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# The diverse functions of the hepatitis B core/capsid protein (HBc) in the viral life cycle: Implications for the development of HBc-targeting antivirals

**Ahmed Diab**[a,b,c], **Adrien Foca**[b,c], **Fabien Zoulim**[b,c,d,e], **David Durantel**[b,c,d,**], and **Ourania Andrisani**[a,*]

[a]Department of Basic Medical Sciences and Purdue Center for Cancer Research, Purdue University, West Lafayette, IN, 47907, USA

[b]INSERM U1052, Cancer Research Center of Lyon (CRCL), Lyon, 69008, France

[c]University of Lyon, Université Claude-Bernard (UCBL), UMR_S1052, UCBL, 69008, Lyon, France

[d]Hepato-Gastroenterology Unit, Croix-Rousse Hospital, Hospices Civils de Lyon (HCL), 69002, Lyon, France

[e]Labex DEVweCAN, 69008, Lyon, France

## Abstract

Virally encoded proteins have evolved to perform multiple functions, and the core protein (HBc) of the hepatitis B virus (HBV) is a perfect example. While HBc is the structural component of the viral nucleocapsid, additional novel functions for the nucleus-localized HBc have recently been described. These results extend for HBc, beyond its structural role, a regulatory function in the viral life cycle and potentially a role in pathogenesis. In this article, we review the diverse roles of HBc in HBV replication and pathogenesis, emphasizing how the unique structure of this protein is key to its various functions. We focus in particular on recent advances in understanding the significance of HBc phosphorylations, its interaction with host proteins and the role of HBc in regulating the transcription of host genes. We also briefly allude to the emerging niche for new direct-acting antivirals targeting HBc, known as Core (protein) Allosteric Modulators (CAMs).

### Keywords

Hepatitis B virus; HBV; HBV core antigen; HBc; HBc structure; HBc phosphorylations; PLK1; Core allosteric modulators; CAMs

*Corresponding author. Department of Basic Medical Sciences and Purdue Center for Cancer Research, Purdue University, West Lafayette, IN, 47907, USA. david.durantel@inserm.fr (D. Durantel). **Corresponding author. INSERM U1052, Cancer Research Center of Lyon (CRCL), Lyon, 69008, France. andrisao@purdue.edu (O. Andrisani).

## 1. Introduction

Hepatitis B virus (HBV) is a major hepatotropic human pathogen, with at least 2 billion people having been exposed to date to the virus according to World Health Organization (Revill and Yuan, 2013). HBV exposure leads to chronic infection in 85–95% of infected neonates/children and 5–15% of infected adults (Hadziyannis, 2011). Chronic infections are associated with a higher risk of developing liver cancer (Revill and Yuan, 2013), with 50% of hepatocellular carcinoma (HCC) attributed to HBV. Viral persistence is due to the ability of the virus to escape the host immune system, and due to establishment of an episome in the nucleus of infected cells, known as covalently closed circular double-stranded DNA (cccDNA). This cccDNA resembles the host genome, as it assumes a chromosome-like structure, and serves as main template for viral transcription. However, cccDNA is devoid of telomeres and replication origins, implying that it can be lost when cells divide; consequently, its reservoir is maintained by recycling of neosynthesized, relaxed-circular DNA (rcDNA) containing nucleocapsids. For an in-depth discussion of cccDNA biology see recent reviews (Allweiss and Dandri, 2017; Lucifora and Protzer, 2016). CccDNA is a 3.2 kb molecule that expresses at least 6 overlapping RNAs, from 4 open reading frames (ORF), leading to production of 7–8 proteins, including viral polymerase (P), regulatory X protein (HBx), envelop proteins (S, M, and L), HBeAg, and core/capsid (HBc). In this review, we focus our discussion on the function of HBc.

HBc, a small versatile protein of 21 kDa, has been classically viewed as a structural protein that self-assembles to form the viral nucleocapsid. In turn, the nucleocapsid initially contains the pregenomic RNA (pgRNA), subsequently converted into rcDNA by reverse transcription. The versatile nature of HBc is primarily a consequence of post-translational modifications occurring at its C-terminal domain (CTD), and thus, this protein directs several viral processes. Accumulated evidence supports that HBc functions extend well beyond this structural role. HBc plays a role in nearly every stage of the HBV life cycle, including: subcellular trafficking and release of HBV genome, RNA metabolism, capsid assembly and transport, and reverse transcription (Fig. 1) (Basagoudanavar et al., 2007; Nassal, 1992; Perlman et al., 2005; Rabe et al., 2003; Zlotnick et al., 2015). Moreover, recent findings implicate an active role for HBc in epigenetic regulation of the viral and host genomes. Collectively, this multiplicity of functions elevates HBc as an excellent target for mechanism-based antiviral therapeutics (Durantel and Zoulim, 2016; Zlotnick et al., 2015). This review will highlight how the unique structure of HBc, its capacity to multimerize, as well as its post-translational modifications, can explain its multiple roles in the HBV life cycle.

## 2. HBc: from translation to capsid structure and self-assembly

The core protein is encoded by the HBV pgRNA which also encodes the viral polymerase, and serves as template for rcDNA synthesis via reverse-transcription. The viral polymerase acts as a molecular switch to regulate these two otherwise competitive processes allowing the virus to replicate efficiently (Ryu et al., 2010, 2008). The 21 kDa HBc protein is the building block or capsomer of the HBV genome-containing icosahedral capsid. The first crystal structure, resolved by Wynne and colleagues, revolutionized our understanding of

HBc biology (Wynne et al., 1999). HBc is a helical protein with five alpha-helices and lacking β-sheets. Helices 2 and 3, making a long alpha-helical hairpin, are responsible for the HBc folding pattern. Interestingly, most nucleocapsid proteins that form icosahedral shells usually fold into barrel structures instead of helical ones, e.g., those seen in the HIV-1 capsid (Wynne et al., 1999). A hydrophobic region makes up the base of the protein. HBc assembles first into dimers via alpha-helical hairpins, which form a four-helix bundle, assuming an inverted T shape. A disulfide bridge between the two Cys-61 residues, stabilizes the association, although this cystine formation is not necessary for dimer assembly (Nassal, 1992; Zheng et al., 1992; Zlotnick et al., 1996). Residues Tyr-132, Arg-127, Pro-129, and Ile-139 appear to be involved in dimer formation (Wynne et al., 1999). Following dimer formation, trimers of dimers assemble into hexamers, eventually forming icosahedral capsid particles (Birnbaum and Nassal, 1990). Multimerization of 120 HBc dimers, i.e., 240 capsomers, leads to production of a 22 nm, icosahedral capsid particle, without need of other viral proteins (Birnbaum and Nassal, 1990). The dimers form the icosahedral capsid with a triangulation number T = 4. Of note, a small fraction of capsids consist of only 90 dimers with a triangulation number T = 3 (Crowther et al., 1994). However, it is unknown whether virions comprised of smaller capsids are infectious or merely represent dead-end products caused by aberrant assembly.

