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Maturation-Associated Destabilization of Hepatitis B Virus Nucleocapsid

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The mature nucleocapsid (NC) of hepatitis B virus containing the relaxed circular (RC) DNA genome can be secreted extracellularly as virions after envelopment with the viral surface proteins or, alternatively, can be disassembled to release RC DNA (i.e., uncoating) into the host cell nucleus to form the covalently closed circular (CCC) DNA, which sustains viral replication and persistence. In contrast, immature NCs containing the viral single-stranded DNA or the pregenomic RNA are incompetent for either envelopment or uncoating. Little is currently known about how mature NCs, and not the immature ones, are specifically selected for these processes. Here, we have carried out a biochemical analysis of the different NC populations upon their separation through sucrose gradient centrifugation. We have found that the maturation of NCs is associated with their destabilization, manifested as increased protease and nuclease sensitivity, altered sedimentation during sucrose gradient centrifugation, and retarded mobility during native agarose gel electrophoresis. Also, three distinct populations of intracellular mature NCs could be differentiated based on these characteristics. Furthermore, mature NCs generated *in vitro* under cell-free conditions acquired similar properties. These results have thus revealed significant structural changes associated with NC maturation that likely play a role in the selective uncoating of the mature NC for CCC DNA formation and/or its preferential envelopment for virion secretion.

Hepatitis B virus (HBV) is a major human pathogen infecting hundreds of millions of people worldwide; annually, nearly a million people die from cirrhosis and hepatocellular carcinoma associated with chronic HBV infections (1, 2). HBV is a member of the *Hepadnaviridae* family, which also includes related viruses infecting mammalian and avian species, such as duck hepatitis B virus (DHBV) (3, 4). All hepadnaviruses contain a small (ca. 3-kb), partially double-stranded (DS), relaxed circular (RC) DNA genome and replicate this DNA genome via an RNA intermediate, the so-called pregenomic RNA (pgRNA), via reverse transcription that is carried out by a multifunctional viral reverse transcriptase (RT). As with retroviruses, hepadnavirus assembly initiates with the formation of a nucleocapsid composed of 240 copies (180 copies for a small fraction of capsids) of a single viral protein, the core or capsid protein (HBc), that packages a copy of RT and pgRNA (5–7) in a process that also depends on host chaperones (8–11). The resulting NC then undergoes a process of maturation whereby the packaged pgRNA is first converted by the packaged RT protein to a single-stranded (SS) DNA and then to the characteristic DS RC DNA (3, 4, 12).

Both the pgRNA- and SS DNA-containing NCs are considered immature, and RC DNA-containing NCs are considered mature, as only the latter, and not the former, are competent for envelopment by host-derived membrane and viral surface proteins for extracellular secretion as enveloped virions (and, hence, hepadnavirus virions do not contain RNA, unlike retroviruses) (3, 13–15). Interestingly, large amounts of empty HBV capsids (i.e., containing no viral RNA/DNA and little to no cellular RNA/DNA) are also assembled in infected cells (16, 17) and, like mature NCs, are efficiently enveloped and secreted such that empty HBV virions (enveloped capsids containing no RNA or DNA genomes) far outnumber the RC DNA-containing ones by orders of magnitude both in cell culture systems as well as in infected animals and patients (17, 18). Apparently, the mature HBV NCs and empty capsids share a structural property that allows their selective envelopment and secretion, or the immature NCs containing either pgRNA or SS DNA may share a different property (the so-called SS blocking signal) (17) that renders them incompetent for envelopment and secretion.

Instead of envelopment and extracellular secretion, mature hepadnavirus NCs can also undergo disassembly (uncoating) and release their RC DNA content into the host cell nucleus for conversion to the covalently closed circular (CCC) DNA episome, which serves as the transcriptional template for the synthesis of all viral RNAs, including pgRNA, sustaining viral replication and persistence (19–22). This so-called intracellular recycling of mature NCs for CCC DNA formation is analogous to CCC DNA synthesis from RC DNA derived from the incoming virion (containing the mature NC) during *de novo* infection.

While it is generally accepted that NC maturation must be accompanied by structural changes that direct the selective envelopment or disassembly of mature NCs, it is not yet clear what the structural changes associated with NC maturation are or whether the same or different structural properties direct the two alternative fates available to mature NCs (envelopment and extracellular secretion versus disassembly/uncoating). NC maturation in DHBV is associated with a dramatic NC dephosphorylation such that mature NCs are completely dephosphorylated, whereas immature NCs are phosphorylated in a heterogeneous manner (23, 24). While NC phosphorylation may be important for pgRNA packaging (25, 26), SS DNA synthesis (25, 27, 28), and subsequent dephosphorylation for RC DNA synthesis (28), the role, if any, of

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the NC phosphorylation state in directing NC envelopment or uncoating is not yet clear. In addition, it has been proposed that mature, but not immature, NCs may expose a nuclear localization signal (NLS) located on the C-terminal domain (CTD) of HBc, which may direct the mature NCs to the nuclear pore, where they can release RC DNA into the nucleus (29–33). Whether this exposure of the CTD NLS is also involved in mature NC envelopment remains unknown.

To explore the structural changes that are associated with HBV NC maturation, we have isolated intracellular NCs and NCs released from extracellular virions and analyzed the properties of the immature and mature NCs, including sedimentation during sucrose gradient ultracentrifugation, mobility during native agarose gel electrophoresis, and sensitivity to proteolysis and nuclease digestion. Our analyses revealed significant structural differences between immature and mature NCs; in particular, mature NCs were found to be significantly less stable than immature ones. Furthermore, subpopulations of mature NCs could be differentiated based on differences in these regards.

MATERIALS AND METHODS

Isolation of HBV NCs from cell lysates and virions by sucrose gradient centrifugation. HepAD38 cells expressing the HBV pgRNA under the control of a tetracycline (Tet)-repressible promoter were derived from the human hepatoblastoma cell line HepG-2 (34) and maintained in Dulbecco modified Eagle–F-12 medium supplemented with 10% fetal bovine serum (FBS) and 5 μg/ml of Tet until induction. Intracellular HBV NCs were isolated as previously described (17, 23, 35), with the following modifications. HepAD38 cells induced for 20 days without Tet were lysed in NP-40 lysis buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% NP-40) containing a protease inhibitor cocktail (Roche) and briefly centrifuged at 14,000 rpm to remove the nuclei and cell debris. The resulting cytoplasmic lysate was then processed in either of two different ways. In the first method, the lysate was incubated with micrococcal nuclease (MNase) (Roche) (150 units/ml) and $CaCl_2$ (5 mM) at 37°C for 90 min to degrade the nucleic acids outside NCs. The MNase was then inactivated by the addition of 10 mM EDTA. The MNase-treated lysate was centrifuged briefly to remove any precipitates before being subjected to ultracentrifugation. In the second method, the MNase treatment was omitted, and the lysate was directly subjected to ultracentrifugation. For sucrose gradient ultracentrifugation, the cytoplasmic lysate prepared as described above was layered onto a 15% to 30% linear sucrose gradient in HCB2 buffer (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM EDTA, 0.01% [wt/vol] Triton X-100, 1× protease inhibitor cocktail [Roche], and 0.1% β-mercaptoethanol) and centrifuged at 27,000 rpm for 4 h at 4°C in a Beckman SW32 rotor. Twenty-three fractions were then collected (from top to bottom) and stored at −80°C until analysis.

