Assembly and export determine the intracellular distribution of hepatitis B virus core protein subunits

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Little is known about the parameters and factors that determine the intracellular distribution of the hepatitis B virus core protein (HBc). In order to study HBc in living cells, HBc was tagged with green fluorescent protein (GFP). Being assembly-incompetent, the GFP-fusion protein was distributed equally throughout the cell. Mutational inactivation of known serine-phosphorylation sites within the C-terminal region led to predominantly intranuclear localization. Phosphorylation of these targets, presumably by an SR domain protein kinase, resulted in a predominantly cytoplasmic localization, which suggests active cytoplasmic export or retention. The phosphoserine itself, and not its negative charge, appears essential for the underlying mechanism. In addition, the arginine-rich, protamine-like domain surrounding these phosphorylation sites does not function as the dominant nuclear-localization signal, as had been assumed previously, because neither deleting nor altering these sequences led to a change in intracellular HBc subunit distribution. Restoring the capability of the fusion protein to form capsids by co-assembly with assembly-competent, sterically uncompromised HBc subunits provided a second assay that gave insight into the effects resulting from capsid formation. Assembly was found to be the dominant factor in the cytoplasmic retention of the GFP–HBc fusion protein. Furthermore, the stability of these empty capsids was influenced by the cell-cycle inhibitor nocodazole. Thus, the intracellular distribution of HBc is dominated by cytoplasmic assembly, which is supported by the active nuclear export of HBc subunits, and modulated during the cell cycle by the instability of capsids.

INTRODUCTION

Hepatitis B virus (HBV) is an enveloped virus with a 3.2 kb genome containing four genes, which encode proteins with multiple functions. This is also exemplified by the 183 aa HBV core protein (HBc). It assembles into the nucleocapsid, interacting with the pregenomic RNA and the viral polymerase. In addition, HBc is involved in the process of reverse transcription and also in the second-strand synthesis of the DNA genome. It targets mature nucleocapsids to the endoplasmic reticulum, where it is believed to interact specifically with the envelope protein (Ganem, 1991; Seeger & Mason, 2000; Seifer & Standring, 1995; Wynne et al., 1999). X-ray analyses and cryo-electron microscopy, as well as mutational analyses, have revealed that HBc is a two-domain protein: a globular assembly domain encompassing aa 10–140, and an unstructured regulatory sequence containing a protamine-like domain (Seifer & Standring, 1995; Wynne et al., 1999), which is essential for the encapsidation of the pregenomic RNA and reverse transcription (Köck et al., 2004; Nassal, 1992) and supposedly for nuclear import (see below).

Newly synthesized HBc remains in the cytoplasm of the infected cell, where it assembles into capsids as a first step in the formation of progeny virions. Accordingly, the core protein is mainly detected in the cytoplasm of hepatocytes in acute HBV infection of the liver. Independent of this main pathway, the question remains as to whether HBc subunits possess a specific intranuclear function similar to those suggested for several RNA viruses, such as hepatitis C, dengue and influenza viruses (Bui et al., 2002; Falcon et al., 2003; Wang et al., 2002). In support of such a nuclear function, it has been demonstrated that HBV core antibodies precipitate nucleosomes and nucleoproteins in
HBc-transfected HepG2 cells. This suggests that HBc could function as a non-histone protein or transcription cofactor (Bock et al., 1994). Furthermore, the core protein of duck hepatitis B virus (DHBc) was found in specific intranuclear bodies, co-localizing with splicing-factor compartments, shortly after infection of primary duck hepatocytes (Mabit et al., 2003). As these bodies form early in infection from the core protein subunits, the mechanism of nuclear entry of the hepadnavirus core protein is probably different from the core protein subunits, the mechanism of nuclear entry has been shown that HBV capsids are not able to cross the nuclear membrane (Ganem, 1991; Guidotti et al., 1994), but rather bind to the nuclear pore complex (NPC) and may even proceed into the nuclear basket, where they probably release the viral genome into the nucleoplasm (Kann et al., 1999, 2007; Pante & Kann, 2002; Rabe et al., 2003). Within the above-mentioned protamine-like domain of HBc are three major serine–proline–arginine phosphorylation motifs (SPRRR, SP motifs) (Albin & Robinson, 1980; Gerlich et al., 1982), characterized by Liao & Ou (1995). Deletion or mutational inactivation of the phosphorylation sites within these arginine-rich SP motifs has been shown to prevent phosphorylation of the core protein (Liao & Ou, 1995), resulting in loss of nuclear HBc accumulation (Eckhardt et al., 1991; Yeh et al., 1990) and preventing virus replication (Schlicht et al., 1989). Therefore, the protamine-like, nucleic acid-binding domain has been assigned to function as a nuclear-localization signal (NLS). Yeh et al. (1993), furthermore, observed differences in the extent of HBc protein phosphorylation between intracellular compartments of fixed cells, the cytoplasmic core protein being hyperphosphorylated compared with the nuclear fraction. They reported a change in intracellular HBc distribution during the cell cycle, with the nuclear fraction being highest in the confluent phase. Exchange of the three major serine targets to alanine abolished this change in HBc distribution, leading to the hypothesis that the nuclear localization of HBc is negatively regulated by phosphorylation (Liao & Ou, 1995).