Focusing on the HBc primary sequence, the full-length protein consists of 183 amino acids with distinct N- and C-terminal domains connected by a hinge region (Nassal, 1992). The N-terminal domain of HBc, spanning amino acid residues 1–140, contains the capsid assembly domain, as truncated HBc 1-140 is sufficient for self-assembly (Birnbaum and Nassal, 1990). On the other hand, the CTD of HBc, spanning amino acids 141–183, is dispensable for *in vitro* viral assembly. Notably, capsids lacking the last 34 amino acids maintain assembly capacity *in vitro*, but do not support genome replication in cells (Nassal, 1992), suggesting a role for the CTD in this process. Of the 34 amino acid residues comprising the CTD, 14 are arginines, and at least seven other residues are either serines or threonines, putative phospho-acceptor sites.

Studies to understand the role of HBc CTD in HBV replication have led to the proposal that electrostatic interactions between the highly basic CTD in assembled capsids and the viral RNA/DNA regulate reverse transcription of HBV DNA (Newman et al., 2009). Charge-driven destabilization of capsids, by either rcDNA maturation and/or changes in post-translational modifications of CTD, for example by phosphorylation, could explain capsid disassembly at the nuclear pores (Basagoudanavar et al., 2007; Perlman et al., 2005; Rabe et al., 2009). In addition, results of a study by Lewellyn and Loeb favor an hypothesis that CTD could serve as a nucleic acid chaperone domain, i.e., CTD could mediate changes in nucleic acid structure hence enabling pregenomic RNA (pgRNA) reverse transcription into rcDNA (Lewellyn and Loeb, 2011). These two models are not necessarily mutually exclusive, since the function of HBc as chaperone may be dependent on the overall charge of its CTD.

A recent study employing cryo-EM scanning has determined the high-resolution 3D structure of assembled HBV capsids (Yu et al., 2013). It was proposed that HBc CTD shuttles between the interior and exterior of capsids, as evidenced by the presence of CTDs

either on the exterior of some capsids or RNA-bound in the interior of other capsids (Yu et al., 2013). This model is attractive as it provides a mechanism by which HBc can be accessible for interaction with host proteins involved in intracellular trafficking, capsid maturation, or capsid disassembly. In this model, the positively charged CTD interacts transiently with the negatively charged Asp2 and Glu43 that line the local three-fold channel, presumably allowing movement of CTD in and out of capsids (Yu et al., 2013). In congruence with the cryo-EM study, biochemical binding assays demonstrate a nucleic acid sensitive and transient exposure of the CTD tail (Chen et al., 2011), further confirming the presence of CTD on the exterior of capsids. Specifically, RNA-filled capsids failed to bind serine-arginine protein kinase (SRPK), a host chaperone that binds full length HBc capsids, but not HBc 1-149 capsids (Chen et al., 2011), confirming the dynamic exposure of CTDs is mediated by CTD-nucleic acid interactions. These results also agree with biochemical studies showing the CTD of assembled capsids is not only sensitive to proteases, but also accessible to host proteins (Chen et al., 2011; Gallina et al., 1989). The exact mechanism of this dynamic CTD shuttling remains unknown, although a role of phosphorylation has been suggested by site directed mutagenesis of HBc (Selzer et al., 2015). However, further detailed analyses of the effect of CTD phosphorylations and dephosphorylations in CTD shuttling are required.

## 3. HBc, a nucleic acid chaperone

Nucleic acid chaperones are proteins that catalyze structural rearrangement of genetic material into a functional conformation (Semrad, 2011). The binding of nucleic acid chaperones to their targets is characteristically weak and transient, lacks sequence specificity, and does not require ATP hydrolysis (Semrad, 2011). An array of viral proteins displays nucleic acid chaperone activity. For example, nucleocapsid proteins of HIV (Levin et al., 2005), dengue virus (Pong et al., 2011), and hepatitis delta virus (Huang and Wu, 1998) are described as nucleic acid chaperones. The CTD of HBc contains an arginine-rich domain resembling histone tails with more than 50% of the residues being arginine (Lewellyn and Loeb, 2011). The CTD binds to nucleic acids *in vivo* due to its positive charge (Nassal, 1992). In addition to its characteristic positive charge, CTD is also structurally disordered (Yu et al., 2013), another key feature of nucleic acid chaperones (Chu et al., 2014). The localization of CTD to the interior of capsids (Zlotnick et al., 1997) and its ability to bind nucleic acids *in vivo* support the model that CTD works as a nucleic acid chaperone. This chaperone activity was recently confirmed *in vitro* using recombinant full length HBc, as well as CTD synthetic peptides (Chu et al., 2014). HBc CTD facilitated annealing and unwinding of DNA and enhanced hammerhead ribozyme cleavage *in vitro*, rendering HBc CTD a *bona* fi*de* nucleic acid chaperone (Chu et al., 2014). However, the high degree of functional complexity associated with the CTD, makes it challenging to demonstrate the nucleic acid chaperone activity of HBc *in vivo*, without disturbing other HBc functions.

## 4. HBc localization: a dynamic process involving active-transport

Reports of subcellular localization of HBc *in vivo* demonstrated both cytoplasmic as well as nuclear distribution. Histologic analysis of tissues from HBV-infected patients indicated a

distribution of HBc/capsids in both cytosolic and nuclear compartments. Sharma et al., as well as Akiba et al., described a predominant nuclear distribution (Akiba et al., 1987; Sharma et al., 2002) while others observed mainly a cytosolic core localization (Michalak and Nowoslawski, 1982; Petit and Pillot, 1985). *In vitro* analysis has shown HBc subcellular localization is in fact cell-cycle dependent (Yeh et al., 1993). The amount of core protein in the nucleus increases in G1 phase, diminishes in S phase, and increases again to detectable levels in non-proliferating cells (Yeh et al., 1993). HBc shuttles between cytoplasm and nucleus rapidly and continuously (Li et al., 2010). These latter points could explain differences described above. Although HBc is 21 kDa, suggesting passive transport to and from the nucleus, this trapping, as exists for human T-lymphotropicvirus-1 (HTLV-1) (Goff, 2007) and human papillomaviruses (Aydin et al., 2014), would be fairly inefficient due to the quasi-quiescent state of hepatocytes. Given that HBc is a phosphorylation target of well-characterized cell cycle regulated kinases, such as cyclin-dependent kinase 2 (CDK2) (Ludgate et al., 2012) and polo-like kinase 1 (PLK1) (Diab et al., 2017), it is highly plausible that such phosphorylation may contribute to HBc/capsid localization. Investigating this question in a physiologically relevant infection model will most certainly expand our understanding of the dynamics of HBc subcellular localization.

