc (including I126A and K96A) were still stimulated to some extent by L for empty virion secretion, it is possible that additional L-HBc interactions outside the MD-MBD (Fig. 7A and B) interaction contribute to secretion of empty virions.

While the role of the NTD in the secretion of complete and empty virions seems clear, the role of HBc CTD in virion secretion remains to be better defined. It has been reported previously that the HBV CTD beyond residue 164 is not essential for DNA-containing virion secretion (34, 72). The role of CTD residues before position 164 (i.e., residues 150 to 164) in complete virion secretion, if any, is difficult to ascertain at present due to their essential role in pgRNA packaging and reverse transcription, stages of the viral life cycle preceding and requisite for complete virion formation. With respect to secretion of empty virions, we have shown here that neither half of the CTD was required for secretion of empty HBV virions, suggesting that the CTD is not essential for interacting with the viral envelope proteins to trigger the formation of empty virions.

Our findings here also indicate that interactions between the pre-S1 MD and HBc MBD (i.e., signal II) can suppress the release of naked capsids in cultured hepatoma cells, in addition to their essential role in secretion of complete HBV virions and their auxiliary role in secretion of empty HBV virions. How this is accomplished remains to be elucidated. One possibility is that virion formation, especially when it is enhanced by L (MD)-MBD (signal II) interactions, directs most or all intracellular capsids toward the envelopment pathway, thus effectively diverting the capsids away from the pathway of naked capsid release (84). This further implies that the apparent abundance of naked capsid release into the culture supernatant of HBV-transfected hepatoma cells, as opposed to the apparent absence of such release during natural infection of humans or experimental infection of chimpanzees (28, 29, 85), may be explained by the much lower expression and secretion levels of HBV envelope proteins than of intracellular capsids by these transformed cells under the current experimental conditions than under *in vivo* infection conditions. Indeed, we have recently found that much lower levels of HBV surface antigen than of virions were released by cultured hepatoma cells than were released into the bloodstream by infected hepatocytes during natural infections *in vivo* (29). As the suppressive effect on the release of naked capsids by the envelope proteins seemed to be dependent on how they were expressed, it seems possible that the exact ratio of envelope proteins to capsid and the ratio of L to M to S may affect the suppressive effect of L on the extracellular release of naked capsids. Indeed, the stoichiometry of empty capsids or mature NCs relative to the envelope proteins and the stoichiometry of the different forms of envelope proteins (L, M, and S) themselves likely also affect the levels of empty and complete virions secreted. Future studies are warranted to clarify these issues.

The function of empty HBV virions remains to be defined. Incomplete viral particles are in fact common occurrences; the so-called light particles of herpes simplex virus (with the envelope and the tegument layers but no capsid or genome) are reported to deliver their interior tegument components to the host cell, which can modulate host function to affect infection outcome by the complete (infectious) virions of the same cell (86, 87). Based on our results here, at least a fraction of empty HBV virions will have the L envelope protein that is essential for viral infection and can thus in principle enter host cells like the complete virions. In analogy with the herpesvirus, the HBV capsid delivered by empty virions into the host cell may modulate host functions to influence infection by the complete virions. Furthermore, as we proposed earlier, serum-empty HBV virions may serve as better biomarkers than serum HBsAg for intrahepatic cccDNA since HBc, an essential component of empty as well as complete virions, can likely be made only from cccDNA and not from integrated HBV, which can direct the production of HBsAg (27, 29, 88). This can be especially useful when serum-complete virions fall to undetectable or unquantifiable levels with potent antiviral therapy targeting the viral RT protein. Empty HBV virions in principle can also serve as the basis for a new

generation of HBV vaccine incorporating all viral structural proteins, not just HBsAg as in the current recombinant HBsAg-based vaccine (4, 27). Findings presented here and further studies to elucidate the mechanisms of virion morphogenesis will have important implications for these potential applications of empty HBV virions.

