Introduction

Chronic infection with hepatitis B virus (HBV) can lead to liver failure and cirrhosis; it is also the leading cause of hepatocellular carcinoma. Approximately 350 million individuals suffer from chronic HBV worldwide, and HBV contributes to 600,000 deaths per year [1]. It is a major public health issue and also a great social concern due to the discrimination against those infected with HBV in the endemic regions [2,3].

HBV is an enveloped double-stranded (DS) DNA virus with an RNA intermediate form. In an infected cell, virion formation is initiated in the cytosol by the binding of a copy of the 3.2 kb “pregenomic” RNA (pgRNA) to the viral reverse transcriptase (RT) and packaging of the pgRNA•RT complex by multiple copies (180 or 240) of the viral capsid protein (Cp183) to form an immature core. Subsequently, the encapsidated single-stranded (SS) pgRNA is reverse transcribed to a full-length minus-stranded DNA that is covalently attached to the priming domain of RT; simultaneously, the RNA template is digested by the RNase H domain of the RT. A complementary, incomplete plus-strand DNA is then transcribed to generate rcDNA [4,5]. These mature cores can then interact with viral surface proteins for envelopment. By studying the lifecycle of HBV we can identify new targets for development of antivirals as well as gain understanding of the function and behavior of these very specialized molecular machines.

The basic building block of the HBV capsid is the core protein homodimer. Most HBV cores are composed of 120 dimers arranged with T = 4 icosahedral symmetry; about 5% are 90-dimer T = 3 icosahedra [6–8]. The core protein has two domains: the N-terminal assembly domain (residues 1–149, which can be expressed as self-assembling Cp149) and the positively charged carboxy-terminal protamine-like domain (residues 150–183, CTD). The assembly domain forms the protein shell of the capsid [8–11]. The CTD is dispensable for capsid assembly but required for packaging RNA [10,12–14], which it binds with high affinity [15]. Structures of empty T = 4, CTD-truncated Cp149 capsid have been solved to high resolution by cryo-EM and X-ray crystallography [9,16,17]. The Cp149 dimer has an overall shape of an inverted capital “T” [8,9,18]. The stem of the “T” is the four-helix bundle dimerization motif, which protrudes outward from the capsid surface. The crossbar of the “T” clusters in a groups of five or six to form the contiguous capsid surface [8,16]. Numerous pores perforating the capsid surface (located at the twofold [i.e. quasi-sixfold], threefold, and quasi-threefold axes) are proposed to allow nucleotides to diffuse in and out of the capsid during reverse transcription [9]. The CTD, localized to fivefold and quasi-sixfold vertices, extends into the capsid interior [18–20].

The capsid affects genome replication through its arginine-rich CTD. Phosphorylation of the CTD is important for RNA packaging and DNA synthesis [13,21,22]. The CTD has three SPRRRR motifs (S155, S162, and S170) identified as the phosphorylation sites critical for pgRNA packaging [23,24]. Mutation of these serines to alanine, to mimic the phosphorylated capsid, suppresses pgRNA encapsidation [22,25–27]. Replacing these serines with aspartate or glutamate to mimic phosphoserine supports pgRNA encapsidation but differentially affects transcription, suggesting that each repeat has an indepen-
Author Summary

Many single stranded RNA virus encapsidate their genome through positively-charged domains of their capsid proteins. Hepatitis B virus (HBV) is a double stranded DNA virus which packages a single-stranded RNA progenome (pgRNA) that is reverse transcribed within the capsid. RNA packaging requires a phosphorylated form of the HBV capsid protein’s RNA-binding carboxy-terminal domain (CTD). Although the capsid has been well studied, the internal structures, the CTDs and the packaged RNA, are poorly characterized. By using in vitro reassembly, we have generated empty and pgRNA-filled capsids using phosphorylation-mimic and unphosphorylated forms of the capsid protein. Using cryo-EM image reconstruction, we have been able to show the structure of encapsidated pgRNA and, independently, the CTD in the absence of RNA to visualize early stages of the HBV assembly. We showed that the structural organization of the CTD changes as a function of the phosphorylation. Changes in CTD structure affect the structure of the encapsidated pgRNA, changing it from thin segments of density in the phosphorylated state, suggestive of single-stranded RNA, to thick rope-like structures consistent with duplex nucleic acid in the unphosphorylated state.