Furthermore, it remains unresolved whether HBc enters the nuclear pore complex (NPC) as a dimer or as a mature capsid. Thus, signals modulating HBc import and export from the nucleus are probably distinct from those that guide transport of mature capsids. An early *in vitro* model for capsid transport by Kann and colleagues suggested mature capsid disassembles into HBc dimers at the cytosolic side of the NPC, releasing the rcDNA-pol complex (Kann et al., 1997). Subsequently, HBc dimers bind importin-β and are transported through a NPC. At this point, HBc dimers dissociate from transport factors and could remain free in the nucleus, eventually playing a role as transcription factors, as described below, or reassemble as empty-capsid forms. On the other hand, as viral polymerase does not contain an importin-β binding (IBB) domain, formation of importin-α/β is required to import the genome complex (Kann et al., 1997). Once at the nuclear side, the rcDNA-pol complex dissociates from importin α/β. Since the viral genome comprises pathogen-associated molecular patterns (PAMPs), it could trigger sensing by cytosolic pattern recognition receptors (PRRs) (Paludan, 2013). This model of genomic nuclear import, showing a release of viral rcDNA in the cytosol could explain the weak innate immunity activation upon HBV infection (Faure-Dupuy et al., 2017; Lucifora et al., 2014; Vanwolleghem et al., 2015). However, this model is based on Woodchuck Hepatitis Virus where capsids were denatured using urea treatment in order to release the rcDNA-pol complex (Kann et al., 1997). Under such conditions, changes in conformation of the viral polymerase could enable its interaction with importin-α.

A more recent alternative model (see Blondot et al., 2016 for in-depth review), suggests a direct interaction between mature capsids and an essential component of the NPC's basket, the nucleoporin 153 (Nup153). It is known that importin-α interacts directly with Nup153, promoting importin-α/β-mediated nuclear import (Ogawa et al., 2012). Of note, it was shown earlier that mature capsids are transported to nuclear pores by an importin α/β complex (Kann et al., 2007). The new model provides two options for mature capsids going through nuclear pores: first, direct binding to Nup153 at the basket of the NPC (cargo size

36 nM) or via coating by importin-α/β and binding to Nup153 (cargo size 38 nM). According to this model, the HBc CTD is not necessary to anchor the capsid at the nuclear basket since importin α/β is required for capsid transport. While both models intend to explain the mechanism of capsid transport through nuclear pores, neither reveals the trigger of such transport.

Studies of the CTD tail revealed a role in directing HBc subcellular localization (Blondot et al., 2016; Li et al., 2010). Several attempts have been made to map the nuclear localization sequence (NLS) to the arginine repeats of CTD. However, the results are conflicting, probably due to the redundancy of arginine repeats within the CTD (Liao and Ou, 1995; Rabe et al., 2003). Recently, an exhaustive mutagenesis analysis of the CTD localized the NLS sequence to the 1st and 3rd arginine repeats, while the 2nd and 4th repeats contained the cytoplasmic retention signals (CRS) (Fig. 2) (Li et al., 2010). The presence of more than one NLS in a protein is known to maximize import efficiency (Mears et al., 1995). Other viral proteins, such as ICP27, of herpes simplex virus type 1 (HSV-1) (Mears et al., 1995) and NS1 of Influenza (Melen et al., 2012), also contain multiple NLS sites. However, one must also consider the accessibility of these sequences in view of the dynamic structural changes of assembled HBc in viral capsids.

The dynamic positioning of HBc CTD in assembled capsids makes it quite challenging to define clearly the different signals regulating HBc transport. Nuclear export of HBc resembles ICP27 of HSV-1 and EBNA1 of Epstein Barr virus (EBV), in that they all bind to the RNA export factor TAP, and all contain the characteristic arginine rich domains (Fig. 2) (Yang et al., 2014). Since subcellular localization of ICP27 and EBNA1 are regulated by arginine methylation (Souki et al., 2009), it is reasonable to envisage a similar mechanism driving HBc localization. However, it is still unknown if the arginine repeats of CTD are methylated, an hypothesis worthy of future investigation. Of note, arginine methyltransferase 5 (PRMT5) was reported as a negative effector of HBV replication and transcription via epigenetic repression of cccDNA transcription and interference with pregenomic RNA encapsidation (Zhang et al., 2017). Interestingly, PRMT5 was shown to bind HBc, and HBc-deficient virus (HBV-ΔHBc) had less PRMT5 bound cccDNA when compared to the wildtype HBV (Zhang et al., 2017).

The contribution of HBc CTD to capsid localization is only beginning to be resolved. The ambiguity is based on the long-standing notion that the CTD localizes to the interior of capsids based on cryo-EM studies (Zlotnick et al., 1997), and has a well-documented role in pgRNA packaging (Nassal, 1992). However, as previously discussed, more recent studies suggest a dynamic model in which HBc CTD shuttles between the interior and exterior of the capsid (Chen et al., 2011; Li et al., 2010; Selzer et al., 2015).