Isolation of NC-associated HBV DNA. To isolate the viral DNA from NCs, the NC-containing sucrose fractions were treated with 0.5% sodium dodecyl sulfate (SDS), with (for isolation of all NC-associated DNA) or without (for isolation of protein-free [PF] DNA) 0.6 mg/ml proteinase K (PK) at 37°C for 1 h. The DNA released from NCs was then recovered by phenol-chloroform extraction and ethanol precipitation.

Endogenous polymerase reaction. NCs (10 μl of the indicated sucrose fraction or cytoplasmic lysate) were incubated with 100 μM each dATP, dGTP, dCTP, and TTP; an EDTA-free protease inhibitor cocktail (Roche); and endogenous polymerase reaction (EPR) buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 0.1% NP-40, 0.1% 2-mercaptoethanol) for 16 h at 37°C for EPR in a final volume of 20 μl, whereby the viral RT packaged within NCs synthesizes DNA by using the endogenous viral RNA and DNA templates packaged within the NCs (3, 36, 37).

Treatment of NCs by PK and nucleases. NCs isolated by sucrose gradient centrifugation (10 μl of the indicated sucrose fraction) or cytoplasmic lysate (10 μl) were treated with 1 mg/ml of PK (Invitrogen) (unless noted otherwise) in EPR buffer in a total volume of 20 μl at 37°C for 1 h. Similarly, for nuclease treatment, MNase (0.25 unit/μl) or DNase I (Roche) (2 mg/ml) was added, and the reaction mixture was incubated at 37°C for 1 h. For MNase digestion, the $MgCl_2$ in the EPR buffer was replaced with $CaCl_2$ (5 mM). For digestion with both PK and DNase, the samples were first treated with PK for 1 h, followed by the addition of DNase I and incubation for another 1 h. The nucleases were inactivated by the addition of 15 mM EDTA. As originally developed by Summers and coworkers and adopted by others to eliminate naked (nonenveloped) DHBV NCs released into cell culture medium (14, 15, 23, 38), DNase I has sufficient time to digest DNA released from NCs under these conditions before it is digested by PK.

Detection of NC-associated viral DNA and RNA. NCs were resolved on a native agarose (1.2%) gel made in 1× TAE buffer (40 mM Tris-acetate and 1 mM EDTA) to detect any DNA released from disrupted NCs as well as intact NCs. To detect all DNA species contained within NCs, they were first disrupted with 0.1% SDS and then resolved on an agarose gel containing 0.1% SDS (21). Following standard Southern blot transfer, viral DNA was detected by using a ^{32}P-labeled HBV DNA probe (11, 21). To detect viral RNA packaged inside NCs, NCs were resolved on native agarose gels and detected by Southern blotting, without the NaOH denaturation step prior to transfer onto a membrane, using a ^{32}P-labeled RNA probe specific for HBV pgRNA (11, 37, 39). Under these conditions, we have shown previously that only pgRNA, and not plus-strand DNA, in HBV NCs is detected (11, 17). HBV capsid proteins were detected by using a mouse monoclonal antibody (MAb) specific for the N terminus of HBc (17, 40).

Gel extraction and identification of viral DNA species in NCs with different mobilities on native agarose gels. NCs with or without prior PK digestion were resolved on a 1% low-melting-point (LMP) agarose gel (Invitrogen). Gel slices containing NCs with different mobilities were melted at 65°C with intermittent agitation, cooled down to 42°C, and then digested with β-agarase I (1 unit per 200 μl of molten LMP gel; NEB) for 2 h at 42°C. Subsequently, the molten gel slices were further treated with 0.5% SDS and 0.6 mg/ml PK for 1 h at 37°C to remove HBc and the viral RT attached to the viral DNA, which was then recovered by phenol-chloroform extraction and ethanol precipitation with linear acrylamide (Ambion). The purified DNA was resuspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and analyzed by Southern blotting. HBV DNA extracted from induced HepAD38 cell lysates and recombinant HBV capsids purified from *Escherichia coli* were resolved in parallel as standards for viral DNA and NC migration on the gel, respectively.

Immunoprecipitation (IP) of viral NCs. NCs were precipitated with either a mouse MAb (catalog number MA1-7607; Thermo Scientific) or a rabbit polyclonal antibody (PAb) (Dako) against HBc, as previously described (35, 41). The nonimmune mouse and rabbit IgGs were used as negative controls for the mouse and rabbit anti-HBc antibodies, respectively. In brief, sucrose gradient fractions containing NCs were incubated at 4°C overnight with the indicated antibody that was prebound to protein A/G resin (Roche). The beads were collected by centrifugation (800 × *g* for 2 min) and washed with Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA (TNE) extensively. The bound NCs were incubated with 0.5% SDS and 0.6 mg/ml PK at 37°C for 1 h to release the NC-associated DNA, which was then resolved on a native agarose gel and detected by Southern blotting.

RESULTS

Mature NCs allow RC DNA escape upon PK digestion. As one potential indication of structural changes during NC maturation, we determined the sensitivities of the different NC populations to proteolysis. We were able to separate HBV NCs of different maturities via sucrose gradient centrifugation using a procedure that we developed previously for fractionating DHBV NCs (23). As judged by the profile of viral DNA resolved on an SDS-containing agarose gel, immature NCs containing SS DNA sedimented slower