Results

Electron cryo-micrographs of empty particles and pgRNA-filled particles

Purified HBV core protein dimers were reassembled into empty capsids (Cp183e-SSS and Cp183e-EEE) and pgRNA-filled capsids (Cp183RNA-SSS and Cp183RNA-EEE) [15]. RNA-filled capsids were dialyzed into buffered 150 mM NaCl while empty capsids were dialyzed into higher salt, 250 mM NaCl, to ensure stability [15]. Cryo-electron micrographs of Cp183e-SSS, Cp183RNA-SSS, Cp183e-EEE, and Cp183RNA-EEE (Figure 1) showed that all four constructs have similar morphology. pgRNA-filled particles noticeably had an inner layer of density characteristic of RNA-filled capsids [8,19,35]. To enhance the signal from the low contrast cryo-images, we translationally aligned particles to generate averaged images (Figure 1, insets). The empty Cp183e-SSS and Cp183e-EEE averages showed a single ring of density, 34 nm in diameter, indicating that they are both hollow spheres (Figure 1A and B). The averages of pgRNA-filled capsids (Figure 1C and D) showed an additional ring, presumably the encapsidated pgRNA; Cp183RNA-EEE appears to have much stronger RNA density than the Cp183RNA-SSS.

Cryo-EM three-dimensional reconstructions

To examine the details of pgRNA structure and the interaction between the capsid and pgRNA, we calculated image reconstructions of $T = 4$ particles to sub-nanometer resolution (Table 1). External views showed that all four types of particle (Cp183e-SSS, Cp183e-EEE, Cp183RNA-SSS, and Cp183RNA-EEE) were very similar (Figure 2A–D); nevertheless, from the central section it appeared that the spikes of the pgRNA-filled capsids adopted a slightly different quaternary structure (Figure 2E–F). As in previously published HBV structures [9,16,18,34,35], the capsids had pores at each twofold, threefold and quasi-threefold axis (Figure 2). Empty capsids, both Cp183e-SSS and Cp183e-EEE, appeared to have extra density partially occluding their twofold

Figure 1. Cryo-micrographs of frozen-hydrated HBV capsids. (A) Cp183e-SSS (e for empty), (B) Cp183e-EEE, (C) Cp183RNA-SSS, and (D) Cp183RNA-EEE particles are shown, frozen hydrated in vitreous ice. These particles show the typical morphology of HBV capsids with characteristic spikes. These samples all have a minor population of smaller, $T = 3$ particles (black arrow). Inserts show translationally averaged images. Empty capsids (A, B) show a single ring corresponding to the protein shell; pgRNA-filled capsids (C, D) show two concentric rings, indicating the presence of an layer of nucleic acid. Note that the RNA ring in Cp183RNA-EEE is thicker than in Cp183RNA-SSS.

doi:10.1371/journal.ppat.1002919.g001
pores (i.e. quasi-sixfolds) (Figure 2A, B, E and F, black arrows). Presumably this density was from free CTDs. pgRNA-filled capsids (Figure 2C, D, G and H) and reconstructions of CTD-truncated particles [8–10,16,17] did not display this density. The central sections of the density maps showed short segments of density (Figure 2E–H, white arrows), presumably the CTDs, tethered from the capsid inner surface. In the empty Cp183-SSS and Cp183-EEE particles, this density was located under each dimer. Under the fivefold vertex, in Cp183-EEE the CTD density condenses to form a funnel-like structure; in Cp183-SSS, the equivalent density is weaker and forms distinct extensions.

The differences between Cp183-EEE and Cp183-SSS CTD organization were also seen in pgRNA-filled particles. However, in RNA-filled capsids, density extending from the capsid inner surface submerged into the internal ring of RNA density (Figure 2G and H). In addition to the fivefold capsid-RNA connection, in Cp183RNA-SSS there were thin density elements connecting the extremities of the dimer to the RNA ring (Figure 3A). A different conformation for connecting density was observed at the Cp183RNA-EEE inner surface (Figure 3B).

The critical observation was that the RNA density in Cp183RNA-SSS and Cp183RNA-EEE adopts different ordered conformations. The strength and order of RNA density was observed in spite of the fact that the asymmetric RNA was subjected to icosahedral averaging (Figure 2G and H); if the RNA was not in part icosahedral, we would have expected a uniform shell of density. In Cp183RNA-SSS, RNA density was localized under twofold and fivefold vertices (Figure 2G); in Cp183RNA-EEE, RNA density was clearly inhomogeneous indicating that the pgRNA has adopted a preferred conformation or constellation of conformations evident even though it has been icosahedrally averaged in these reconstructions. White arrows indicate the CTD tails tethered from the capsid inner surface. Oval, triangle, and pentagon indicate locations of twofold, threefold and fivefold axes, respectively.