Transport of cargo through the nuclear envelope is often facilitated by regulatory phosphorylation/dephosphorylation of specific residues (Nardozzi et al., 2010). Earlier studies reported phosphorylated HBc was only observed in the cytoplasm, based on subcellular fractionation of *in vivo* $^{32}$P-radiolabed cell lysates (Yeh et al., 1993). However, the molecular mechanism regulating subcellular localization of monomeric/dimeric vs. assembled HBc in capsids remains ambiguous. The potential contribution of HBc

phosphorylation to the nuclear import of capsids comes from known import mechanisms for other viral proteins, including EBNA-1 (Kitamura et al., 2006) and SV40 T-antigen (Hubner et al., 1997). Specifically, phosphorylation of EBNA-1 increases its affinity for the import adaptor importin-α5 which mediates the nuclear transport via interaction with importin-β (Kitamura et al., 2006). SV-40 large T antigen has the best characterized NLS, and phosphorylation upstream of the classic NLS enhances nuclear import (Hubner et al., 1997). Thus, the phosphorylation status of HBc CTD could facilitate interaction with nuclear pore complex components. Indeed, in pulldown experiments using HBc CTD-GST fusion proteins, serine to alanine substitution at the three serine/proline (S/P) sites (Fig. 3) showed increased binding to importin-α (Liu et al., 2015), suggesting phosphorylation impedes trafficking of HBc to the nucleus. While the *in vivo* relevance of these observations remains to be determined, other groups have reported that HBc phosphorylated *in vitro* by PKC in assembled capsids, preferentially bound importin-β (Kann et al., 1999). However, the phosphorylation sites of HBc required for interaction with importin-β have not been mapped (Kann et al., 1999). Furthermore, it is unresolved whether protein kinase C (PKC) is the *in vivo* kinase that phosphorylates HBc CTD (Daub et al., 2002). Thus, further studies are necessary to address this issue, employing well-characterized and functionally documented mutants of HBc.

Alternatively, as shown by Schmitz et al., capsid maturation could be a driving force of HBV nuclear import (Schmitz et al., 2010). At the nuclear pore, HBc capsids dissociate into dimers followed by entry into the nucleus (Rabe et al., 2009). Mature capsids directly interact with Nup153, an essential component of the nuclear basket that facilitates nuclear import via importin-β (Schmitz et al., 2010). Interaction of mature capsids with Nup153 resulted in capsid disintegration followed by release of viral genomes into the nucleus (Schmitz et al., 2010). Interestingly, capsids with immature genomes, i.e., RNA-filled capsids, accumulate at the nuclear pore. Recently, it was reported that serine to glutamate substitutions at the 3 S/P sites of HBc CTD (Fig. 3) reduced CTD exposure to the exterior of capsids suggesting a role for these phosphorylations in directing CTD exposure– inside or outside the capsid (Selzer et al., 2015). However, the mechanism responsible for distinguishing mature capsids *vs.* RNA-filled capsids remains unclear (Hu and Liu, 2017). Phosphorylation of HBc, shuttling of the HBc CTD to the interior *vs.* exterior of capsids, and maturation of the genome, may regulate exposure of different localization signals to host transport machinery. Such a multilayer and dynamic regulation network can best explain the difficulty in deciphering the mechanism of HBc subcellular localization during the HBV life cycle. To understand these important dynamic regulatory networks, it is essential to gain better understanding of HBc structure, HBc interacting partners, and HBc post-translational modifications.

## 5. HBc phosphorylation

Since the HBV genome does not encode a protein kinase, endogenous/cellular kinases must mediate post-translational HBc modifications (Lanford and Notvall, 1990). PKC (Kann and Gerlich, 1994; Wittkop et al., 2010), serine arginine protein kinase 1 (SRPK1) (Daub et al., 2002) and CDK2 (Ludgate et al., 2012) have been suggested as endogenous kinases that associate with or are packaged within viral capsids. The robust phosphorylation of HBc has

been demonstrated *in vitro* by phospho-proteomic approaches (Chen et al., 2011), and *in vivo* by labeling with $^{32}$P-orthophosphate (Jung et al., 2014). The HBc CTD contains multiple serine/threonine phospho-acceptor sites which are remarkably well-conserved among related viruses (Jung et al., 2014). Phospho-proteomic analyses of HBc have identified at least seven conserved serine and threonine sites that are phosphorylated *in vivo* (Chen et al., 2011). Notably, proline-directed serine (S/P) sites at positions 155, 162 and 170, are highly conserved and several S/P kinases have been reported to phosphorylate these sites (Fig. 3) (Daub et al., 2002; Ludgate et al., 2012). Other putative kinases, including: SRPKs, Glyceraldehyde-3-phosphate dehydrogenase protein kinase (GAPD-protein kinase), PKC, and an unknown kinase of 46 kDa, have also been reported (Daub et al., 2002; Duclos-Vallee et al., 1998; Kann et al., 1999; Ludgate et al., 2012) (Table 1). Yet none of these studies have mapped phosphorylation to specific sites, with the exception of SRPK1 and SRPK2 at the 3 S/P sites (Daub et al., 2002). However, the exact contribution of the SRPK-mediated phosphorylation to viral replication is enigmatic, because, although overexpression of SRPK1 or SRPK2 impaired HBV replication, this effect was independent of kinase activity (Zheng et al., 2005). These results support that SRPK-mediated phosphorylation of HBc observed *in vitro* is due to promiscuous substrate specificity of these kinases (Ludgate et al., 2012), and that SRPKs exert other functions affecting viral replication. For example, SRPKs could act as non-canonical chaperones in capsid assembly (Chen et al., 2011). Regarding PKC-mediated HBc phosphorylation, inactivation of PKC led to defective capsid envelopment, but had no effect on genome maturation (Wittkop et al., 2010). There is, however, lack of consensus regarding PKC being the candidate host kinase since specific inhibition of PKC had no effect on CTD phosphorylation (Daub et al., 2002; Ludgate et al., 2012). CDK2 was shown to phosphorylate the core protein of HBV both *in vitro* and *in vivo* (Ludgate et al., 2012). Intriguingly, CDK2 inhibitors had no observable effect on viral replication (Ludgate et al., 2012). The absence of such effect could be due to lack of specificity of the chemical inhibitors (Sakurikar and Eastman, 2016) or, more likely, due to redundancy among host kinases in phosphorylating HBc. Knockdown cell lines for CDK2 or other kinases via expression of shRNAs could elucidate their contribution to HBc phosphorylation and viral replication.

Although the kinases that phosphorylate HBc are still to be determined, phosphorylations of HBc do regulate its function at many levels (Gazina et al., 2000; Jung et al., 2014; Kann et al., 1999; Ludgate et al., 2011). Specifically, phosphorylation and de-phosphorylation of the HBc protein is required for capsid maturation as demonstrated by mass spectrometric analyses and site-directed mutagenesis studies of HBc (Basagoudanavar et al., 2007; Perlman et al., 2005). Phosphorylation of different serine residues of HBc CTD regulates reverse transcription, pgRNA encapsidation, DNA synthesis, subcellular localization, and virion secretion (Basagoudanavar et al., 2007; Gazina et al., 2000; Lan et al., 1999; Perlman et al., 2005). HBc phosphorylation is also required for the chaperone activity of CTD (Chu et al., 2014). It was also reported that the HBc phosphorylation status influences capsid stability (Ludgate et al., 2016), since capsids with glutamate phosphomimetic substitutions at the 3 S/P sites (S/P to E/P) were more stable than wild type capsids. It is likely that during various stages in the life cycle of HBV or as a function of the metabolic and proliferative status of the infected hepatocyte, distinct kinases target HBc phosphorylation, perhaps in an

overlapping manner. The identification of those kinases, and the mechanisms by which they regulate HBV replication, is of great significance and will provide valuable targets for mechanism-based therapeutics.