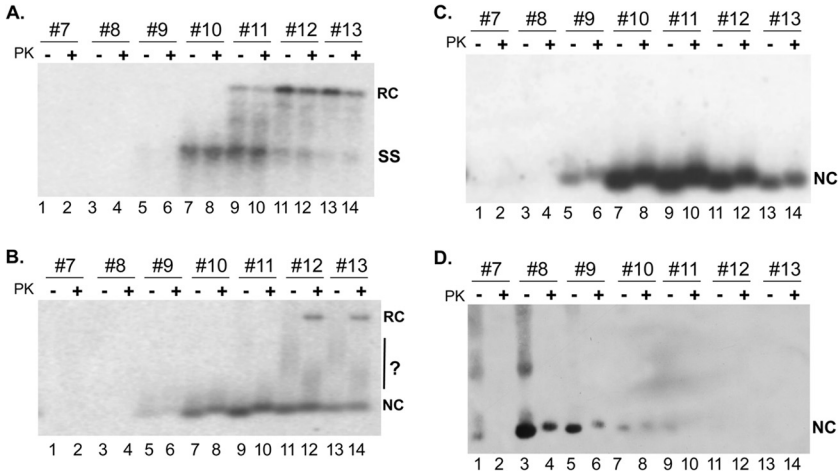

FIG 1 Isolation of HBV NCs from MNase-treated lysates by linear sucrose gradient centrifugation and detection of NC-associated viral DNA, RNA, and capsid protein. Induced HepAD38 cells were lysed by using NP-40, and the cytoplasmic lysate was treated with MNase. The supernatant from the MNase treatment was fractionated by linear sucrose gradient centrifugation as described in Materials and Methods. (A and B) Aliquots from fractions 7 to 13 were treated with or without PK and resolved on an agarose gel with (A) or without (B) 0.1% SDS, which was also added to the samples loaded in panel A. Viral DNA released from the SDS-disrupted NCs (A) or associated with native NCs (B) was detected by Southern blotting with an HBV-specific DNA probe. In both panels A and B, the agarose gel was treated with NaOH to remove RNA as well as to denature DS DNA. (C) To detect capsid-associated pgRNA, the NaOH treatment was omitted, and viral RNA was detected by using a riboprobe specific for the plus strand of the HBV genome. (D) Capsid protein was detected by using and anti-HBc MAb specific for the HBc N terminus (see Materials and Methods for details). NC, nucleocapsid; RC, relaxed circular DNA; SS, single-stranded DNA. The question mark in panel B denotes putative NCs with altered mobility on the gel.

than mature NCs containing RC DNA on these gradients (Fig. 1A), likely due to the difference in mass and density (contributed by the second DNA strand) of the mature versus immature NCs, as observed for DHBV NCs (23). Treatment of the different NCs with PK indicated that some mature NCs were disrupted such that their RC DNA content was released following PK digestion (Fig. 1B, lanes 12 and 14). The identity of the RC DNA released from mature NCs following PK treatment, which comigrated exactly with the RC DNA purified from NCs following standard SDS-PK digestion and phenol-chloroform extraction, was further verified by digestion with the restriction enzyme EcoRI, which converted the RC DNA to the linear form (Fig. 2C). In contrast, no SS DNA release was apparent following PK treatment (Fig. 1B; see also Fig. 3A). In addition, immature NCs containing pgRNA were found to be resistant to PK digestion, as no loss of RNA signal associated with NCs was noted after PK treatment (Fig. 1C). Thus, maturation-associated NC destabilization was indeed demonstrated.

As we reported previously (17), large amounts of empty (nucleic acid-free) HBV capsids were also detected, concentrating in

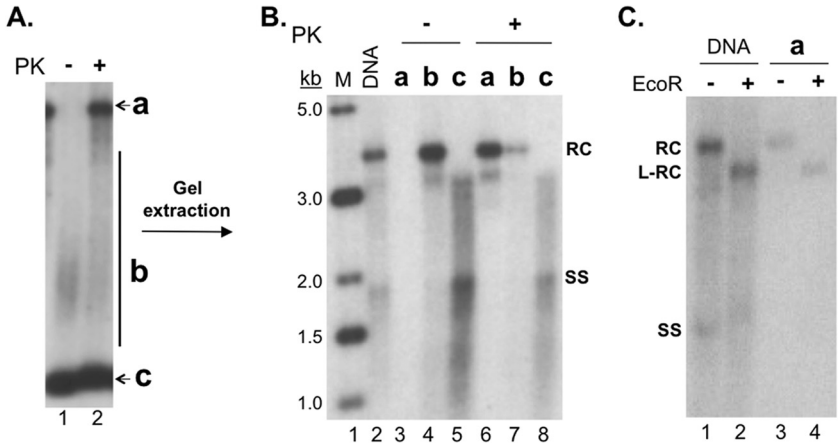

FIG 2 Identification of viral DNA species from different NC populations. (A) Aliquots from sucrose fraction 12 (Fig. 1) were resolved on an agarose gel, with (lane 2) or without (lane 1) PK pretreatment, and detected by Southern blotting using an HBV DNA probe. (B) DNA from the distinct NC band (lanes 5 and 8) (c in panel A), the smear between 3-kb DNA and the NC band (lanes 4 and 7) (b in panel A), and the putative RC DNA released from NCs by PK (lanes 3 and 6) (a in panel A) were recovered from a 1% LMP agarose gel and then resolved on another native agarose gel, along with the DNA released from the same sucrose fraction by digestion with SDS (0.5%) and PK (0.6 mg/ml) (lane 2). Note that DNA recovery following gel extraction was not necessarily quantitative, and panel B is meant to show, qualitatively, the DNA pattern from the different gel positions shown in panel A. (C) The viral DNA extracted from cytoplasmic NCs (lanes 1 and 2) or gel piece "a" in panel A (lanes 3 and 4) was detected by Southern blotting with (lanes 2 and 4) or without (lanes 1 and 3) prior digestion by EcoRI, which cleaves the viral DNA once and converts RC DNA to the linear form. M, DNA molecular weight marker.

the top fractions that contained little to no viral or cellular nucleic acid (Fig. 1A to C and D, lanes 3 and 5). As the bottom fractions containing the mature NCs were well separated from empty capsids (and thus less contaminated by them) and contained only picogram amounts (per lane loaded) of viral DNA or pgRNA, it was no surprise that the capsid proteins in these fractions were undetectable by the anti-HBc antibody used, which has a lower limit of detection of ca. 1 ng (data not shown). Interestingly, like the mature NCs, a major portion of empty capsids was also sensitive to PK digestion, suggesting that the empty capsids and mature NCs may share a property that rendered them protease sensitive. On the other hand, a slight upshift of the NC band was observed following PK digestion with empty capsids (Fig. 1D), pgRNA-containing NCs (Fig. 1C) (as we reported previously [35]), SS DNA-containing NCs (Fig. 1B; see also Fig. 4), as well as recombinant HBV capsids purified from bacteria (data not shown).

Mature NCs migrated heterogeneously and slower than immature NCs on native agarose gels. We noted that in the bottom fractions of the sucrose gradient that contained mostly mature NCs (fractions 12 and 13) (Fig. 1B), some viral DNA, presumably associated with NCs (see below), migrated as a smear (denoted by the "?") and slower than the fairly distinct NC band, which comigrated with empty capsids (Fig. 1D) and recombinant HBV capsids purified from bacteria (17). The following results suggested to us that mature NCs might migrate as the smear but that immature NCs might migrate as the faster-migrating distinct band. First, those fractions that contained the smear contained mostly mature RC DNA (fractions 12 and 13) (Fig. 1A). Second, the fraction containing only the SS DNA (fraction 10) did not show the smear (Fig. 1B, lane 7). Third, no RNA was detected in the smear (Fig. 1C). Fourth, the mobility of the smear but not that of the NC band was significantly affected by PK digestion (Fig. 1B). To identify the DNA species in the NCs with different mobilities, DNA was recovered from separate gel slices containing the NC band and the smear and was detected by Southern blotting. Indeed, RC DNA was detected exclusively in the smear (Fig. 2Ab and B, lane 4) and not in the NC band, which instead contained SS DNA and immature DS DNA (Fig. 2Ac and B, lane 5). Thus, mature NCs did display altered mobility on native agarose gels relative to immature NCs as another indication of structural alterations accompanying NC maturation. We also observed that a portion of mature NCs migrating as the smear remained resistant to PK (Fig. 2A, lane 2, and B, lane 7), suggesting that the smear observed contained two populations of mature NCs: one (mature population 1 [M1]) resistant to PK that did not release their RC DNA content following PK digestion and the other (M2) sensitive to PK that did release their RC DNA after PK digestion. Sometimes, PK-resistant M1 migrated as a somewhat faster smear following PK digestion (Fig. 1B; see also Fig. 5B), but this was not always observed (e.g., Fig. 2A and 3A), possibly depending on the exact gel electrophoresis conditions.

