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

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Running Title: Requirements for empty HBV virion secretion

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Abstract

During the morphogenesis of hepatitis B virus (HBV), an enveloped virus, two types of virions are secreted: (1) a minor population of complete virions containing a mature nucleocapsid with the characteristic, partially double-stranded, relaxed circular DNA genome and (2) 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 for, but could stimulate empty virion secretion. Also, substitutions in L that eliminate secretion of complete virions reduced but did not eliminate empty virion secretion. S mutations that block 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 vs. mature nucleocapsids interact with the S and L proteins during the formation of complete vs. empty virions.

47 **Importance**

48 Hepatitis B virus (HBV) is a major cause of severe liver diseases including cirrhosis
49 and cancer. In addition to the complete infectious virion particle, which contains an outer
50 envelope layer and an interior capsid that, in turn, encloses a DNA genome, HBV infected cells
51 also secrete non-infectious, incomplete viral particles in large excess over the complete virions.
52 In particular, the empty (or genome-free) virion share with the complete virion the outer
53 envelope and interior capsid but contain no genome. We have carried out a comparative study
54 on the capsid and envelope requirements for the secretion of these two types of virion particles
55 and uncovered both shared and distinct determinants on the capsid and envelope for their
56 secretion. These results provide new information on HBV morphogenesis, and have implications
57 for efforts to develop empty HBV virions as a novel biomarker and a new generation of HBV
58 vaccine.
59

60 **Introduction**

61 Hepatitis B virus (HBV) infects chronically ca. 300 million people worldwide and remains
62 a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1, 2). HBV is a
63 member of the *Hepadnaviridae*, a group of retroid viruses harboring a small (ca. 3.2 kbp),
64 partially double-stranded (DS), relaxed circular (RC) DNA genome that is replicated via reverse
65 transcription of an RNA intermediate called pregenomic RNA (pgRNA) (3, 4). The HBV DNA
66 genome is enclosed within a protein capsid composed of 240 (for the major population) or 180
67 (for the minor population) copies of one viral protein, the core or C (HBc) protein (5-7), which is
68 in turn enclosed by an outer envelope consisting of a host-derived lipid bilayer studded with
69 three viral envelope or surface proteins, S, M, and L (8-10).
70
71 Upon entry into human hepatocytes, HBV delivers its RC DNA to the host cell nucleus,
72 which is converted to a covalently closed circular (CCC) DNA that serves as the transcriptional
73 template able to generate all viral RNA species required for productive viral replication (11-13),
74 including the aforementioned pgRNA. In addition to serving as the template for viral reverse
75 transcription (i.e., the precursor to RC DNA), pgRNA also serves as the mRNA for the viral C
76 and polymerase (P) proteins. pgRNA is specifically packaged into an immature nucleocapsid
77 (NC) in a process also dependent on the P protein and host factors (14-18). Viral reverse
78 transcription within immature NCs, carried out by P, a specialized reverse transcriptase (RT),
79 converts pgRNA to RC DNA found in mature NCs (3, 13, 19, 20). Mature NCs, but not immature
80 ones that contain pgRNA or the single-stranded (SS) DNA intermediate, are then selected for
81 envelopment by the viral envelope proteins and secreted into the bloodstream as complete and
82 infectious virions (3, 21-26).
83
84 In addition to the complete RC DNA-containing virions, HBV replication also leads to the
85 production and release of two classes of incomplete or subviral particles, neither of which is

infectious (27). The first is the classical Australian antigen, HBsAg sphere or filament that is composed of only the viral envelope proteins - devoid of either the viral capsid or genome – and is present in the blood of infected patients in 100-100,000-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 DNA-containing virions by HBV in cell cultures, experimentally infected chimpanzees, and naturally infected humans (28-30).

It remains unknown how either RC DNA-containing mature NCs or empty capsids are selected for envelopment but immature, SS DNA or pgRNA-containing NCs are excluded. In particular, the signal(s) on mature NCs or empty capsids that direct 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 three 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 SS DNA or pgRNA in immature NCs trigger a “SS Blocking Signal” to actively prevent the envelopment of immature NCs (28). As genome maturation occurs within the capsid and 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 SS Blocking Signal, which prevents envelopment of immature NCs, remains unclear, as does the relationship between these and the secretion signal on empty capsids.

112

113 The capsid does play an integral role in viral assembly and replication. The N-terminal
114 two thirds (the assembly domain or N-terminal domain, NTD; amino acids 1-140) of the core
115 protein (7, 31-33) provides the protective shell for the viral genome, while its C-terminal domain
116 (CTD; 150-183), connected to NTD via a short linker peptide (141-149), plays essential roles in
117 pgRNA packaging and the ensuing reverse transcription (34-39). The highly basic CTD is
118 shown to have non-specific RNA and DNA binding activity (40, 41). While traditionally thought to
119 be dispensable for capsid assembly, CTD has been shown to be also important to facilitate
120 capsid assembly under physiological (low) protein and salt concentrations in vitro and in human
121 cells (42).