To this end, our recent study has identified the mitotic PLK1 as another cellular kinase that phosphorylates HBc *in vitro* (Diab et al., 2017). PLK1 substrates are usually primed by phosphorylation at S/P directed sites mediated by CDKs, including CDK1 or 2 (Elia et al., 2003). Recombinant HBc, as well as immuno-purified HBc from transfected mammalian cells serves as robust PLK1 substrate *in vitro* (Diab et al., 2017). We have mapped the phospho-acceptor sites to S168, S176, and S178 (Fig. 3) (Diab et al., 2017), all of which have been identified as *in vivo* phospho-acceptor sites by an unknown kinase (Jung et al., 2014). Remarkably, inhibition of PLK1 by specific chemical inhibitors or siRNA-mediated knockdown of PLK1 inhibited viral replication, suggesting HBc phosphorylation by PLK1 is functionally important in virus biosynthesis (Diab et al., 2017). Significantly, we made the interesting observation, in accordance with the known substrate preference of PLK1, that alanine substitutions at the 3 S/P sites (S/P to A/P) resulted in loss of PLK1 phosphorylation *in vitro.* By contrast, PLK1 phosphorylation was robustly recovered in phospho-mimetic mutants, containing serine to aspartic acid substitutions at all three S/P sites (Diab et al., 2017). This suggests that CDK2 functions as the priming kinase for HBc, mediating the phosphorylation of the S/P sites which in turn enables the subsequent phosphorylation by PLK1 (Elia et al., 2003). Based on our results that PLK1 is a positive effector of HBV replication, a mechanistic understanding of the role of PLK1 phosphorylation of HBc in HBV replication has promising therapeutic potential. So far, PLK1 is the sole host-kinase whose chemical inhibition results in impaired HBV replication *in vivo* (i.e., in liver humanized mouse model) at concentrations comparable to other known anti-HBV agents (Diab et al., 2017). Finally, based on this dependence of PLK1 phosphorylation of HBc on its prior phosphorylation by CDK2, we reason combination treatment with inhibitors for CDK2 and PLK1 kinases will be effective in suppressing these modifications and thus effectively inhibit virus biosynthesis. This hypothesis is particularly attractive since safety of PLK1 (Gjertsen and Schoffski, 2015) and CDK2 (Asghar et al., 2015) inhibitors has been established in clinical trials for different types of human cancer.

## 6. HBc and interaction with host factors

HBc modulates nearly every stage of the HBV life cycle. With such extensive and intricate functions, HBc has evolved to modulate several host-protein functions. For example, HBc dimers interact with HSP90, and HSP90 facilitates formation of HBV capsids both *in vitro* and *in vivo* (Shim et al., 2011). On the other hand, the host HSP40 protein also binds, but de-stabilizes HBc by accelerating its degradation (Sohn et al., 2006). The binding of HBc to other cellular factors, such as NIRF, an E3 ubiquitin ligase, is implicated in HBc degradation (Qian et al., 2012). Thus, dynamic interactions of HBc with host chaperones regulate both HBc stability and capsid assembly (Qian et al., 2012; Shim et al., 2011; Sohn et al., 2006).

Viruses hijack host RNA processing machinery (Salvetti and Greco, 2014) and HBV is no exception. HBc utilizes the nuclear export factor 1 (NXF1/p15) and transcription export (TREX) machinery for nuclear export as a ribonucleoprotein complex along with the viral

pgRNA (Yang et al., 2014); however, TREX-mediated export of HBc and pgRNA are independent of each other (Yang et al., 2014). Other viruses that hijack the NXF1/p15 pathway for mRNA processing and export include EBV (Juillard et al., 2009) and HSV-1 (Johnson et al., 2009). Interestingly, depletion of different TREX components has no apparent effect on accumulation of viral DNA, suggesting redundancy among host factors utilized by the virus.

Another class of host proteins commonly targeted by viruses is serine, arginine rich-proteins (Howard and Sanford, 2015) which, along with the kinases (SRPKs) that phosphorylate them, play central roles in alternative RNA splicing (Howard and Sanford, 2015). Intriguingly, HBc CTD resembles serine, arginine-rich proteins and it is not surprising that SRPKs associate with and potentially phosphorylate HBc (Chen et al., 2011; Daub et al., 2002; Zheng et al., 2005). Interestingly, mass spectrometry analyses of HBc interacting proteins has identified SRPK1 as well as several known SRPK1 interacting partners, including: SRSF1, SRSF9, DHX9, CDKN2A, DDX21, and DDX50 (Diab et al. unpublished data). The functional significance of these HBc interactions in the viral life cycle is unknown and worthy of investigation. We speculate that HBV usurps the host RNA-processing machinery for production and processing of its own viral RNA. It is also likely that such interactions may allow HBc to interfere with host gene expression. This has been evident in the case of E1E4 protein of the human papilloma type 1 virus (HPV1), which not only binds to SRPK1, but also inhibits phosphorylation of its host serine arginine substrates (Prescott et al., 2014). A yeast two-hybrid screen of a human liver cDNA library identified the human protein GIPC1 as another HBc interaction partner (Razanskas and Sasnauskas, 2010), but the significance of such interaction remains to be determined. GIPC1 contains a PDZ domain, a protein-protein interaction domain (reviewed in Hung and Sheng, 2002). PDZ-containing host proteins are commonly targeted by viral proteins; examples include the Tax protein of the T-cell leukemia virus type 1 (Rousset et al., 1998) and the E6 protein of the human papilloma virus type 18 (Favre-Bonvin et al., 2005).

It is reasonable to speculate that HBc exerts its various roles by interacting with specific host factors at different stages of the viral life cycle in a phosphorylation dependent manner. A better dissection of host factors that interact with HBc could facilitate development of new antiviral strategies or help elucidate core assembly modulators (CAMs).