Mature RC DNA-containing NCs formed *in vitro* under cell-free conditions also display altered migration on agarose gels and protease sensitivity. The above-described results suggested that NC maturation in the cells was associated with structural changes that could be detected by their altered mobility on agarose gels and increased sensitivity to proteolysis. To ascertain further if these properties were indeed induced by NC maturation, rather than resulting from the manipulations used to purify the NCs, we decided to mature the purified NCs *in vitro* by using classical EPR

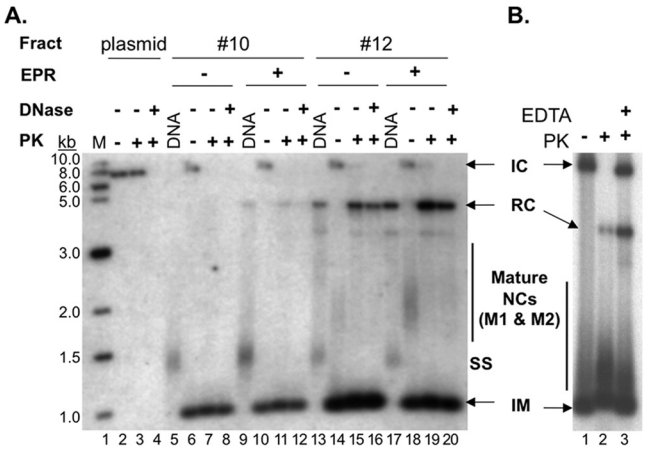

FIG 3 Generation of slow-migrating and unstable mature NCs *in vitro* by EPR. (A) HBV NCs from sucrose fractions 10 and 12 (Fig. 1) were resolved on a native agarose gel and detected by Southern blotting with (lanes 10 to 12 and 18 to 20) or without (lanes 6 to 8 and 14 to 16) prior EPR followed by mock digestion (lanes 6, 10, 14, and 18), PK digestion (lanes 7, 11, 15, and 19), or PK and DNase I digestion (lanes 8, 12, 16, and 20). DNA purified from NCs from sucrose fraction 10 (lanes 5 and 9) or sucrose fraction 12 (lanes 13 and 17) by SDS-PK digestion and phenol-chloroform extraction, with (lanes 9 and 17) or without (lanes 5 and 13) prior EPR, was loaded as DNA controls. An HBV DNA-containing plasmid was treated with PK (lane 3) or PK plus DNase I (lane 4) or was untreated (lane 2) and detected in parallel. Lane 1 shows the DS DNA marker (M). This gel was run longer (than the one in Fig. 1B) to separate the SS DNA from the immature NCs. (B) HBV NCs from sucrose fraction 12 (Fig. 1) were treated with PK (lane 2) or with PK in the presence of 10 mM EDTA (lane 3) or were untreated (lane 1). Viral DNA was detected by Southern blotting as described above for panel A. IC, internal control (HBV DNA-containing plasmid); IM, immature NC; M1, PK-resistant mature NC; M2, PK-sensitive mature NC.

(3, 36, 37), whereby the packaged RT protein carries out viral DNA synthesis using the (endogenous) viral RNA and DNA templates within the NCs. Both fraction 10 (containing immature NCs) and fraction 12 (containing mostly mature NCs) were used for EPR to allow immature NCs to mature and mature NCs to further elongate the second (plus) strand of DNA *in vitro*. The anticipated DNA synthesis during EPR was verified by purifying the NC DNA, followed by Southern blot detection. Thus, readily detectable RC DNA was produced by immature NCs from fraction 10 *in vitro* after EPR (i.e., mature NCs formed from immature ones) (Fig. 3A, lane 9 versus lane 5), and the RC DNA level was further increased in fraction 12 containing both mature and immature NCs (lane 17 versus lane 13). Furthermore, when the NCs were resolved on the native agarose gel, it was apparent that a stronger smear, representing mature NCs, was detected in fraction 12 following EPR, indicating that the mature NCs formed *in vitro* also underwent structural changes similar to those formed in cells, resulting in their altered mobility (Fig. 3A, lane 18 versus lane 14). A smear representing mature NCs migrating above the distinct NC band also emerged in the immature NC fraction (fraction 10) following EPR (Fig. 3A, lane 10 versus lane 6, and data not shown), although it was much weaker than that detected in the mature NC fraction, indicating that mature NCs formed from immature NCs in this fraction also displayed altered mobility on the agarose gel.

Moreover, mature NCs formed *in vitro* by EPR also acquired protease sensitivity, just like those formed in cells. Thus, the small amounts of RC DNA synthesized in fraction 10 by EPR were re-

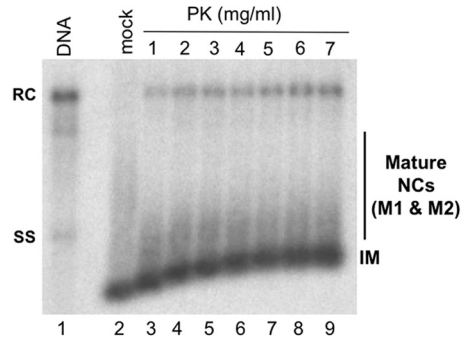

FIG 4 Digestion of HBV NCs with increasing concentrations of PK. HBV NCs from sucrose fraction 12 (Fig. 1) were treated with the indicated concentrations of PK (lanes 3 to 9) or mock treated (lane 2), resolved on a native agarose gel, and detected by Southern blotting as described in the legend of Fig. 1B. The samples shown in lanes 2 to 9 contained the same buffer components as those in the highest concentration (7 mg/ml) (lane 9) of PK digestion (3.3 mM Tris-HCl [pH 7.5], 17% glycerol). EDTA (10 mM) was also included in all digestion mixtures to block the apparent endogenous nuclease activity induced by PK treatment and preserve all viral DNA released from the NCs by PK (Fig. 3). In lane 1, the same amount of NCs as those used in lanes 2 to 9 (5 μl of sucrose fraction 12) was treated with PK in the presence of SDS (0.5% SDS and 0.6 mg/ml PK) to release all the DNA species, which were resolved directly on the gel without phenol extraction and ethanol precipitation and served as the quantitative standard for the amounts of RC DNA released by PK from the same NCs.