Table 1. Image reconstruction data.

|                  | Cp183-SSS | Cp183-EEE | Cp183RNA-SSS | Cp183RNA-EEE |
|------------------|-----------|-----------|--------------|--------------|
| Number of particles (in reconstruction/total) | 27489/36676 | 14416/16967 | 7201/10502 | 7439/9196     |
| Number of CCD frames | 594       | 294       | 193          | 394          |
| Nominal magnification | 80,000 x  | 80,000 x  | 40,000 x    | 80,000 x    |
| Pixel size (Å)   | 1.484     | 1.484     | 2.940        | 1.484        |
| Defocus ranges (µm) | 0.16–4.10 | 0.60–3.13 | 0.27–3.80    | 0.54–4.87    |
| Resolution (Å)   | 5.5       | 5.8       | 8.0          | 7.0          |

Resolution was estimated based on a Fourier Shell Correlation of 0.5.

Figure 2. Cryo-EM 3D reconstructions of empty and pgRNA-filled Cp183 capsids. Surface shaded exterior maps of T = 4 (A) Cp183-SSS, (B) Cp183-EEE, (C) Cp183RNA-SSS, (D) Cp183RNA-EEE and their related central sections (E–H). Insets show enlarged views of the twofold (i.e. quasi-sixfold) vertex. All four maps have a similar external appearance with 120 spikes decorating a fenestrated capsid surface; the outer layer extends from a radius of 125 to 170 Å. In (A) Cp183-SSS and (B) Cp183-EEE, a thin layer of electron density partially occludes the central opening pore at the twofold axis (A, B, E, F, black arrows). This density is unique to the empty capsids. The pgRNA-filled capsids (C, D, G, H), on the other hand, lack the density across the twofold pore but display a substantial internal layer of density at the radii between 100–120 Å. In central sections (G, H), this density, corresponding to the co-assembled pgRNA, is clearly inhomogeneous indicating that the pgRNA has adopted a preferred conformation or constellation of conformations evident even though it has been icosahedrally averaged in these reconstructions. White arrows indicate the CTD tails tethered from the capsid inner surface. Oval, triangle, and pentagon indicate locations of twofold, threefold and fivefold axes, respectively.

doi:10.1371/journal.ppat.1002919.g002
Eee, the RNA density appeared as radially arrayed segments (Figure 2H). Radial density maps (Figure 3C and D) revealed similar core protein density distributions in Cp183RNA-SSS and Cp183RNA-EEE (at radii of 167, 153, 144 Å). However, the density shell at 113 Å showed that the RNA of Cp183 RNA-SSS forms an icosahedral cage where fivefold pentamers are connected across twofolds; whereas in Cp183RNA-EEE, the RNA shell displayed a complicated mesh of density with the strongest density surrounding the fivefold vertices and comparatively weaker segments at the twofold vertices (Figure 3D).

A close comparison between Cp183RNA-SSS and Cp183RNA-EEE revealed the respective differences in the interactions between their CTDs and pgRNA (Figure 3A and B). In Cp183RNA-EEE, strong and continuous density originated from the A subunit and projected towards the fivefold axis and into the pgRNA density (Figure 3B). This stalactite-shaped density, also seen in the empty particle (compare Figure 2G to Figure 2H), is evident in the radial density map at radius of 126 Å (Figure 3D). Additional density, located under the CD dimer, connected down to the pgRNA layer at the quasi-threefold location. From the AB dimer, density projected straight into the pgRNA layer (Figure 3A and C); whereas in CD dimer, the density projected inward, obliquely toward the threefold axis and eventually immersed into pgRNA density (Figure 3C).