## 7. HBc as a regulator of transcription

In chronically infected patients, HBc is detected in both cytoplasm and nucleus of hepatocytes (Chu and Liaw, 1987). *In vitro* models of HBV infection also show nuclear as well as cytoplasmic localization of HBc. Nuclear localization of HBc, in biopsies from HBV infected patients, has been linked to high viremia and deficient immune response (Chu and Liaw, 1987; Nguyen et al., 2009). These results prompted investigations of the nuclear function of HBc, in relation to virus biosynthesis and influence upon host gene expression.

In the nucleus of infected cells, the viral cccDNA assumes chromatin-like structure in association with histone and non-histone proteins, along with viral HBx and HBc proteins (Bock et al., 2001; Lucifora and Protzer, 2016). Association of HBc with the cccDNA mini-

chromosome was reported to alter nucleosome spacing (Bock et al., 2001). Furthermore, it was reported that HBc preferentially associated with CpG island 2 of the HBV genome that overlaps enhancers (Enh) I and II involved in viral transcription; HBc binding to CpG island 2 positively correlated with the rcDNA/cccDNA ratio and serum levels of HBV DNA in infected patients (Guo et al., 2011). HBc may also act as transcriptional activator of the HBV pre-Core promoter by enhancing binding of NF-kB upstream of viral Enh II (Kwon and Rho, 2002). However, how HBc modulates the function of the cccDNA mini-chromosome remains to be determined. Since HBc is also essential for viral DNA replication, a definitive genetic approach involving mutagenesis or depletion of HBc is not feasible as a means to delineate its role in cccDNA mini-chromosome functions. Therefore, novel approaches are required to target nuclear HBc, influencing its capacity to associate with the cccDNA mini-chromosome, without affecting HBc cytoplasmic functions.

Regarding effects of HBc on host gene expression, HBc enhanced cAMP-Response-Element (CRE)-mediated transcription via the CRE/CREB/CBP pathway (Xiang et al., 2015) in a manner similar, but weaker to previously reported HBx-mediated CRE activation (Andrisani, 1999; Williams and Andrisani, 1995). HBc may also function as a transcriptional repressor of host genes. For example, binding of HBc to E2F1 reduced the DNA-binding ability of E2F1 at the p53 promoter, thereby inhibiting p53 transcription (Kwon and Rho, 2003). In hepatoma cell lines expressing the core protein, HBc blocked the human death receptor 5 (DR5) by repressing its promoter, and inhibition of DR5 desensitized hepatocytes to TRAIL-induced apoptosis (Du et al., 2009). These results suggest a role for HBc in development of chronic infection by preventing hepatocyte death via blocking DR5 expression. The gene regulatory function of HBc may have evolved as a means whereby the virus may evade host immunity. HBc has been shown to downregulate IFN-induced host antiviral responses in hepatoma cell lines, by interacting with the promoter of MxA, an INF-inducible gene widely implicated in antiviral response (Fernandez et al., 2003). However, these conclusions must be further validated by determining *in vivo* association of HBc with the MxA promoter, in a physiological cellular context of HBV infection. Interestingly, MxA has been shown to inhibit HBV replication by direct interaction with HBc, preventing capsid formation (Li et al., 2012), and providing yet another example of antagonism between viral and host proteins. Ectopic expression of HBc in HepG2 cells and primary hepatocyte cultures sensitized cells to TNF-α induced apoptosis, by disrupting the interaction between mitogen-activated protein kinase kinase 7 (MKK7) and receptor of activated protein kinase C 1 (RACK1) (Jia et al., 2015). This suggests a direct role of HBc in driving liver pathogenesis in chronically infected patients. These results require validation in a rigorous experimental model.

In continuing this effort to understand the effect of HBc on host gene expression, recent studies have generated the genome-wide profile of HBc in HBV-infected hepatocytes, employing chromatin immunoprecipitation microarray studies (ChIP-on-chip). These studies revealed that promoters of nearly 3100 host genes exhibited increased binding to HBc (Guo et al., 2012). However, further studies are necessary to understand the functional significance of HBc binding to the host genome in affecting HBV-mediated disease pathogenesis. Toward this goal, Lucifora et al. recently demonstrated interaction of nuclear deaminases APOBEC3A and APOBEC3B with HBc-bound cccDNA in host nucleus, leading to cccDNA deamination and degradation (Lucifora et al., 2014). Whether HBc recruits these

nuclear deaminases to host genes is unknown and of great importance, given the role of APOBEC proteins as cancer driver genes in humans (Harris, 2015; Morganella et al., 2016; Nik-Zainal et al., 2012). Muli-omics analyses of HBc transfected cells linked HBc over expression up-regulated glycolysis and amino acid metabolism, providing further insights into the role of HBc in the molecular pathogenesis of HBV-mediated tumorigenesis (Xie et al., 2017).

## 8. HBc as a target for clinical intervention against chronic HBV infection

Due to the multiple functions of HBc in the HBV life cycle, this protein has become a preferential target for developing novel, direct-acting agents against HBV replication. Molecules targeting capsid assembly have been known for some time with the discovery BAY41-4109, a heteroaryldihydropyrimidine derivative (Deres et al., 2003), and AT130, a phenylpropenamide derivative (Delaney et al., 2002). This class of inhibitors were subsequently abbreviated core assembly/allosteric modulators (CAMs), due to their mode of action, which is mainly based on allosteric modulation (Zlotnick et al., 2015). Interestingly, these molecules were shown to be active against nucleoside analogue resistant strains, which render them very attractive when viral resistance was a genuine clinical challenge (Billioud et al., 2011; Delaney et al., 2002; Wang et al., 2012). Moreover, CAMs are active against most HBV genotypes (Berke et al., 2017). Surprisingly, CAM therapy has not reached the clinic –probably due to safety or pharmacological issues. Since this early time, active research on structural and biological features of the HBc protein has led to the discovery of many other CAMs. Currently there are 3 types of CAMs according to their different molecular architectures, including heteroaryldihydropyrimidine, phenylpropenamide, and sulfamoylbenzamides derivatives (Fig. 4) (Zlotnick et al., 2015). These CAMs are thought to inhibit nucleocapsid-associated reverse transcription of pgRNA into rcDNA by preventing encapsidation of pgRNA, by accelerating formation of HBV nucleic-acid-free, yet normal-in-shape capsids, or by promoting formation of abnormal capsids. This defines two classes of CAMs based on these *in vitro/in tubo* structural criteria. In addition to their role in nucleocapsid formation and resulting conversion of pgRNA into rcDNA, CAMs have other modes of action. CAMs have been shown to prevent, in a post-entry manner, formation of cccDNA (Berke et al., 2017). This could be due to retention of rcDNA at the nuclear basket; in this respect, CAMs could bind to nucleocapsid and stabilize the structure, thus preventing its disassembly. CAMs also prevent the release from hepatocytes of RNA-containing and genome-free viral particles (Lam et al., 2015; AASLD abstract). Very recently it has been reported that specific CAMs prevent release of the HBe antigen (Lahlali et al., HBV meeting 2017). HBeAg, a secreted antigen sharing primary structure with HBc, has potential immune-modulatory functions. An active and fast inhibition of HBeAg secretion by CAMs could promote HBeAg seroconversion, an immunological process that occurs during natural history of HBV infection, and considered an important clinical end-point during therapy.