leased by PK digestion (Fig. 3A, lanes 9 and 11), and more RC DNA was released from fraction 12 following EPR due to increased amounts of mature NCs formed *in vitro* that were also PK sensitive (Fig. 3A, lane 19 versus lane 15). Also, in Fig. 3A, the SS DNA was separated better from the NC band, which showed more clearly that no SS DNA was ever released by PK digestion, as suggested initially by the results shown in Fig. 1B. Surprisingly, the "released" RC DNA was still partially resistant to DNase digestion (Fig. 3A, lanes 12, 16, and 20). Incidentally, PK digestion seemed to activate some contaminating nuclease in the sucrose fractions, which degraded the internal control plasmid DNA, but again, the "released" viral RC DNA persisted (Fig. 3A, lanes 7, 11, 15, and 19). Control reactions with the plasmid DNA alone verified that the PK itself was not contaminated with DNase activity (Fig. 3A, lane 3), and the PK-plus-DNase I procedure was able to efficiently eliminate the plasmid (Fig. 3A, lane 4). Also, the plasmid DNA control as well as some of the released viral RC DNA were rescued from the PK treatment when EDTA (which inactivates DNase) was included during the PK digestion (Fig. 3B), thus confirming the presence of endogenous DNase activity in the sucrose fractions that was activated by PK treatment. As discussed further below, these results suggested that the RC DNA in the mature NCs was still partially protected by the residual NC structure even after PK digestion.

To measure the efficiency of RC DNA release by PK digestion, we added EDTA during PK digestion to prevent the degradation of the released viral RC DNA from endogenous nuclease, and we increased the PK concentrations from 1 to 7 mg/ml. The total amounts of RC DNA, and other DNA species, within NCs were detected following PK digestion in the presence of SDS, which disrupts all NCs. As shown in Fig. 4, even at 7 mg/ml, PK released only approximately 50% of the RC DNA (lane 9 versus lane 1). Also, RC DNA release did not increase appreciably further above 3 mg/ml PK. Also consistent with the survival of some mature NCs following digestion with high concentrations of PK, some smear signal representing mature NCs above the immature NC band remained. The band of immature NCs containing the SS DNA (Fig. 2) or pgRNA (Fig. 1C) was not disrupted by PK concentrations as high as 7 mg/ml, except for the fact that its upward mobility shift was more pronounced at higher PK concentrations (Fig. 4), consistent with what we reported previously (35). The upward mobility shift was not due to alterations of buffer/salt conditions in the digested samples, as the PK stock was diluted (3-fold in Fig. 4 or 20-fold in all other experiments) into the digestion reaction mixture, and there was minimal change in the buffer/salt conditions with or without PK digestion. Furthermore, all digestion mixtures, including the mock digestion in Fig. 4, contained the same buffer components, and the mobility upshift was clearly related to the concentrations of PK (Fig. 4), as we showed previously (35).

A population of intracellular mature NCs was sensitive to exogenous nuclease. The fact that some mature NCs were sensitive to PK digestion suggested that some NCs might even be sensitive to the MNase treatment commonly used during NC isolation (to remove nucleic acids not packaged within NCs) and thus were eliminated by MNase and escaped our detection so far. To detect these putative, nuclease-sensitive NCs, an NC-containing cytoplasmic lysate was directly subjected to linear sucrose gradient centrifugation without the MNase treatment step. A new smear, which was absent from the previous gradient when the lysate was treated with MNase before gradient centrifugation (Fig. 1B), migrating even slower than RC DNA on the agarose gel, appeared in the top fractions of the gradient (Fig. 5B, lanes 1, 3, 5, and 7). PK treatment of these top fractions caused the smear to migrate as a distinct band comigrating with RC DNA (Fig. 5B), indicating again that these PK-digested mature NCs released their RC DNA during electrophoresis. That these slow-migrating smears in the top fractions contained RC DNA was also verified by resolving them on an SDS-containing agarose gel, which revealed RC DNA in these fractions in the absence of PK treatment (Fig. 5A). Furthermore, when the DNA content in the slow-migrating smear present in fraction 6 was extracted from the gel piece, it was confirmed to be RC DNA (Fig. 5C). These results thus confirmed our supposition that a portion of mature NCs (dubbed M3), with the slowest sedimentation on sucrose gradients and the slowest mobility on native agarose gels, was indeed present intracellularly and was removed by the nuclease treatment used to prepare the lysate.

To verify that RC DNA found in the top gradient fractions was in fact still associated with some sort of capsid structure, we performed immunoprecipitation using two different antibodies against HBc. The precipitated HBc/capsid, if any, was digested with SDS and PK to release its DNA content, which was then detected by Southern blotting. We found that both a mouse MAb and a rabbit PAb against HBc were able to precipitate immature and mature NCs, as evidenced by the presence of SS and RC DNAs in the precipitates when a fraction (fraction 12) containing both NC species was used for IP (Fig. 6, lanes 4 and 6). When a top sucrose fraction (fraction 6) harboring only the RC DNA-containing, slow-migrating smear was used for IP, both HBc antibodies were able to precipitate RC DNA (Fig. 6, lanes 10 and 12). These results thus confirmed the presence of the HBc protein, and likely some capsid structure, that was associated with RC DNA in the M3 NCs.

The nuclease sensitivity of the M3 NCs in the gradient fractions

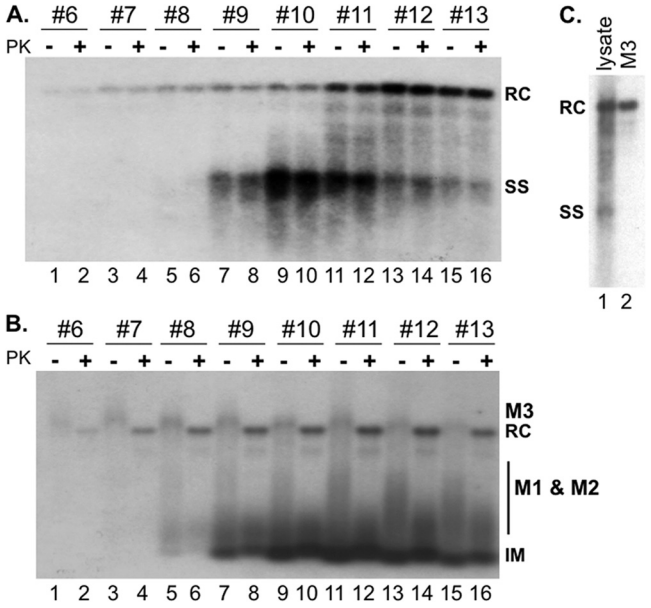

FIG 5 Isolation of HBV NCs by sucrose gradient centrifugation from cell lysates without MNase treatment. The preparation of the HepAD38 cell lysate and sucrose gradient centrifugation were carried out as described in the legend of Fig. 1, except that the lysate was not first treated with MNase. (A and B) Aliquots from sucrose fractions 6 to 13 were treated with (+) or without (−) PK, followed by resolution on an agarose gel with (A) or without (B) 0.1% SDS and detection by Southern blotting. (C) Viral DNA purified from the cytoplasmic lysate (lane 1) or gel extracted (lane 2) from the M3 NC spot in fraction 6 (lane 1 in panel B) was detected by Southern blotting. M1, PK-resistant mature NC; M2, PK-sensitive mature NC; M3, nuclease-sensitive mature NC.