Organization of the CTD in the HBV capsid

Difference maps (e.g. Cp183-SSS less a molecular model of Cp149) revealed the overall CTD organization of empty HBV capsids in the unphosphorylated and phosphorylation-mimic states (Figure 4). Both maps were rendered at density levels needed to

Figure 3. Interaction between the HBV capsid and pgRNA. A radially color-coded isosurface rendering of AB dimers and related pgRNA of (A) Cp183RNA-SSS and (B) Cp183RNA-EEE viewed from a 90° rotation of the region identified by an arrow in the rightmost inset. The Cp183RNA-EEE forms a massive pentagonal density under the fivefold vertex and correlating with a thicker layer of pgRNA than seen with the Cp183RNA-SSS reconstruction. Panels of radially cued densities of (C) Cp183RNA-SSS and (D) Cp183RNA-EEE, viewed along an icosahedral twofold axis at radii of 167, 153, 144, 126, 113 Å. In these images, the protein is presented in white with the high-to-low densities indicated by the gray scale. The density distribution patterns corresponding to capsid are very similar (three leftmost elements). CTDs are expected to be dominant features through radii of 117 to at 128 Å. At 126 Å the high-density features at CD dimer in Cp183 RNA-SSS are tilted toward to the threefold axis, but the related density in Cp183RNA-EEE remains at the dimer position. Notably, the CTD density correlating with the A subunit is much weaker in the Cp183RNA-SSS, whereas Cp183RNA-EEE shows a strong propeller of density along the fivefold axes. At lower radius, pgRNA density shows distinct distributions. In the Cp183RNA-SSS, the density is strongest along the twofold edge connecting fivefold axis, which forms an icosahedral cage. In the Cp183RNA-EEE the density, while thinner than in Cp183RNA-SSS, forms a more evenly distributed sphere.

doi:10.1371/journal.ppat.1002919.g003
obtain the expected volume of the core protein (we note the signal was \( >1 \sigma \)). The locations of the CTDs within Cp183, -SSS (Figure 4A) and Cp183, -EEE (Figure 4B) shared a similar distribution, unsurprising as they erupt from the same regions of the contiguous capsid. However the organization and strength of the CTD density varied substantially. The CTD of the Cp183, -SSS formed five pillars of density surrounding the fivefold axis (Figure 4D), a density cluster under the quasi-threefold vertices (Figure 4C), and density that partially occluded the twofold opening.

In Cp183, -EEE the fivefold CTD density, extending from each A subunit, formed a large stalactite-like structure extending to lower radius (Figure 4F). This structural characteristic was also observed in the Cp183RNA-EEE, where the fivefold stalactite density impinged on the pgRNA layer (Figure 3B and D at 126 Å). The last visible residue in the atomic model (Protein Data Bank (PDB) entry 1QGT) was close to the CTD density in both difference maps (Figure 5). However, the mutation of just three residues resulted in the shift of the CTDs from a relatively difference maps (Figure 5). The locations of the CTDs in both Cp183 forms are overlaid (C, shows the superimposition side views of A and B), the movement of the peptide and the increased degree of interaction in Cp183 RNA-EEE is immediately obvious, implying that the EEE mutation modulates a subunit-subunit interaction.

doi:10.1371/journal.ppat.1002919.g005

Structural comparison of pgRNA in Cp183RNA-SSS and Cp183RNA-EEE

Difference maps of the empty capsids subtracted from the pgRNA-filled capsids show that pgRNA adopted dramatically different structures in unphosphorylated and phosphorylated capsids (Figure 6). Density assigned to pgRNA in the Cp183RNA-SSS resembled an icosahedral cage (Figure 6A), closely matching the ds rcDNA structure found in the native (presumably unphosphorylated) virion [34]. The RNA density under the fivefold vertices was connected along the icosahedral twofolds. A branch of the RNA density extended from the twofold edge and terminated near the center of the threefold axis. The structural similarity between the pgRNA observed here and the dsDNA in the native virion, along with the thickness of the RNA density suggests that the icosahedrally ordered pgRNA in Cp183RNA-SSS may be largely DS RNA (Figures S2 and S3).

In striking comparison, the pgRNA in Cp183RNA-EEE formed a complicated mesh-like density network (Figure 6B, see Figure S2 for additional radial density maps). The pgRNA in Cp183RNA-SSS appeared to be condensed at the twofold position with a diameter of \( \sim 20 \AA \) consistent with double stranded nucleic acid. The pgRNA in Cp183RNA-EEE resembled a net stretched over a sphere, where each segment in the mesh was approximately 7–8 Å thick, about the diameter of single-stranded RNA. Other analyses of the data supported this view. In the circularly averaged image (Fig. 1D) we observed that the pgRNA was stronger than capsid density. In the central cross-section of the three-dimensional map we observed that the pgRNA density was composed of short segments of strong density (Figure 2H). Furthermore, the Fourier shell correlation (FSC) at the radii corresponding to the pgRNA shell (90–118 Å) indicated a resolution of 6 Å at the 0.5 cutoff (Figure S4), which is slightly better than the overall resolution estimated for the whole 3D model (7 Å at FSC 0.5 cutoff). These metrics indicated that the pgRNA within Cp183RNA-EEE had substantial structural order. We suggest that the conformational rearrangement of the pgRNA between expanded and condensed forms depends on Cp183 phosphorylation state.