To study the intracellular distribution, regulation mechanisms and cytonucleoplasmic transport of HBc subunits within living cells, we used green fluorescent protein (GFP) fusion constructs. These constructs were similar to those used earlier to investigate the bidirectional nucleocytoplasmic transport of DHBc (Mabit et al., 2001). In HBc, the GFP fusion eliminated its assembly ability, allowing the study of core subunits only in a simplified cell-culture system. Co-expression of unfused core protein restored the assembly capability of the GFP fusion, thus making it possible to investigate the distribution of HBc subunits with and without the effects of capsid assembly.

RESULTS

HBc is unable to form capsids when it is fused to GFP

In cells transfected with pHbc, a plasmid encoding wild-type HBc, the immunoreactive protein was detected predominantly in the cytoplasm (Fig. 1a). This observation is consistent with data from HBV-infected tissue with a higher proliferation rate (Chu et al., 1995; Naoumov et al., 1990). In contrast, the expression of plasmid p2GFP-HBc, in which two tandemly repeated GFP genes are linked to the N terminus of the Hbc sequence, resulted in an equal distribution of GFP fluorescence throughout the cell, including the cytoplasm and nucleus (Fig. 1b). The distribution of the HBc fusion protein was homogeneous, as seen by anti-HBc immunostaining in fixed cells (data not shown); this is in contrast to the patterns observed in earlier work with internally integrated GFP (Kratz et al., 1999; Mabit et al., 2001, 2003). In order to test whether the homogeneous

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**Fig. 1.** Intracellular distribution of GFP-fused HBc differs from that of wild-type HBc. (a) Anti-HBc immunostain of fixed cells transfected with pHBc; the core protein is localized preferentially in the cytoplasm. (b) Fluorescence micrograph of HeLa cells transfected with p2GFP-HBc; the fusion protein shows an even nucleocytoplasmic distribution pattern. Anti-HBc immunostain from 2GFP-HBc-expressing cells showed the same diffuse distribution pattern (not shown). Both pictures were taken at day 4 post-transfection. Bars, 50 μm.
distribution seen reflected protein equilibrium, 2GFP–HBc-expressing cells were permeabilized by adding digitonin (see Methods). As a result of this treatment, fluorescence diffused completely out of the cell within 2–3 min. As digitonin permeabilizes only the plasma membrane (Kutay et al., 1997), this indicates that 2GFP–HBc was freely distributable throughout the cell and was not attached stably to intracellular structures.