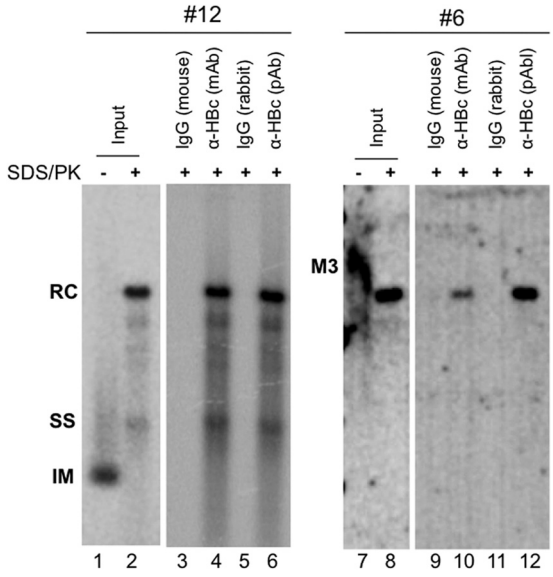

FIG 6 Immunoprecipitation of NC-associated HBV DNA. Sucrose gradient fractions 6 and 12 from Fig. 5 were immunoprecipitated with either anti-HBc MAb (lanes 4 and 10) or PAb (lanes 6 and 12) or control antibodies (lanes 3, 5, 9, and 11). The precipitates were incubated with 0.5% SDS and 0.6 mg/ml PK to release the NC-associated DNA, which was then resolved on a native agarose gel and detected by Southern blotting. NCs from the input sucrose fractions, with (lanes 2 and 8) or without (lanes 1 and 7) prior treatment with SDS and PK, were also resolved in parallel to visualize the NCs or DNA species released from the NCs.

was further confirmed when they were first treated with MNase or DNase I before the SDS and PK treatment to release their DNA content, which was subsequently resolved on an agarose gel. Southern blot analysis showed that the RC DNA in fractions 6 and 9 was completely eliminated by the nuclease treatment (Fig. 7A, lanes 2, 3, 5, and 6). Furthermore, the nucleases preferentially degraded RC DNA but not SS DNA in fraction 9 (Fig. 7A, lanes 5 and 6). A slight decrease of the RC DNA level was also noticeable in fraction 12 (Fig. 7A, lanes 8 and 9), suggesting that some nuclease-sensitive (M3-like) NCs were also present in this fraction, consistent with the presence of a smear migrating above RC DNA on the native agarose gel even in this bottom fraction (Fig. 5B), although it contained mostly M1/M2 NCs that were resistant to nuclease but sensitive to protease (Fig. 1 to 4). Thus, some heterogeneity existed in the nuclease-sensitive M3 NCs in their sedimentation on the sucrose gradient, spreading throughout the gradient (fractions 6 to 13) (Fig. 5B and 7). Possibly, some heterogeneity in their migration on the agarose gel might also exist, in that some M3 NC smears clearly migrated slower than or comigrated with the RC DNA band (fractions 6 to 13) (Fig. 5B), and others might comigrate with the M1/M2 NCs (fractions 8 to 10 and potentially 11 to 13) (compare Fig. 5B with 1B).

To confirm the presence of nuclease-sensitive and PK-sensitive mature NCs in the unfractionated cytoplasmic lysate, we treated the lysate directly with PK or MNase. The MNase-treated lysate was further incubated with SDS and PK to release the viral DNA from NCs, which was resolved on a native agarose gel and detected by Southern blotting (Fig. 7B, lanes 1 and 2). The PK-treated lysate was also resolved on a native agarose gel to detect any RC DNA released (Fig. 7B, lanes 3 and 4). Indeed, approximately one-third (average, 35%; standard deviation, 11%) of the RC DNA (but no SS DNA) in the lysate was reproducibly eliminated by MNase, verifying the presence of nuclease-sensitive mature NCs. Also, some RC DNA was released from mature NCs during gel electro-

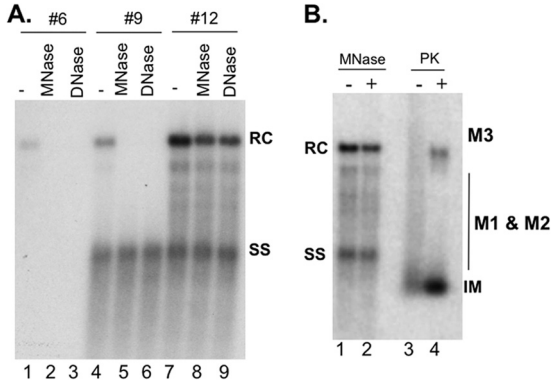

FIG 7 Nuclease and PK digestion of fractionated and unfractionated NCs. (A) Sucrose gradient fractions 6, 9, and 12 prepared from lysates without prior MNase treatment (Fig. 5) were digested with MNase or DNase I as described in Materials and Methods. The reaction mixtures were incubated with 0.5% SDS and 0.6 mg/ml PK to release the NC-associated DNA, which was then resolved on a native agarose gel and detected by Southern blotting. (B) Cytoplasmic lysate prepared from induced HepAD38 cells was treated with MNase (lane 2) or PK (lane 4) or was mock treated (lanes 1 and 3). Samples loaded onto lanes 1 and 2 were subsequently treated with 0.5% SDS and 0.6 mg/ml PK. All samples were then resolved on a native agarose gel and subjected to Southern blotting to detect HBV DNA.

phoresis following PK digestion of the lysate, confirming the presence of PK-sensitive mature NCs in the lysate. These results thus indicated that nuclease and protease sensitivities of a portion of intracellular mature NCs were intrinsic properties of these mature NCs and did not result from the manipulations (e.g., ultracentrifugation) used to isolate the NCs. It was noticeable that with the crude cell lysate (as opposed to the sucrose gradient fractions), there was more of a smeary signal throughout the lane (Fig. 7B, lane 3). It was possible that under these conditions, some immature NCs might have slower and heterogeneous mobility, possibly due to the presence of cellular components in the crude lysate that were removed by the gradient fractionation. Following PK digestion, which presumably eliminated the putative cellular components, the immature NCs migrated as a more distinct and fast-running band that appeared stronger in signal (Fig. 7B, lane 4).