Discussion

In this study, we determined the 3-D structures of the CTD and pgRNA of \textit{in vitro} assembled HBV particles using cryo-EM and 3-D image reconstruction. Based on cell culture studies [22,25,26], we used Cp183-EEE to mimic the effects of phosphorylation.
Through difference map imaging, subtracting x-ray coordinates of an HBV capsid of C-terminally truncated capsid proteins [9] from selected Cp183 capsids, we found that the inclusion of the EEE mutation in the CTDs had profound effects on the CTD structure. The altered CTD organization resulted in an equally dramatic reorganization of packaged pgRNA.

Conformational changes of the CTD and RNA associated with phosphorylation

The phosphorylation state of HBV is believed to change during the course of assembly and reverse transcription. The initial assembly reaction involves a phosphorylated form of the core protein [36,37]. In a related hepadnavirus, duck hepatitis B virus, the immature phosphorylated cap particle becomes hypophosphorylated as it matures [36,37]. Dephosphorylation likely occurred at the point when the plus-stranded DNA was synthesized [38,39]. In surface representations (using a density cutoff based on the capsid volume, Figure 6), the calculated volumes of the pgRNA in Cp183RNA-EEE and Cp183RNA-SSS were about the same, 1.1 x 10^6 A^3, a volume consistent with the 3.2 kb pgRNA assuming an average RNA density of 1.7 g cm^-3. This suggests both particles encapsidated the same amount of pgRNA. Indeed, sucrose gradient velocity sedimentation suggested that the majority of the Cp183RNA-SSS capsid contain one pgRNA (Figure S6). Based on charge, Cp183-EEE has less capacity for RNA binding than Cp183-SSS; electrophoretic mobility shift titrations of RNA by Cp183-EEE saturated only when there was sufficient protein for one RNA per capsid [15].

To test for differences in RNA order in our reconstructions, we examined the volume of the RNA density at different contour levels. We found that the relative pgRNA volume in Cp183RNA-SSS decreased faster than in Cp183RNA-EEE; the ratio of RNA volume to capsid volume is shown in Table S1. Thus, we suggest that the difference in the strength of the RNA density is due to the relative disorder of RNA in Cp183RNA-SSS.

The difference in RNA order appears to correlate with differences in structure. In the Cp183RNA-SSS structure, the surface shaded RNA density was thick enough to accommodate DS RNA. In Cp183RNA-EEE the narrow strands of density could only fit single-stranded RNA (Figures 6B, S2 and S3). We suggest that a single-stranded pgRNA structure is more favorable for reverse transcription. The ability of the CTD to affect RNA structure was consistent with the hypothesis that the core protein itself (via the CTDs) can act as an RNA chaperone [21,40].

Thus, our data indicate that the CTDs change conformation in response to phosphorylation and transduce a conformational change in the packaged nucleic acid. We propose that these changes are linked to reverse transcription. A similar modulation on the nucleic acid structure by reversible phosphorylation has similar density formation was observed beneath the quasi-sixfold vertex. It is possible that the larger opening at the quasi-sixfold pore provides extra space to increase CTD mobility.

The distinct structures of the encapsidated pgRNA in Cp183RNA-SSS and Cp183RNA-EEE suggest a novel response by RNA to the phosphorylation state of hepadnavirus core protein (Figure 7). The pgRNA density in Cp183RNA-SSS shares a common organization with the nucleic acid density observed in rcDNA-filled particles (from transgenic mice and human sources) and virus-like particles containing RNA from an E. Coli expression system [8,34]. These disparate particles both show an icosahedral cage of nucleic acid stretching from fivefold to fivefold. The structural similarity suggests that the nucleic acid was organized by the CTDs. Conversely, the RNA density in the Cp183RNA-EEE capsid was visibly stronger in both the 2-D averaged image (Figure 1D, inset) and the central section of the 3-D reconstruction (Figure 2H).