MATERIALS AND METHODS

Plasmids. DNA sequences encoding the WT and NTD mutant HBc proteins were cloned from the pSVcore constructs (genotype A) (54) into the envelope-defective pCMV-HBV/Env$^-$ or envelope-proficient pCIdA-HBV/pgRNA (genotype D) construct as described previously (83). These constructs direct the expression of the HBV pgRNA, expressing either WT or mutant HBc, under the cytomegalovirus (CMV) promoter. The HBc protein expressed from this construct is a chimera between genotype D (positions 1 to 28 and 145 to 183) and genotype A (positions 29 to 144), which was used as a wild-type (WT) reference for all HBc mutants harboring NTD mutations (all single-residue substitutions relative to the reference). Expression of the WT envelope proteins in pCIdA-HBV/pgRNA is driven from the native HBV promoters (83). pSVHBV1.5C$^-$ expresses an HBc-defective HBV genome (genotype A) (54), which is capable of supporting viral replication upon complementation with HBc. HBV pgRNA is driven by the simian virus 40 (SV40) early promoter in this construct. pCI-HBc, -HBc-164, and -HBc-164Cys were constructed by placing the coding sequences for the full-length (genotype D) HBc or for HBc CTD truncations downstream of the CMV promoter in pCI (Promega) and were used to express, respectively, WT HBc, HBc truncated at position 164, and HBc truncated at 164 but with the addition of a terminal Cys residue. pCI-HBcΔ150–164 expresses an HBc mutant with a deletion of the N-terminal portion of the CTD (residues 150 to 164).

The following constructs were used to express the WT or mutant HBV envelope proteins. pSVBX24H, pSV33H, and pSVB45H express the HBV S, M plus S, and L plus M plus S proteins, respectively (genotype A) (63, 75, 89, 90). pSVLS is identical to pSVB45H except for an ATG-to-ACG point mutation of the pre-S2 start codon. Constructs for expressing all three envelope proteins with the L mutations A1 to A7 have been described before (61). The WT reference for this series of L mutants contained an N-terminal deletion from position 2 to 30 (designated pSV45-31L) (61). pT7HB2.7 is another construct used to express the WT L, M, and S envelope proteins under the native HBV promoters (genotype D) (91) or their mutant versions S195–197AAA with A substitutions at residues 195 to 197 (78) and W196–201A with substitutions of three As for Ws at W196/199/201 (76), with both sets of substitutions being present in all three envelope (L, M, and S) proteins.

Transient transfection. Transfection of HepG2 and Huh7 cells was done as previously described (12, 92). Briefly, HepG2 cells in 60-mm dishes were transfected with 4 μg (total) of plasmid using FuGENE6 (Roche). Huh7 cells seeded in 60-mm dishes were transfected with 10 μg (total) of plasmid using a CalPhos mammalian transfection kit (Clontech). When two plasmids were used for transfection, they were used at a 1:1 mass ratio. Cells and culture supernatant were harvested on day 7 posttransfection. All transfection experiments were repeated between two and five times, and representative images are shown.

Analysis of viral particles and proteins and CsCl density gradient centrifugation. Culture supernatant containing HBV virions and naked NCs was concentrated by polyethylene glycol precipitation and digested with DNase I (1 mg/ml at 37°C for 1 h) to eliminate residual plasmid DNA before analysis for virion secretion. For Fig. 2, the treated culture supernatant was further fractionated by isopycnic CsCl gradient ultracentrifugation (22, 28, 46) to separate virions from naked (nonenveloped) NCs, which were also released into the cell culture supernatant. Purified virion fractions or DNase-digested concentrated medium samples were analyzed by native agarose gel electrophoresis as described previously (28, 93). Upon transfer of viral particles to nitrocellulose membrane, encapsidated DNA in viral particles was detected using ^{32}P-labeled HBV DNA probe, followed by detection of core proteins associated with virions or naked NCs on the same membrane using a rabbit polyclonal (Dako) or mouse monoclonal (28) anti-HBc antibody. Goat (Dako) or rabbit (Virostat) polyclonal anti-HBV surface protein was then used to detect the viral envelope proteins after the membrane was stripped. Viral capsids in cytoplasmic lysate were detected similarly following resolution by agarose gel electrophoresis (28, 30). Total HBc protein in the lysate was detected by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis, as previously described (28, 30). The CsCl gradient fractionation experiment was repeated using samples from three separate transfections. Representative images are shown.

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