However, bearing in mind some of the other functions described in this review (i.e. regulation of host gene expression, modulation of host and viral RNA metabolism, etc.), one might anticipate CAMs could play a role in HBV replication inhibition and HBV-induced pathogenesis. To date several CAMs are undergoing clinical trials: NVR 3–778 (NCT02112799; Novira Therapeutics), ABI-H0731 (NCT02908191; Assembly

Biosceinces), JNJ-56136379 (NCT02662712; Janssen), and GLS-4 (trial with China-CFDA; Morphothiadine Mesilate, HEC Pharm), and many others are the focus of pre-clinical evaluations.

## 9. Unanswered questions and future directions

As discussed in this review, HBc is involved in various aspects of HBV life cycle: formation of the viral capsid, pgRNA encapsidation followed by its reverse transcription, and as a component of the viral mini-chromosome, contributing to its chromatin structure. The unique structure of HBc CTD and its modifications, mainly phosphorylation, are essential for viral biosynthesis. Accordingly, HBc appears to be an ideal target for mechanism-based therapeutics. Yet many questions remain unanswered when it comes to HBc biology. A definitive answer to the identity of the kinases responsible of HBc phosphorylation *in vivo* is yet to be determined. Additionally, systematic investigation of HBc-host interactions, in the context of physiologic infection as well as well as details of its molecular properties, will expand our knowledge of how HBc is regulated by post-translational modifications, as well as how HBc regulates host functions. Moreover, given the unique structure of the CTD, the seemingly promiscuous affinity of HBc for RNA and nucleic acids in general, (Birnbaum and Nassal, 1990; Nassal, 1992), suggests potential interactions between HBc and host RNA metabolism. This corroborates our recent findings of long-noncoding (lnc) RNAs NEAT1 and NEAT2 as HBc interacting partners in cellular models of HBV replication and infection (Diab et al., HBV meeting 2017). Ribonucleoprotein immunoprecipitation (RIP) of HBc confirmed HBc associates with NEAT1 and NEAT2, known scaffolds of ribonucleoprotein complexes that constitute nuclear speckles (Hutchinson et al., 2007). Employing affinity purification of HBc, combined with mass spectrometry-based proteomics, we identified nuclear speckle proteins heterogeneous nuclear ribonucleoprotein M (HNRNPM), splicing factor proline/glutamine-rich (SFPQ), and non-POU domain containing protein (NONO) as HBc interacting partners. Thus, we propose that HBc, as an RNA-interacting protein, associates and regulates host RNAs and specifically lncRNAs. Accumulating evidence supports a regulatory role of lncRNAs in cellular gene expression (Hutchinson et al., 2007). Whether HBc/lncRNA interactions also exerts a role in hepatocyte physiology and virus biosynthesis is presently unknown (Zhang et al., 2016) and these topics merit further investigation.

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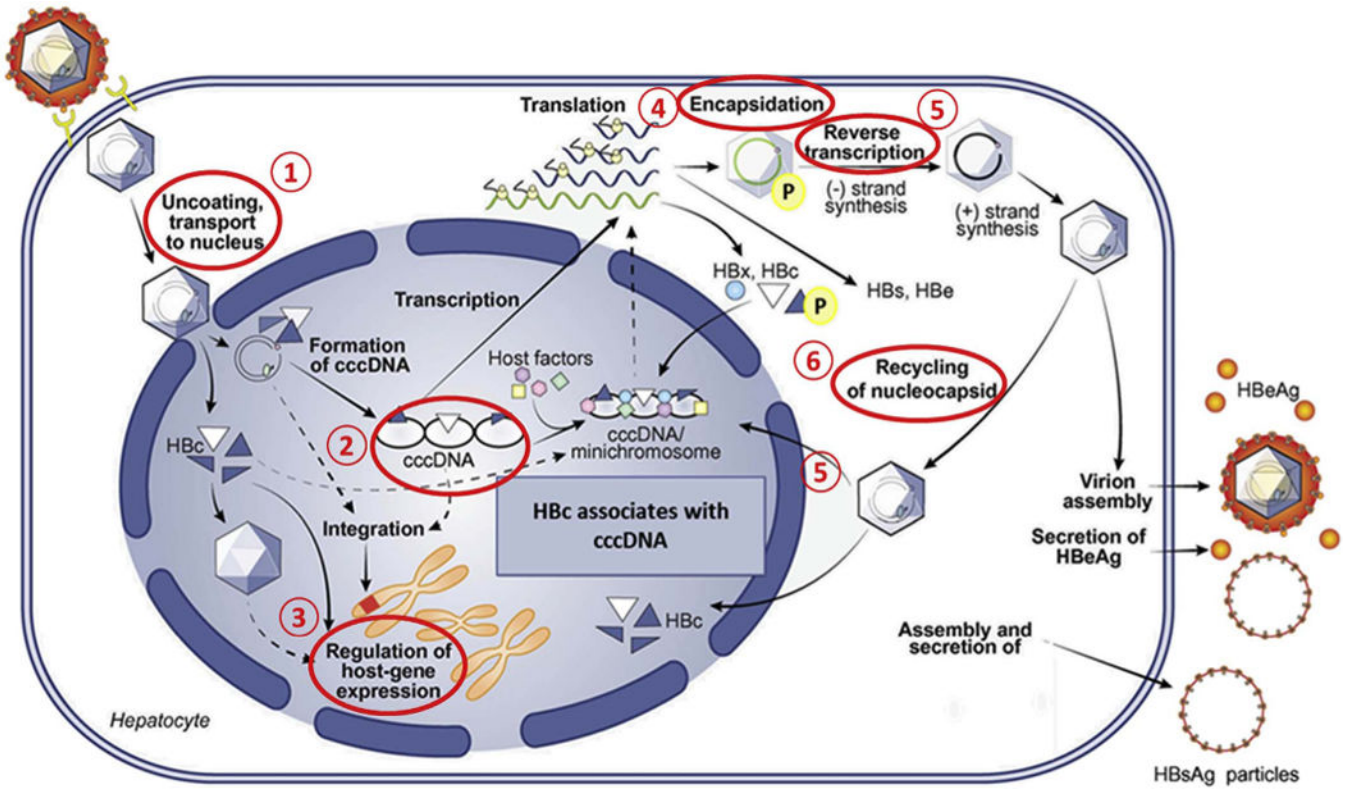