So, what is the basis of the difference in the strength of the RNA density? We cannot rule out the possibility that some empty capsids may be accidently selected in Cp183-SSS or Cp183-EEE reconstructions though these were not prevalent in sucrose gradient experiments (Figure S6). The pgRNA density in Cp183RNA-SSS is similar to previously published HBV structures with unphosphorylated core protein where the RNA density is always weaker than capsid density [3,8,18,34], even where the amount of encapsidated RNA is about the size of a genome [18]. In surface representations (using a density cutoff based on the capsid volume, Figure 6), the calculated volumes of the pgRNA in Cp183RNA-EEE and Cp183RNA-SSS were about the same, 1.1 x 10^6 A^3, a volume consistent with the 3.2 kb pgRNA assuming an average RNA density of 1.7 g cm^-3. This suggests both particles encapsidated the same amount of pgRNA. Indeed, sucrose gradient velocity sedimentation suggested that the majority of the Cp183RNA-SSS capsid contain one pgRNA (Figure S6). Based on charge, Cp183-EEE has less capacity for RNA binding than Cp183-SSS; electrophoretic mobility shift titrations of RNA by Cp183-EEE saturated only when there was sufficient protein for one RNA per capsid [15].

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Thus, our data indicate that the CTDs change conformation in response to phosphorylation and transduce a conformational change in the packaged nucleic acid. We propose that these changes are linked to reverse transcription. A similar modulation on the nucleic acid structure by reversible phosphorylation has
also been reported for histone H1 protein, which contains SPXX repeats (where X can be K or R) that are similar to the SPRRR motifs found in the HBV CTD [41,42]. The phosphorylation states of the histone H1 protein seem to affect its secondary structure and are involved in both condensation and decondensation of the chromatin at different stages during the process of DNA replication [43–46]. While the Cp183RNA-EEE is a novel structure, the pgRNA in the Cp183-SSS capsid resembles previous unphosphorylated structures containing RNA and DNA. The conformational similarity between the pgRNA in Cp183RNA-SSS and previous rcDNA structures, as well as the dimensions of the pgRNA density, suggests that at least part of the single-stranded pgRNA is condensed into a duplex architecture. It is notable that the ability of a virus capsid to control the structure of packaged RNA has also been observed in Pariacoto virus and flock house virus, even with non-native RNA [47,48]. Thus, our findings suggest that the conformation of the icosahedrally arrayed CTDs and the packaged nucleic acid represents a mobile compromise of electrostatics and the equilibrium and non-equilibrium thermodynamics that is vital to virus function.

**Functional implication of the CTD surface exposure in negatively regulating the signal for the viral envelopment**

Although the majority of the density corresponding to the CTD was found internally at the quasi-sixfold, threefold and fivefold vertices (Figure 4A and B), it has been long postulated that the CTD is partially exposed to the capsid surface for the purpose of signaling during viral replication [9,28,49–51]. Our results (here and previously [28]) suggest that the CTD may be accessible through the large quasi-sixfold pore (Figure 7). We observed CTD-associated density passing through the quasi-sixfold pore in empty capsids (Figure 2A and B inserts, black arrows); similar density has not been observed in CTD-truncated or genome-filled HBV capsids. Indeed, probing empty Cp183e-SSS capsids with the CTD-specific SRPK results in the capsid decorated by SRPK at every quasi-sixfold vertex [28]. The failure of capsids filled with *E. Coli* RNA to bind to SRPK columns [28] indicates that CTDs are not readily accessible when associated with RNA.

Ning et al. suggested that single-stranded nucleic acid (either pgRNA or single-stranded DNA) in the immature capsids negatively regulates HBV core trafficking by preventing CTD exposure [52]. They observed that secreted enveloped particles contained either empty capsids (over 90%) or DS rcDNA-filled cores. Thus, the blocking hypothesis predicts that mature DS rcDNA-containing capsid shares structural characteristics with the empty capsid [52]. This hypothesis is supported by EM reconstructions. First, in empty capsids, the CTDs were partially exposed through the quasi-sixfold pore (Figure 2E and F). Second, in the presence of the pgRNA, CTDs strongly interacted with the genome; the single-stranded genome obstructed exposure of CTD-associated signals [28]. Third, the partially DS rcDNA in the