122

123 Subtle structural differences have been observed by cryo-electron microscopy (EM)
124 between virion-derived HBV NCs, which were presumed at the time to be all mature and contain
125 RC DNA, and recombinant HBV capsids derived from bacteria containing non-specific RNA
126 (43). It is now clear, however, that the majority of the virion-derived capsids used for that and
127 other EM studies (9, 10) were almost certainly empty capsids and only a small minority were in
128 fact mature NCs. The structures observed therefore likely represent a mixture of empty capsids
129 and mature NCs. More recent cryo-EM imaging and biophysical studies also revealed some
130 structural differences among recombinant, in vitro assembled capsids that were empty or
131 packaged artificially with pgRNA or SS or DS DNA (but not the P protein) (44, 45). The role, if
132 any, of these putative structural differences in directing NC envelopment remains to be
133 determined. In addition, NC maturation is associated with a dramatic dephosphorylation at the
134 CTD of the capsid protein such that mature NCs are dephosphorylated whereas immature NCs
135 are heavily phosphorylated (30, 46, 47). CTD phosphorylation is important for pgRNA packaging
136 (48, 49) and DNA synthesis (39, 46, 50, 51). At least for DHBV, subsequent CTD
137 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 RC DNA synthesis but 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 SS DNA 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 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 part, with the N-terminal extension of M (preS2) being also entirely contained in L, which has an additional N-terminal extension (preS1). Specifically, the so-called matrix domain (MD) in L, a short linear sequence in the C-terminal part of preS1 and N-terminal part of preS2, 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 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 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 use 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 vs. those for complete virions. Our results showed that mutations in HBc or L that completely block 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 block HDV secretion didn't inhibit secretion of either complete or empty HBV virions. These results thus demonstrated that morphogenesis of empty vs. complete HBV virions involve both common and distinct signals on both HBc and envelope proteins, and S requirement for HDV secretion was also different from that of HBV empty virions.

187 **Materials and Methods**

188

189 **Plasmids.** DNA sequences encoding the WT and NTD-mutant HBc proteins were cloned from
190 the pSVcore constructs (genotype A) (54) into the envelope-defective pCMV-HBV/Env$^-$, or
191 envelope-proficient pCIdA-HBV/pgRNA (genotype D) as described (72). These constructs direct
192 the expression of the HBV pgRNA, expressing either WT or mutant HBc, under the
193 cytomegalovirus (CMV) promoter. The HBc protein expressed from this construct is a chimera
194 between genotype D (position 1-28 and 145-183) and genotype A (29-144), which was used as
195 a "wildtype" (WT) reference for all HBc mutants harboring NTD mutations (all single residue
196 substitutions relative to the reference). Expression of the WT envelope proteins in pCIdA-
197 HBV/pgRNA is driven from the native HBV promoters (72). pSVHBV1.5C$^-$ expressing a HBc-
198 defective HBV genome (genotype A) (54), which is capable of supporting viral replication upon
199 complementation with HBc. HBV pgRNA is driven by the simian virus 40 (SV40) early promoter
200 in this construct. pCI-HBc, -HBc-164, -HBc-164Cys were constructed by placing the coding
201 sequences for the full-length (genotype D) HBc or HBc CTD truncations downstream of the
202 CMV promoter in pCI (Promega) and were used to express, respectively, WT HBc, HBc
203 truncated at position 164, HBc truncated at 164 but with the addition of a terminal Cys residue.
204 pCI-HBc Δ150-164 expresses an HBc mutant with a deletion of the N-terminal portion of the
205 CTD (from 150 to 164).

206

207 The following constructs were used to express the WT or mutant HBV envelope
208 proteins. pSVBX24H, pSV33H, and pSVB45H expresses the HBV S, M+S, L+M+S proteins
209 respectively (genotype A) (63, 73-75). pSVLS is identical to pSVB45H except for an ATG to
210 ACG point mutation of the preS2 start codon. Constructs for expressing all three envelope
211 proteins with the L mutations A1-A7 have been described before (61). The "WT" reference for
212 this series of L mutants contained an N-terminal deletion from position 2-30 (designated as

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) (76) or their mutant versions S195-197AAA with A substitutions at 195-197 (77) and W196-201A with substitutions of three W for A at W196/199/201 (78), both sets of substitutions being present in all three envelope (L, M, S) proteins.