RC DNA in all mature NCs remained protein linked. As studies using DHBV NCs suggested that elongation of the plus strand in RC DNA (induced by EPR) may result in RC DNA deproteination (i.e., removal of the RT protein from RC DNA), which in turn triggers NC uncoating (33), we were interested in determining whether the RC DNA associated with the destabilized HBV NCs was linked to protein (RT) or not. To this end, HBV NCs were harvested from the sucrose gradient prepared from the cell lysate that was not pretreated with MNase (so as to retain all mature NCs, including the nuclease-sensitive M3 species) as well as from the unfractionated (and not nuclease-treated) cell lysate itself and were further subjected to EPR to elongate the DNA strands within the NCs. NC-associated DNA was subsequently purified by using phenol-chloroform extraction with or without prior PK digestion to isolate total NC DNA or protein-free (PF) (i.e., with no linked RT protein) (21, 22) DNA, respectively. No DNA was extracted without prior PK digestion from any of the NCs, even after EPR, indicating that the RC DNA associated with the destabilized mature HBV NCs remained covalently attached to the RT protein, and further elongation of the plus strand of RC DNA did not result in RC DNA deproteination either (Fig. 8).

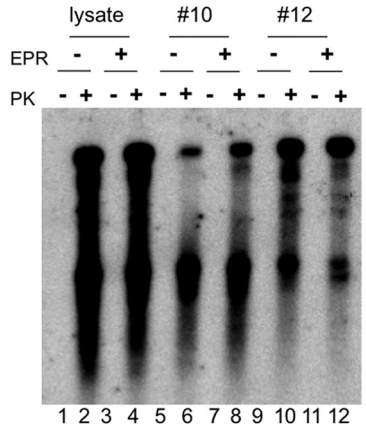

FIG 8 Detection of protein-linked but not PF HBV DNA from NCs. HBV DNA was extracted by phenol-chloroform extraction from the induced HepAD38 cell lysate or sucrose gradient fractions prepared from the lysate without MNase pretreatment. To extract PF DNA (from which the covalently linked RT protein was removed) in the NCs, NCs were disrupted with SDS (but without PK digestion) before phenol extraction (odd-numbered lanes). To extract both protein-linked and PF DNA, the lysate or fractions were treated with SDS and PK before phenol extraction (even-numbered lanes). EPR was also performed with the lysate or the fractions before DNA extraction for the samples shown in lanes 3, 4, 7, 8, 11, and 12. All samples were then resolved on native agarose gels, and viral DNA was detected by Southern blotting.

DISCUSSION

We have found here that HBV NC maturation is associated with significant structural changes that could be detected by differences in NC sedimentation on sucrose gradients, mobility on native agarose gels, and, in particular, sensitivity to protease and nuclease digestion (summarized in Fig. 9). Whereas the exact structural basis for these observed differences remains to be elucidated, some suggestions can be made here. As sucrose gradient centrifugation separates macromolecules/particles based on their size and density, it might be predicted that mature NCs, with their mature plus-strand DNA contributing to their mass and density, will sediment faster than immature ones. This was indeed observed with DHBV mature NCs previously (23) and most mature HBV NCs here (the M1 and M2 subpopulations). The fact that a population of mature HBV NCs (M3) revealed here sedimented slower, instead of faster, than immature NCs is consistent with rather dramatic structural changes of this population, which is also suggested by their dramatically slowed mobility on the agarose gel and their failure to protect their RC DNA content. On the other hand, a major determinant of macromolecular migration on native agarose gels is the surface charge. We have found previously that immature NCs of duck hepatitis B virus (DHBV) were heavily phosphorylated at the DHBc CTD, whereas mature NCs were dephosphorylated (23, 28). If at least some copies of CTDs are exposed on the NC surface, their dephosphorylation on mature NCs would indeed render them more positively charged than the (phosphorylated) immature NCs and could thus be, at least in part, responsible for the slower mobility of all the mature HBV NCs found here (M1, M2, and M3) on agarose gels. However, the topology of the CTDs during NC maturation remains to be clearly defined, with some, but not all, reports suggesting that they are exposed preferentially on mature NCs (29, 31–33, 42–44).

The release of RC DNA, but not SS DNA, from some mature NCs (M2 and M3) during agarose gel electrophoresis after PK treatment indicates that HBV NC maturation is associated with a gain of PK sensitivity and decreased stability. One unifying explanation that could account for the protease sensitivity, as well as the decreased mobility on agarose gels and sedimentation on sucrose gradients, of mature NCs may be some sort of structural weakening and loosening of the capsid shell such that protease cleavage sites become more accessible from the exterior of the capsid, the particles become less dense, and more positive charges (e.g., from the dephosphorylated CTDs) are exposed on their surface. On the other hand, the RC DNA remained largely protected from nuclease digestion even after PK treatment, suggesting that PK did not completely disrupt the integrity of these mature NCs but presumably caused some defects in the NC shell to allow their RC DNA content to escape during electrophoresis (but not to allow exogenous nuclease to enter the NCs). Thus, PK-treated mature NCs appear to behave like parvoviruses that can release their DNA without complete capsid disassembly (45, 46). We also acknowledge that it is difficult at present to exclude completely the possibility that the differential stability of the different NC populations might have been induced by the isolation procedure, admittedly a potential concern with any biochemical experiments involving isolation of materials from cells. However, as the same procedures

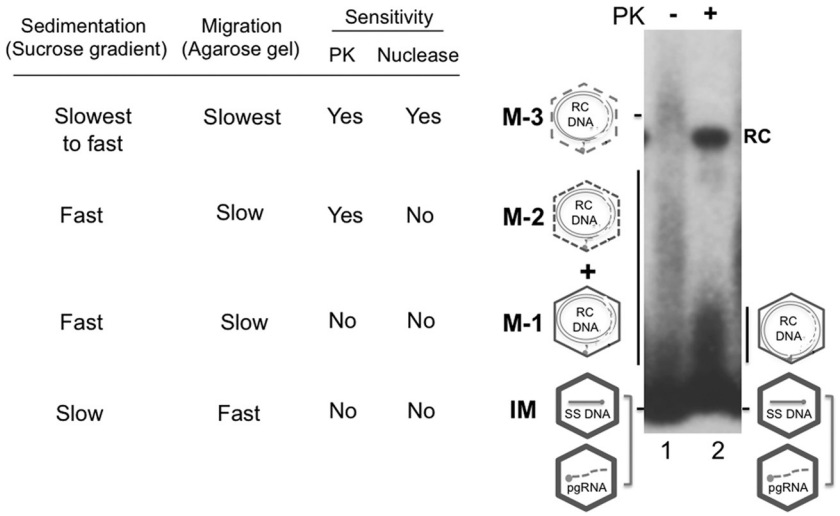

FIG 9 Summary of the diverse populations of HBV NCs. The sedimentation profile during sucrose gradient ultracentrifugation, migration on native agarose gels, and sensitivity to PK and nuclease digestion of the different HBV NCs are summarized. Shown at the left is a representative Southern blot image displaying the various NC populations on a native agarose gel, with (lane 2) or without (lane 1) prior PK digestion. The thickness and the broken versus solid lines of the capsid shell in the cartoons denote the decreasing stability from the immature NCs to the M3 NCs. IM, immature NC (containing either SS DNA or pgRNA).