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**Figure 7. An assembly schema for HBV Cp183 capsids.** Assembly of Cp183 dimers in the absence of RNA results in empty capsids with CTDs transiently exposed through the pores on the icosahedral twofold axes. Co-assembly of Cp183 dimers with pgRNA results in RNA-filled capsids where the RNA structure is responsive to the phosphorylation states of the CTD. doi:10.1371/journal.ppat.1002919.g007
mature core is expected to be much less flexible and may not be able to interact with all of the CTDs, which suggests that a fraction of the CTDs may regain their mobility in mature BS RNA cores [53]. Thus, nucleic acid-regulated exposure of CTDs through quasi-sixfold pores is a likely mechanism for signaling by the HBV core. In summary, we report sub-nanometer resolution structures of the full-length empty and pgRNA-filled HBV capsids assembled from unphosphorylated and phosphorylation-mimic core proteins. The structures show that the configurations of the RNA-binding CTDs and pgRNA respond to changes in CTD phosphorylation. Our data indicate that phosphorylation affects the structure of CTDs and the CTDs affect RNA organization. Such functional correlation of the CTD implies that the HBV core has nucleic acid chaperone activity. We further provide direct evidence of partially exposed CTDs on the capsid exterior, suggesting how they may play a role in intracellular trafficking and secretion of HBV cores. Even though we cannot draw a complete structural description of HBV maturation yet, the substantial changes of the CTD and pgRNA we observed in this study indicated that HBV is a highly dynamic molecular machine. Nevertheless, in the authentic capsid the viral RT, and possibly host factors, take critical parts in pgRNA packaging; their impact on the structure of encapsidated pgRNA is currently under investigation.

Materials and Methods

Purification of HBV capsids

The pgRNA production, the plasmids coded for HBV Cp183-SSS and Cp183-EEE, and the capsid purification were described previously [15]. Capsids stored at −80°C were disassembled by dialysis at 4°C in the disassembly buffers (1.5 M guanidine HCl, 0.5 M LiCl, 50 mM HEPES at pH 7.5, 10 mM DTT for Cp183-SSS, and 1.5 M guanidine HCl, 1.5 M LiCl, 50 mM Tris at pH 9.5, 10 mM DTT for Cp183-EEE). The encapsidated heterogeneous RNA packaged from E. Coli cells was precipitated by a spin of 20,000 × g for 15 min at 4°C. Protein dimers were recovered from the supernatant and purified by size exclusion chromatography using an analytical grade Superose 6 column (GE Lifesciences) equilibrated in disassembly buffer. Fractions containing core protein were identified by SDS-PAGE. Cp183-SSS and Cp183-EEE dimers were either used for the reassembly experiments immediately or stored at 4°C for a short period. Formation of the empty capsids (Cp183-SSS and Cp183-EEE) was approached by dialyzing the purified dimers in the reassembly buffer (250 mM NaCl, 50 mM HEPES at pH 7.5, 2 mM DTT for Cp183-SSS and 250 mM NaCl, 50 mM Tris pH 7.4, 2 mM DTT for Cp183-EEE). The pgRNA-filled capsids (Cp183RNA-SSS and Cp183RNA-EEE) were prepared by reassembling the purified dimers with in vitro transcribed HBV pgRNA at a molar ratio of protein dimer to RNA polymer = 120:1 in the reassembly buffer (150 mM NaCl, 50 mM HEPES at pH 7.5, 2 mM DTT for Cp183-SSS and 150 mM NaCl, 50 mM Tris at pH 7.4, 2 mM DTT for Cp183-EEE) overnight. Samples for cryo-EM were further concentrated by Amicon Ultra centrifugal filter units (Millipore, MA). The quality and the concentration of the sample were routinely checked by negative stained EM using 2% uranyl acetate.

Cryo-electron microscopy

The sample preparation and cryo-EM operation were followed well established procedures described previously [28]. Briefly, a drop of 3.5 µl sample solution was applied on a glow-discharged Quantifoil holey-carbon grid (R2/2), blotted with filter paper from both sides for 4 s to produce a thin layer of specimen solution across the holes. The grids were quickly plunged into liquid ethane bath cooled by liquid nitrogen in a cryo-container. All processes described above were performed by a FEI Vitrobot. The vitrified specimen on the grid was then transferred to a Gatan 626DH cryo-holder and kept at the low temperature environment (<= −176°C) for the subsequent processing. The cryo-holder was then rapidly inserted into a JEOL-3200FS EM (JEOL Ltd., Japan) operated at 300 kV with an in-column energy filter using a 20-eV slit except for Cp183RNA-SSS. Digitized images were recorded under the low-dose condition (<20 e−/Å²) on an UltraScan 4000 4k×4k CCD camera (Gatan Inc., Oxford, UK) at a nominal magnification of 80,000× (equal to 0.1484 nm at the specimen space) for Cp183-SSS, Cp183-EEE, and Cp183RNA-EEE and 40,000× (equal to 0.294 nm at the specimen space) for Cp183RNA-SSS. Images were taken at multiple defocuses to compensate the effect from the contrast transfer function of the EM.