Transient transfection. Transfection of HepG2 and Huh7 cell was done as previously described (12, 79). 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 CalPhos Mammalian Transfection Kit (Clontech). When two plasmids were used for transfection, they were used at 1:1 mass ratio. Cells and culture supernatant were harvested on day seven post-transfection. All transfection experiments were repeated between 2-5 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 37^0C for 1 h) to eliminate residual plasmid DNA before analysis for virion secretion. Where indicated, the treated culture supernatant was further fractionated by isopycnic CsCl gradient ultracentrifugation (22, 28, 46) to separate virions from naked (non-enveloped) NCs, which are 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 (28, 80). 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

239 detect the viral envelope proteins after stripping the membrane. Viral capsids in cytoplasmic
240 lysate were detected similarly following resolution by agarose gel electrophoresis (28, 30). Total
241 HBc protein in the lysate was detected by sodium dodecyl sulfate (SDS)-polyacrylamide gel
242 electrophoresis (PAGE) and western blot analysis, as previously described (28, 30). The CsCl
243 gradient fractionation experiment was repeated using samples from three separate
244 transfections. Representative images are shown.
245

246 **Results**

247

248 **HBc NTD mutations that block complete virion secretion did not block empty virion**
249 **secretion.**

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

267

268 Culture supernatant from transfected human hepatoma cells able to support HBV
269 replication and secretion (Huh7 or HepG2, as indicated in the figures) was collected and
270 concentrated. Viral particles including (enveloped) virions as well as naked capsids (non-
271 enveloped) were then resolved by native agarose gel electrophoresis. Virion-associated DNA

was detected by Southern blotting to detect 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 negligible). We could confirm that, in contrast to WT HBc that supported secretion of complete (DNA-containing) and empty virions (Fig. 1A, lanes 1 & 5), no DNA-containing virions were detectable by the L60A, L95A, K96A, or I126A HBc NTD mutant (Fig. 1B and 1C, lanes 2, 6). In contrast, secretion of empty virions was readily detectable by all these NTD mutants (Fig. 1B and 1C, lanes 10, 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 mutant (Fig. 2B-E, lanes 1-3). In contrast, secretion of empty virions was readily detectable by all these NTD mutants, as evidenced by the presence of HBc protein in the virion fractions (Fig. 2B-E, lanes 5-7).

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 HBc CTD in their secretion. However, as we reported recently (42), a CTD truncation construct (C149) with the entire CTD (150-183) 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

298 HBc truncated at 164 (C164, i.e., deleting the C-terminal part of CTD) remains competent for
299 secretion of DNA-containing virions (34, 81). Thus, we were interested in determining whether
300 C164 could support secretion of empty virions. In addition, since the C-terminal Cys (i.e.,
301 Cys183) is involved in cross-linking HBc dimers in the capsids and can stabilize the capsids (32,
302 82, 83), we constructed another mutant, C164Cys, which expresses C164 plus a terminal Cys
303 residue. An expression construct for the WT HBc, C164, or C164Cys was cotransfected with a
304 second construct expressing all HBV proteins except HBc into HepG2 and Huh7 cells in the
305 aforementioned *trans*-complementation assay. Both naked capsids and virions were released
306 by the transfected HepG2 cells (Fig. 3A, lanes 1-3) or Huh7 cells (Fig. 3A, lanes 5-7) in all three
307 co-transfection conditions, indicating that deletion of the C-terminal part of HBc CTD (i.e., from
308 165-183) did not block secretion of empty virions.
309
310 It was noticeable that the levels of virions (or naked capsids) containing C164 were
311 lower than those containing the WT HBc in both cell lines, and addition of the C-terminal Cys
312 residue in C164Cys enhanced the levels of both virions and naked capsids (Fig. 3A, top). These
313 results suggested that the deletion of 165-183 from the HBc CTD may partially impair HBc
314 expression and/or assembly in hepatoma cells. We thus measured the amount of intracellular
315 HBc in the lysate of transfected cells by both agarose gel electrophoresis to detect assembled
316 capsids (Fig. 3B, top) and SDS-PAGE to detect total HBc proteins (assembled and non-
317 assembled) (Fig. 3B, bottom) expressed in the cell. Indeed, the levels of intracellular capsids
318 matched those released into the culture supernatant (compare Fig. 3B, top with Fig. 3A, top).
319 The total intracellular C164 or C164Cys levels were also lower than WT HBc, especially in Huh7
320 cells (Fig. 3B, bottom). Thus, there was a partial defect of C164 in either expression and/or
321 assembly in human hepatoma cells. The addition of a C-terminal Cys residue, as normally found
322 in WT HBc, was able to enhance capsid assembly and/or stability. Thus, while the total core
323 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., from 165-183) was not required for secretion of DNA-containing (34, 81) or empty virions (Fig. 3), we were interested in determining if the N-terminal portion of CTD (i.e., 150-164) was required. Therefore, we constructed HBcΔ150-164, the reciprocal of HBc164, and tested its ability to support virion secretion in the trans-complementation 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 sequences from 150-164 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 (165-183) nor N-terminal (i.e., 149-164) 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, as compared to secretion of complete virions, we tested the effects of mutations in MD of L that have been shown to block secretion of complete virions. WT HBc and all mutants were expressed in the context of the HBV replicon defective in envelope protein expression, which were complemented with another construct expressing the WT Env or A3 or A4 L mutants (Fig. 1). 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, 3). These L proteins were tested in combination with all 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, 5-8; Fig. 1B & 1C, lanes, 9-16). We then decided to test a number of additional L mutants, which are defective in secretion of complete virions, in combination with WT HBc and the L95A HBc mutant. As reported before (61), the L mutations (A1, 2, A5-7) all eliminated secretion of complete virions that was supported by the WT L (Fig. 4A, lanes 1-12, 13); in contrast, they all allowed secretion of the WT or the L95A mutant capsids as empty virions (Fig. 4B, lanes 1-12). 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 9-12; Fig. 1B and 1C, lanes 17-24; Fig. 4C).