**Fig. 1.**

Stages of the HBV life cycle regulated by HBc. 1) HBc regulates transport and nuclear release of the viral genome (Blondot et al., 2016). 2) HBc associates with cccDNA (Guo et al., 2011). 3) HBc modulates host gene expression (Xie et al., 2017). 4) HBc is required for pgRNA encapsidation (Nassal, 1992). 5) HBc is required for reverse transcription (Lewellyn and Loeb, 2011), and 6) persistence of cccDNA involves recycling of nucleocapsids into the nucleus (Nassal, 2015).


**Fig. 2.**

Arginine-rich motifs of different viral proteins exhibit an abundance of arginine residues within short 10–20 residues. The arginine-rich domain of HBc has distinct nuclear localization (NLS) and cytoplasmic retention signals (CRS) (Li et al., 2010).

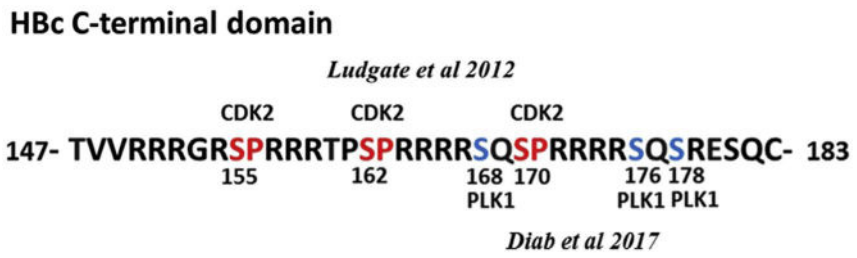


**Fig. 3.**

HBc C-terminal domain (CTD) is a phosphorylation substrate for CDK2 (SP sites at positions 155, 162 and 170) and PLK1 (phosphorylation sites at positions 168, 176 and 178), as shown by Ludgate et al. (2012) and Diab et al. (2017), respectively.

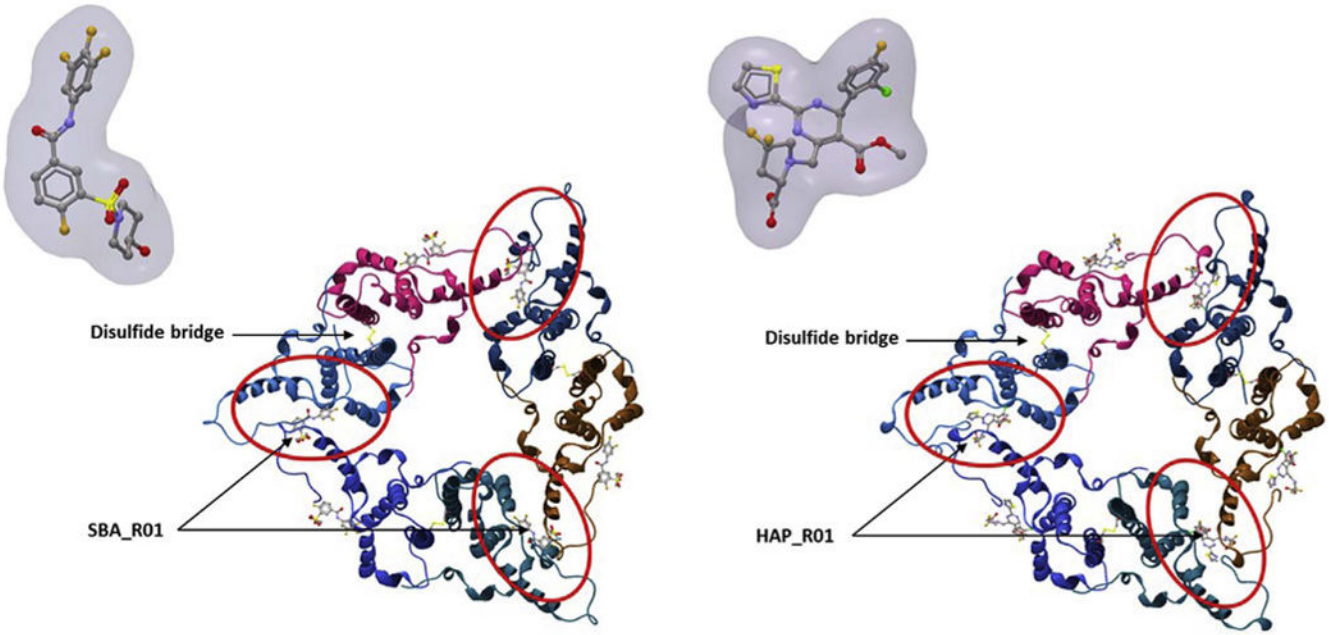


**Fig. 4.**

Top view of an HBc hexamer in complex with CAM sulfamoylbenzamide SBA_R01, PDB: 5T2P (Left) and CAM heteroaryldihydropyrimidine HAP_R01 PDB: 5WRE (Zhou et al., 2017) (Right).

**Table 1**

Kinases that phosphorylate HBc.

| Kinase | Effect on viral replication | Immuno-detected in capsids | Phosphorylation sites | References |
|---|---|---|---|---|
| PKC | Required for envelopment | Yes | ND | Kann and Gerlich, 1994; Wittkop et al., 2010 |
| GAPD-PK | ND | No | ND | Duclos-Vallee et al., 1998 |
| SRPK1/2 | Decrease replication | ND | S155,S162,S170 | Daub et al., 2002 |
| CDK2 | No effect | Yes | S155,S162,S170 | Ludgate et al., 2012 |
| PLK1 | Enhance replication | ND | S168,S176,S178 | Diab et al., 2017 |