Image processing

Selected images which fulfilled the criteria of the suitable particle concentration, optimal ice thickness and minimal specimen drift, were used for analysis. Particle images were semi-automatically boxed using program e2boxer.py from EMAN2 software [54]. The defocus level of each micrograph was estimated using RobEM (http://cryoem.ucsd.edu/programs-old.shm) and only the phase reversal was corrected in the subsequent data processing. The initial starting model for each specimen was reconstructed by an ab initio random model method [55]. Origin and orientation were determined and refined using AUTO3DEM [56]. The refinement processed iteratively with a successively improved 3-D model from each refinement until a stable 3-D reconstruction had been achieved. The resolution of each 3-D reconstruction was estimated by Fourier shell correlation using a threshold value of 0.5. The final 3-D maps of Cp183-SSS, Cp183-EEE, Cp183RNA-SSS and Cp183RNA-EEE were reached at the resolutions of 5.5 Å, 5.8 Å, 8.0 Å, and 7.0 Å, respectively (Figure S7). The 3-D reconstructions were visualized using RobEM and Chimera [57].

Difference map analysis

A CTD-truncated HBV capsid, Cp149 model, was calculated from the atomic coordinates (Protein Data Bank entry: 1QGT) using e2pdb2mrc.py and low-pass filtered to 10 Å. The difference map of CTD was calculated by subtracting Cp149 model from Cp183-SSS or Cp183-EEE; the difference map of pgRNA was calculated by subtracting Cp183-SSS from Cp183RNA-SSS or Cp183-EEE from Cp183RNA-EEE. Prior to the subtraction, all maps were normalized based on their average density and the standard deviation because the map calculated from the crystal structure generally has different density values than the cryo-EM reconstruction. The sizes of the maps were scaled, and the difference observed here was less than 1% in all cases. The region corresponding to the capsid shell (at the radii between 125–160 Å) was then used to calibrate the density. The resulting difference map was rendered at the contour level that is equivalent to that component rendered at the estimated full mass of the parental 3-D model.

Accession codes

The cryo-EM density maps have been deposited to EMDataBank.org. The EMDataBank accession number for Cp183-SSS, Cp183-EEE, Cp183RNA-SSS, and Cp183RNA-EEE are EMD-2057, EMD-2058, EMD-2059, and EMD-2060, respectively.
Reassembled Cp183 RNA-SSS capsids were initially centrifuged at 39,000 rpm (190,000 x g) for 2 h at 4°C. Fractions were manually collected from the bottom and assayed by an HPLC system equipped with a diode array UV-vis detector (Shimadzu) using a Bio SEC-5 HPLC column with a 500 Å pore diameter (Agilent). Light scattering-corrected UV absorbances at 260 nm, 280 nm, and the corrected 260 nm/280 nm ratios were calculated [58] and plotted for each fraction. Selected fractions were analyzed by negative stained EM using 2% uranyl acetate. The results showed a major peak containing unaggregated particles (Fractions 21–28) and a minor peak (Fractions 19–20) containing aggregated particles. In further analysis of the major peak we found that the fractions near the top of the gradient (Fractions 25-28) contained a mixture of T = 3 and T = 4 particles. Based on absorbance, the average nucleotide per dimer in this region was about 32, suggesting a mixture of 33% of T = 4 particles and 67% of T = 3 particles; although some empty capsids could also have sedimented in this region. Fractions 21–24 contained mainly T = 4 particles and the calculated average of 27 nucleotides per dimer, or 3240 nucleotides per T = 4 capsid, suggested that there was one pgRNA per capsid.

Acknowledgments
Microscopy data were collected at the IU Cryo-Transmission Electron Microscopy Facility, part of the Nano-Materials Characterization Center at Indiana University-Bloomington. We thank Dr. David Morgan for the EM support. We also thank Zhenning Tan for the help on the sucrose gradient/HPLC experiments and Dr. Sarah Katen for the constructive criticism and comments.

Author Contributions
Conceived and designed the experiments: JCW AZ. Performed the experiments: JCW MSD. Analyzed the data: JCW AZ. Contributed reagents/materials/analysis tools: MSD. Wrote the paper: JCW AZ.
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