The small surface protein was sufficient for basal level secretion of empty HBV virions.

Since none of the L mutants tested, which abolish secretion of complete virions, blocked empty virion secretion significantly, we were led to the possibility that L may not be needed for empty HBV virion secretion, in contrast to the secretion of complete HBV virions but similar to the secretion of HDV virions. To test this possibility, the WT or L95A HBc mutant, plus different combinations of the envelope proteins, were used for determining complete and empty HBV virion secretion. As expected, secretion of complete virions required L (Fig. 5A, lanes 1-5) and the L95A HBc mutation abolished secretion of complete virions (Fig. 5A, lanes 6-10). 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, 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 co-expressed with L (Fig. 5C), consistent with the well-known effect of intracellular S retention by L (74).

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.

375 Close inspection of the empty virion secretion results showed that L+M, and to a lesser
376 extent, L, could enhance empty virion secretion that was supported by S alone at a lower level.
377 Thus, although the L+S, and L+M+S, combination had less overall HBsAg secretion (due to the
378 retention property of L) than S or M+S (Fig. 5C, lanes 1, 3, 6, 8 vs. 2, 4, 7, 9), they actually
379 showed more empty virion secretion when combined with the WT HBc (Fig. 5B, lanes 1, 3, vs.
380 2, 4; Fig. 5D). Furthermore, L mutations (A1-3, A5-7) that blocked complete virion secretion
381 mostly eliminated this enhancement of virion secretion with WT HBc although they still allowed
382 empty virion secretion with WT HBc at a reduced level (Fig. 1A, lane 5 vs. 6-7; Fig. 4B, lane 13
383 vs. 1-5). Interestingly, the stimulatory effect of WT L on empty virion secretion was also mostly
384 lost when the mutant, instead of WT, HBc proteins were used. Thus, levels of empty virion
385 secretion by the L60A, K96A, and I126A HBc mutants were not stimulated or only stimulated
386 weakly by the WT L as compared to the mutant L proteins (Fig. 1B, lanes 10-12; 1C, lanes 10-
387 12, 14-16). The effect of L95A on empty virion secretion seemed to be somewhat variable.
388 Empty virion secretion by this mutant was still stimulated by the WT (but not mutant) L protein
389 (Fig. 1B, lanes 14-16; Fig. 4B, lanes 7-11, 14) and its secretion with WT L was only slightly
390 (within 2-fold) less than the WT HBc (Fig. 4B, lanes 13, 14). On the other hand, in other
391 experiments, levels of empty virions formed by L95A were clearly less than WT HBc and
392 stimulated little by the presence of WT L as compared with S alone (Fig. 5B, lanes 6-9).
393 Interestingly, the M and S envelope protein combination appeared to support somewhat less
394 efficient secretion of empty virions than S alone (Fig. 5D) although the difference didn't reach
395 statistical significance. On the other hand, in the absence of M, L plus S supported less
396 secretion of empty virions than L plus M plus S (Fig. 5D) (see more on the role of M in
397 Discussion).
398
399 It was also notable that LMS (and to a lesser degree, L plus S), but not S alone or M
400 plus S, reduced dramatically the naked capsid signals in the culture supernatant (Fig. 1A, lanes

1, 5; Fig. 4A and 4B, lane 13; Fig. 5A and 5B, lanes 1, 3). Mutations in either L (A1-A3, A5-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, 6; 1B, lanes 2, 4, 6, 8, 10, 12, 14, 16; 1C, lanes 2, 4, 6, 8, 10, 12, 14, 16; Fig. 4A and 4B, lanes 1-5, 7-11, 14). Thus, L apparently interacted with capsids to suppress naked capsid release in a process requiring the same preS1 (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, 7 vs. 1, 5) and also failed to suppress the release of naked capsids (Fig. 1A, lanes 3, 7; Fig. 1B, lanes 3, 7, 11, 15; Fig. 1C, lanes 3, 7, 11, 15), 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, 5; and 3C, lanes 1, 3; also see below Fig. 6, lane 1 vs. 2 and 5 vs. 6 when the envelope proteins were expressed from another expressing construct different from the ones used in Fig. 1, 4 and 5) (see also Discussion below).