(including the relatively mild and fast one-step sucrose gradient centrifugation) were used to isolate all the NCs, we consider it likely that the observed differences do reflect differences in the intrinsic properties of the different NC populations in cells, which is further supported by the fact that M2 or M2-like mature NCs could be generated *de novo* by using isolated NCs under cell-free conditions through EPR *in vitro*.

How might maturation cause NC destabilization and the putative structural alterations detected here? It is clear that NC maturation *in vitro* (mainly the elongation of the plus strand of RC DNA) was sufficient to generate PK-sensitive mature NCs that also migrated slowly and heterogeneously on agarose gels, suggesting that these are intrinsic properties of mature NCs that can be acquired with few or no extrinsic factors. Recently, it was reported that HBc can bind SS DNA or RNA with high affinity and readily forms NCs *in vitro*, packaging (albeit nonspecifically) these nucleic acid species, whereas DS DNA is poorly assembled together with HBc (47). It is also well accepted that RNA packaged in immature NCs stabilizes the capsids (48, 49). Thus, the change in the nucleic acid content as NCs mature (from pgRNA or SS DNA to DS DNA) will result in a significant decrease in the interaction between the capsids and their nucleic acid content, which could at least partially account for the maturation-associated NC destabilization. The dynamics of CTD phosphorylation during NC maturation (23, 27, 28, 39, 41), which modulates HBc nucleic acid binding activities (44, 50, 51), may also modulate NC stability. Indeed, mature, but not immature, NCs of DHBV are selectively destabilized by phosphomimetic (S/T-to-D or -E) substitutions at the DHBc CTD (28, 52). In addition, the irregular complexes observed to form *in vitro* between HBc and DS DNA (47) are consistent with the slow and smeary migration on gels and slow sedimentation on sucrose gradients of a subpopulation of mature NCs (M3). Furthermore, we note that the genome content inside viral capsids has been implicated in dictating capsid surface properties in a number of other viruses, e.g., the externalization of an internally located domain of a parvovirus capsid protein induced by DNA packaging (53, 54) and the selective nuclear export of DNA-containing (but not empty) capsids in herpes simplex virus (55).

The maturation-associated NC structural changes that we have uncovered here have obvious implications for disassembly or uncoating that occurs selectively with mature NCs. Uncoating is an essential process during viral infections but remains ill understood for most viruses, particularly for HBV. It is thought that the HBV NCs (with a diameter of 32 to 36 nm) are too large to traverse the nuclear pore and have to undergo disassembly on the cytoplasmic side in order to release the RC DNA into the nucleus during both *de novo* infection and intracellular amplification of CCC DNA (21, 22, 29, 33, 56, 57). On the other hand, premature destabilization of the immature NCs may be detrimental to viral DNA synthesis and may preclude NC maturation. Thus, the selective destabilization of mature NCs that we detected here is most likely linked to NC uncoating mechanistically. It is also consistent with previous reports showing that mutations in both the N-terminal domain and CTD of HBc and DHBc selectively destabilize mature NCs (28, 52, 58–60). Interestingly, retroviral DNA synthesis is also associated with destabilization of the viral capsids to initiate uncoating (61), and mature adenovirus capsids become less stable than immature ones (62), suggesting that destabilization of mature capsids may be a common theme in virus uncoating.

The maturation-associated NC structural differences detected here might also be relevant for the selective secretion of mature NCs as enveloped virions (3, 13–15, 17). On the one hand, as HBV virions can contain partially DS DNA with relatively long SS gaps, which migrated as a smear below the RC DNA band (e.g., see reference 17), it is possible that the NC structural changes that we have detected here may not be absolutely required for NC envelopment and virion secretion. On the other hand, it is possible that RC DNA-containing NCs (i.e., more or fully mature), with the structural changes detected here, may have an enhanced envelopment efficiency compared to that of NCs containing longer SS gaps (i.e., less mature). This was shown to be the case for DHBV

(15; our unpublished data) but is not yet known definitively for HBV. Furthermore, the finding that some empty capsids were also protease sensitive, like some mature NCs, and were competent for both envelopment and secretion suggests that the signal directing the envelopment of empty capsids and mature NCs may also be related in certain aspects. In particular, the lack of any RNA (or DNA) in empty capsids may result in their instability, just as the generation of DS DNA in mature NCs weakens the capsid-genome interaction and triggers NC destabilization. Alternatively, the tight association of the capsids with the SS DNA or pgRNA in immature NCs may trigger a structural change (reflected by protease resistance and fast migration and tight banding on agarose gels) corresponding to the putative single-strand retention signal (17), blocking their envelopment.

The relationship between the different mature NCs (M1 to M3) (Fig. 9) remains to be defined. There may be a continuum of protease sensitivity between the M1 and M2 NCs, and even harsher proteolysis than that attempted here may also disrupt M1. The nuclease-sensitive mature NCs (i.e., M3), which also displayed heterogeneity in their sedimentation on the sucrose gradient and possibly migration on the agarose gel, may represent the most extensively "uncoated" mature NCs detectable here that are ready to release RC DNA for nuclear entry and CCC DNA formation, with those sedimenting and migrating the most slowly being the most uncoated. Protease digestion *in vitro* rendered some, but not all, RC DNA-containing NCs unable to protect their DNA content from nuclease digestion. Perhaps the efficient generation of the M3 NC species requires some factors missing in the *in vitro* system. One or more populations of mature NCs detected here may also represent "dead-end" species unable to undergo either uncoating or envelopment. In addition, it is not yet clear whether the escape of RC DNA from mature NCs following protease digestion *in vitro* (M2 and M3) represents a bona fide step during uncoating *in vivo* or if proteolysis is indeed needed for uncoating *in vivo*. Some studies have suggested that DHBV RC DNA deproteination (i.e., removal of the RT protein) may be required to trigger uncoating (33). However, our results here and another recent report (57) show that destabilized mature HBV NCs, even those failing to protect their RC DNA, contain only protein-linked DNA, suggesting that RC DNA deproteination likely occurs after rather than before (at least partial) uncoating in HBV and thus may not be a prerequisite for uncoating. Whether this discrepancy represents an intrinsic difference between HBV and DHBV or is simply a reflection of the experimental differences remains to be resolved. Further detailed characterization of the mature and immature NC structures, including the use of higher-resolution methods such as cryo-electron microscopy and tomography (44, 62), should reveal more details of the NC structural dynamics during maturation that are critical for regulating viral reverse transcription, NC uncoating, and NC envelopment and virion secretion.

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