S mutations defective in HDV secretion didn't block complete or empty HBV virion secretion.

Since S alone allowed secretion of empty HBV virions, similar to HDV, 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, W196-201A (W196/199/201A) (78, 84) and 195-197AAA (77), 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 1-4) or from an HBc-expressing plasmid that expresses only the HBc protein (Fig. 6, lanes 5-8). In neither case was the secretion of empty HBV virions affected by the S CYLII mutations. The S mutations also didn't affect secretion of complete virions (Fig. 6, lanes 2-4). These results indicated that the S requirements for secretion of empty (or complete) HBV virions were distinct from those for HDV virion secretion.

436 **Discussion**

437 Secretion of both empty and complete HBV virions requires interactions between the
438 viral capsid and one or more viral envelope proteins. On the capsid side, we have shown here
439 that substitutions of the HBc NTD residues on the surface of the capsids in the previously
440 defined MBD (Fig. 7A), which block the secretion of DNA-containing virions, did not prevent the
441 secretion of empty virions. Furthermore, the HBc CTD was not essential for empty virion
442 secretion, either. On the envelope side, substitutions in MD (Fig. 7B) in the preS1 domain of L
443 that block secretion of complete virions also did not block empty virion secretion. Furthermore,
444 among the three viral envelope proteins, the smallest, S, alone was sufficient for empty virion
445 secretion at a basal level and the L (and possibly M) could enhance empty virion secretion, in
446 contrast to the secretion of complete virions, which absolutely requires both S and L. Thus, the
447 secretion requirements of empty HBV virions are distinct from those of complete virions and
448 share some similarity to its satellite virus, HDV, whose secretion is also supported by S alone.
449 However, S mutations that block HDV secretion (in the CYL II, Fig. 7B) had no effect on
450 secretion of either empty or complete HBV virions. In addition, residues in MBD on the capsid
451 surface as well as those in MD of preS1, while not essential for secretion of empty virions, could
452 enhance empty virion secretion, and could also suppress the release of naked (non-enveloped)
453 capsids from hepatoma cell cultures.
454
455 Our results indicate that the secretion of complete vs. empty HBV virions is mediated via
456 distinct, as well as shared, signals. These results, together with the information available in the
457 literature, allow us to propose a two-signal model for HBV virion secretion (Fig. 7C). While
458 additional biochemical and structural studies will be required to confirm directly the interactions
459 as predicted here, the genetic evidence provided by our mutagenesis experiments strongly
460 support this model. First, empty capsids display a basal and constitutive positive signal
461 (Secretion Signal I) that induces their envelopment, which is mediated, at a basal level, via

462 interactions with S (Fig. 7C, top). Although the structural basis of Signal I remains to be defined,
463 we have recently obtained evidence supporting a critical role of the HBc linker in empty virion
464 secretion (KC Liu, et al, submitted). Thus, the linker peptide may harbor Signal I or is involved in
465 generating the signal. In support of the proposed capsid-S interactions, peptides derived from S
466 were shown to interact with capsids in vitro (85, 86). However, the specific site on S that
467 interacts with empty capsids to direct empty virion secretion remains to be defined. Our results
468 here and previous findings (78, 84) do indicate that the S requirements for interacting with HBV
469 capsids to secrete empty (or complete) HBV virions are distinct from those for interacting with
470 the HDV ribonucleoprotein (RNP) to secrete HDV virions. Additional comparative studies to
471 define the HBV S requirements for empty vs. complete HBV virion formation, and those for HDV
472 virion formation, should provide important insights into the molecular details driving HBV and
473 HDV morphogenesis, in particular, how HBV S recognizes two seemingly rather different
474 substrates (the HBV capsid vs. the HDV RNP).
475
476 Second, a positive signal distinct from Signal I, which we call Secretion Signal II and
477 likely consists of the previously defined MBD within the HBc NTD and located spatially on the
478 capsid surface, emerges on mature, RC DNA-containing NCs, which primarily interacts with the
479 MD of L, to mediate secretion of DNA-containing or complete virions. Assuming that the
480 constitutive Secretion Signal I remains on immature NCs, we hypothesized that immature NCs
481 acquire a negative (retention) signal that is dominant over Secretion Signal I so as to inhibit their
482 envelopment (28). The emergence of Signal II on mature NCs could, by itself, overcome the
483 negative effect of the inhibitory signal on envelopment (Fig. 7C, IIa). For example, the preS1
484 (MD)-mature NC interactions, alone, without any contribution of S, may be sufficient to direct
485 secretion of complete virions. If the negative signal of envelopment on immature NCs indeed
486 represents the sequestration of a positive secretion signal (which could be the Secretion Signal I
487 suggested here), as we proposed earlier (28), the emergence of a different positive signal

488 (represented by Signal II) could be sufficient to trigger secretion of complete virions.
489 Alternatively, additional structural changes of mature NCs, distinct from the emergence of Signal
490 II, may be required to remove the Blocking Signal, e.g., by de-sequestration (exposing) of the
491 secretion signal (Signal I) previously hidden in immature NCs upon NC maturation, which,
492 together with Signal II, facilitate the envelopment and secretion of mature NCs (Fig. 7C, IIb).
493
494 We have also shown here that the same interactions between the MBD on capsids
495 (Signal II) and the MD on L, though not absolutely required for secretion of empty virions, could
496 stimulate empty virion secretion (Fig. 7C, top). HBc MBD mutants were less competent for
497 empty virion secretion than WT HBc when complemented with WT envelope proteins. Also, MD
498 mutations in L mostly eliminated the stimulatory effect of L on empty virion secretion with WT
499 HBc. Indeed, peptides related to MD in preS1 were shown to bind mostly empty capsids
500 isolated from HBV-infected human liver (85, 86) and CTD-deleted capsids (C144) from insect
501 cells (86). Thus, it appears that Signal II postulated to emerge (inducibly) on mature NCs, may
502 also be present on empty capsids (constitutively), which, like Signal I, would also have to be
503 suppressed on immature NCs by the presence of pgRNA or SS DNA, or overwhelmed by the
504 secretion inhibitory signal, to prevent their secretion. We emphasize that the structural basis for
505 the emergence or sequestration of either Signal I or II on empty capsids and mature NCs
506 remains to be elucidated. As discussed in the Introduction, although CTD dephosphorylation is
507 correlated with NC maturation, it is not necessary for secretion of either complete or empty
508 virions (30). A possible structural correlate of the secretion signals is the dynamic stability of the
509 capsids: both mature NCs and empty capsids, which are competent for virion formation, are less
510 stable than immature NCs, which are incompetent for virion formation (87).
511
512 The role of the M envelope protein in the secretion of virions appears to be complex. On
513 one hand, M plus S was not better (and may even be worse) than S alone in supporting

514 secretion of empty virions (Fig. 5), suggesting that the preS2 domain of M, in an N-terminal
515 open configuration and perhaps its preS2-linked N/O-glycosylation (1), does not stimulate, and
516 may even interfere with, empty virion secretion. On the other hand, the less efficient secretion of
517 empty virions by L plus S vs. L plus M plus S suggests that M is needed to enable L to stimulate
518 secretion of empty virions. We note that the absence of M (i.e., comparing L plus M plus S vs. L
519 plus S) also led to somewhat reduced secretion of complete virions (Fig. 5A), consistent with a
520 previous report (88), suggesting that M might also play an auxiliary role in the secretion of
521 complete virions. It is also possible that the absence of M indirectly affected virion secretion by
522 increasing the ratio of L:S(+M), which would decrease secretion of virions as well as HBsAg
523 particles due to the intracellular retention function of L. The somewhat lower levels of HBsAg in
524 the culture supernatant secreted by LS compared with LMS (Fig. 5C) would be consistent with
525 this possibility. Future studies will be required to resolve these possibilities.
526
527 The effect of the L95A mutation on the secretion of empty virions appeared to be weaker
528 (and more variable) as compared with the other HBc MBD mutants. This may suggest that it
529 could still interact with L (MD) to stimulate empty virion secretion though clearly defective in
530 secretion of complete virions. So, this mutant may impair secretion of complete virions in a way
531 that is somewhat different from the other HBc MBD mutants. Interestingly, we showed recently
532 that this mutant also does not cause hyper-destabilization of mature NCs to increase CCC DNA
533 formation, in contrast to the other MBD mutants (72). Also, since other MBD mutants of HBc
534 (including I126A and K96A) were still stimulated to some extent by L for empty virion secretion,
535 it is possible that additional L-HBc interactions outside of the MD-MBD (Fig. 7A and 7B)
536 interaction contribute to secretion of empty virions.
537
538 While the role of the NTD in the secretion of complete vs. empty virions seems clear, the
539 role of HBc CTD in virion secretion remains to be better defined. It has been reported previously

that HBV CTD beyond residue 164 is not essential for DNA-containing virion secretion (34, 81). The role of CTD residues before 164 (i.e., 150-164) 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 preS1 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 auxiliary role in secretion of empty HBV virions. How this is accomplished remains to be elucidated. One possibility is that virion formation, esp. when it is enhanced by L (MD) – MBD (Signal II) interactions, directs most or all intracellular capsids towards the envelopment pathway, thus effectively depriving the capsids from the pathway of naked capsid release (89). 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, 90), may be explained by the much lower expression and secretion of HBV envelope proteins, relative to the levels 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, relative to levels of virions, were released by cultured hepatoma cells compared to those released into the blood stream 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 ratio of L:M:S, may affect the suppressive effect of L on the

566 extracellular release of naked capsids. Indeed, the stoichiometry of empty capsids or mature
567 NCs relative to the envelope proteins and stoichiometry of the different forms of envelope
568 proteins (L, M, and S) themselves likely also affects the levels of empty and complete virions
569 secreted. Future studies are warranted to clarify these issues.
570
571 The function of empty HBV virions remains to be defined. Incomplete viral particles are
572 in fact common occurrences; the so-called light particles of herpes simplex virus (with the
573 envelope and the tegument layers but no capsid nor genome) are reported to deliver their
574 interior tegument components to the host cell, which can modulate host function to affect
575 infection outcome by the complete (infectious) virions of the same cell (91, 92). Based on our
576 results here, at least a fraction of empty HBV virions will have the L envelope protein that is
577 essential for viral infection and can thus in principle enter host cells like the complete virions. In
578 analogy with the herpesvirus, the HBV capsid delivered by empty virions into the host cell may
579 modulate host functions to influence infection by the complete virions. Furthermore, as we
580 proposed earlier, serum empty HBV virions may serve as a better biomarker than serum HBsAg
581 for intrahepatic CCC DNA since HBc, an essential component of empty as well as complete
582 virions, can likely be made only from CCC DNA and not from integrated HBV, which can direct
583 the production of HBsAg (27, 29, 93). This can be especially useful when serum complete
584 virions fall to undetectable or un-quantifiable levels with potent antiviral therapy targeting the
585 viral RT protein. Empty HBV virions in principle can also serve as the basis for a new generation
586 of HBV vaccine incorporating all viral structural proteins, not just HBsAg as in the current
587 recombinant HBsAg-based vaccine (4, 27). Findings presented here and further studies to
588 elucidate the mechanisms of virion morphogenesis will have important implications for these
589 potential applications of empty HBV virions.
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855 **Figure legend**
856

857 **Figure 1. Analysis of complete and empty virion secretion by HBc NTD mutants in**
858 **combination with preS1 mutants.** Huh7 cells were transfected with a HBV genomic construct
859 defective in envelope protein expression (pCMV-HBV/Env$^-$), expressing either WT (A) or the
860 indicated HBc NTD mutations (B, L60A, K95A; C, L96A, I126A), complemented with a 2nd
861 plasmid expressing all three envelope proteins with WT or mutant preS1 (A3, A4) sequences
862 (pSV45-31L). Seven days later, the concentrated culture supernatant containing both secreted
863 virions and naked NCs was analyzed for virion secretion by native agarose gel electrophoresis.
864 Following transfer to nitrocellulose membrane, viral DNA was detected by ^{32}P-labeled HBV DNA
865 (A, lanes 1-4; B, lanes 1-8; C, lanes 1-8), followed by detection of HBc (core) protein using an
866 HBc-specific polyclonal antibody (A, lanes 5-8; B, lanes 9-16; C, lanes 9-16) and envelope
867 proteins using an anti-HBs polyclonal antibody (A, lanes 9-12; B, lanes 17-24; C, lanes 17-24).
868 HBV virions (V), HBsAg particles (HBs), and capsids (Ca) are indicated.
869

870 **Figure 2. CsCl density gradient analysis of virion secretion by HBc NTD mutants.** HepG2
871 cells were transfected with the indicated HBV genomic constructs (pCIdA-HBV/pgRNA)
872 containing the WT HBc (A) or indicated HBc mutants (B, L60A; C, L95A; D, K96A; E, I126A).
873 Seven days later, the concentrated culture supernatant was analyzed for virion secretion by
874 CsCl gradient ultracentrifugation. Fractions containing HBV virions as well as cytoplasmic lysate
875 (lysate) containing intracellular NCs (NC) were resolved by native agarose gel electrophoresis.
876 Following transfer to nitrocellulose membrane, viral DNA was detected by ^{32}P-labeled HBV DNA
877 (lanes 1-4), followed by detection of HBc (core) protein using an HBc-specific polyclonal
878 antibody (lanes 5-8). HBV virions (V) and capsids (Ca) are indicated.
879

880 **Figure 3. Analysis of empty virion secretion by HBc CTD deletion mutants.** HepG2 (A and
881 B, lanes 1-4; C, lanes 1, 2) and Huh7 (A and B, lanes 5-8; C, lanes 3, 4) cells were transfected
882 with a plasmid expressing the WT HBc (pCI-HBc) (A and B, lanes 1, 5; C, lanes 1, 3), or the
883 CTD deletion mutant C164 (pCI-HBc-C164) (A and B, lanes 2, 6), C164cys (pCI-HBc-C164Cys)
884 (A and B, lanes 3, 7), or Δ150-164 (pCI-HBc Δ150-164) (C, lanes 2, 4), together with an HBV
885 genomic construct expressing all viral proteins except HBc (pSVHBV1.5C$^-$). The samples in
886 lanes 4 and 8 of panels A and B were from cells transfected with the HBc-defective genomic
887 construct alone. Seven days later, the concentrated culture supernatant was analyzed for virion
888 secretion by native agarose gel electrophoresis (A and C). The cytoplasmic lysate containing
889 intracellular NCs was also resolved by native agarose gel electrophoresis (B, top) or SDS-
890 PAGE (B, bottom). Following transfer to nitrocellulose (A; B, top; C) or PVDF (B, bottom)
891 membrane, HBc (core) was detected using an HBc-specific polyclonal antibody (A, top; B, top;
892 C, bottom) or monoclonal antibody (B, bottom). The viral envelope proteins were detected using
893 an anti-HBs polyclonal antibody (A, bottom). Viral DNA was detected by ^{32}P-labeled HBV DNA
894 probe (C, top). HBV virions (V), HBsAg particles (HBs), and capsids (Ca) are indicated, as are
895 the full-length HBc (C) and truncated C164 and C164cys proteins.
896
897 **Figure 4. Analysis of complete and empty virion secretion by WT HBc and the NTD L95A**
898 **mutant in combination with preS1 mutants.** Huh7 cells were transfected with a HBV genomic
899 construct defective in envelope protein expression (pCMV-HBV-Env$^-$), expressing either WT or
900 the L95A HBc NTD mutation, complemented with a 2nd plasmid (pSV45-31L) expressing all
901 three envelope proteins with WT or mutant preS1 (A1, A2, A5-A7) sequences. Seven days later,
902 the concentrated culture supernatant was analyzed for virion secretion by native agarose gel
903 electrophoresis. Following transfer to nitrocellulose membrane, viral DNA was detected by ^{32}P-
904 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.

Figure 5. Analysis of complete and empty virion secretion by WT HBc and the L95A mutant supported by different combinations of the three envelope proteins. Huh7 cells were transfected with a HBV genomic construct defective in envelope protein expression (pCMV-HBV-Env$^-$), expressing either WT or the L95A NTD mutant, complemented with a 2nd plasmid expressing all three envelope proteins (LMS) (pSVB45H), S alone (S) (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 signal in virions (relative virion secretion) were quantified and compared to that with LMS, which was set to 1.0. Statistical analysis was performed using the Student t-test, two-tailed and unpaired. ns, $P>0.05$; **, $P<0.01$; ***, $P<0.001$.

Figure 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$^-$) (lanes 1-4) or a plasmid expressing HBc alone (pCI-HBc) (C) (lanes 5-8), together with a 2nd plasmid expressing all three envelope proteins with 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 (top), followed by detection of HBc (core) protein using an HBc-specific

931 polyclonal antibody (middle) and envelope proteins using an anti-HBs polyclonal antibody
932 (bottom). HBV virions (V), HBsAg particles (HBs), and capsids (Ca) are indicated.
933
934 **Figure 7. Schematics of HBc and HBV envelope protein domain structure and a working**
935 **model for HBV virion secretion. A. HBc domain structure.** The boundaries of the HBc NTD,
936 linker, and CTD domains are indicated on the top. The MBD (scattered within the NTD on the
937 linear sequence but located on the capsid surface spatially) that is involved in interactions with
938 the L envelope protein for secretion of complete virions is indicated at the bottom. The positions
939 of HBc mutations used in this study are also indicated. **B. Domain structure of the HBV**
940 **envelope proteins.** The boundaries of preS1, preS2, and S domain are indicated on the top,
941 with the positions within the S domain also indicated (i.e., position 175 as 1 or S1 within the S
942 domain). The MD at the junction between preS1 and preS2 (mostly within preS1), which is
943 involved in interactions with the HBc MBD for secretion of complete virions, is indicated in the
944 middle (A1-A7 denoting mutants within the MD that were used in this study). Also indicated is
945 the CYL-II involved in secretion of HDV. The bottom three lines denote the viral L, M, and S
946 envelope proteins, with L containing all three (preS1, preS2, and S) domains, M containing the
947 preS2 and S domains, and S containing the S domain only. **C. Model for two distinct signals**
948 **in the secretion of empty vs. complete HBV virions.** The diamonds denote the capsids, and
949 outer circle the viral membrane, with the S, preS2, and preS1 domains of the envelope protein
950 denoted by the vertical bar, the small and large filled balls, respectively. Secretion Signal I or II
951 are proposed to exist on the surface of empty capsids or mature NCs to direct their interactions
952 with the S or L envelope protein respectively. Immature NCs display a Blocking Signal that
953 negatively regulates NC envelopment and virion formation, which can be overwhelmed by
954 Secretion Signal II in pathway IIa but has to be eliminated (and re-exposure of Secretion Signal
955 I) also in pathway IIb for secretion of complete (i.e., RC DNA-containing) virions. Grey lettering
956 in parenthesis 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 preS domain (75, 94). The preS1 MD in L can only interact with the capsid in the L-i topology. The alternative topology, L-e, with the PreS 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 text for details.