To test whether the altered intracellular distribution of the GFP fusion was related to loss of capsid formation, lysates of HBc-expressing cells were separated according to assembly state by sedimentation through a sucrose gradient followed by Western blotting, stained for HBc (Fig. 2a, b) or GFP (Fig. 2c). In the control with wild-type HBc (Fig. 2a), the core protein was found mostly in the position of capsids, as reported previously (Kratz et al., 1999). In contrast, 2GFP–HBc was found almost completely in the top fractions (Fig. 2b, c), the position of unassembled subunits, demonstrating that the GFP-fusion protein lacked the ability to form stable capsids. Further analysis of the top fractions from the sucrose gradients described in Fig. 2 by non-reducing SDS-PAGE (Nassal & Rieger, 1993; Nassal et al., 1992) suggests that the 2GFP–HBc fusion was already impaired at the level of HBc dimer formation. As shown in Fig. 3(a) (see also Supplementary Fig. S1, available in JGV Online), the GFP-fusion protein migrated essentially as a single band to a position of about 80 kDa (the molecular mass of the protein monomer being 84 kDa). In contrast, wild-type HBc migrated as two major species (Fig. 3b), one band corresponding to the molecular mass of the HBc monomer (21.5 kDa) and the other at approximately 120 kDa, probably representing a distinct higher assembly intermediate, possibly a trimer of HBc dimers (Belnap et al., 2003; Nassal et al., 1992). This latter result apparently reflects the strong tendency of wild-type HBc to rapidly form highly stable dimers, which in turn support the efficient formation of intra-dimer S–S links by post-work-up oxidation. The absence of an analogous signal by non-reducing SDS-PAGE in lysate from 2GFP–HBc-transfected cells thus indicates that the GFP fusion causes minor perturbations in the HBc structure that destabilize the core protein dimer, or at least prevent intra-dimer S–S links from forming.

**Fig. 3.** 2GFP–HBc subunits do not form stable dimers. Fractions 02 of the gradients shown in Fig. 2 were run on a non-reducing SDS-PAGE gel. A Rainbow molecular mass marker (Amersham) was used as a protein marker. (a) 2GFP–HBc migrates mainly as a single band to a position of about 80 kDa, as estimated for this protein. (b) In contrast, wild-type HBc migrates to mainly two distinct positions. The calculated molecular mass of one HBc subunit is 21 kDa; the higher single band at about 120 kDa is unlikely to be equivalent to dimers. The weaker bands around 46 kDa may reflect dimers. The distinct higher band at about 120 kDa presumably represents higher aggregates, possibly trimers of dimers.

**Fig. 2.** 2GFP–HBc and HBc form different bands in sedimentation gradients. Cell lysates from transfected HeLa cells obtained at day 4 post-transfection were separated by sedimentation in a sucrose gradient. Western blots of the sedimentation fractions from top (01) to bottom (14) were probed with anti-HBc antibodies and detected with peroxidase. (a) Wild-type HBc was detected in fractions 01–03 (subunits and dimers) and 07–09 (empty HBV capsids). (b) Sedimentation of 2GFP–HBc; the core protein is detected predominantly in the top fractions and not in the capsid position, indicating its inability to form capsids. (c) Blot (b) probed with a mAb against GFP; the core protein is visualized in more fractions, probably due to a higher sensitivity of the anti-GFP antibody.

**GFP fusions are capable of co-assembling with wild-type HBc**

To test whether the loss of assembly competence of the fusion protein was due to sterical interference by the bulky GFP extension, the protein was co-expressed with wild-type HBc by mixed transfection of the HBc and 2GFP–HBc plasmids. As shown in Fig. 4(a), this led to a predominantly cytoplasmic distribution of the GFP fluorescence. Cell lysates were analysed by sedimentation in a sucrose gradient followed by Western blot analysis in order to confirm that this change was caused by restored capsid assembly (Fig. 4b–d). As expected, a significant fraction of the GFP fusion was detected, together with the wild-type protein, by antibodies against either HBc (Fig. 4b, c) or GFP (Fig. 4d) in the capsid position. Thus, although compromised sterically by its GFP moiety from forming capsids on its own, the HBc part of the fusion protein was still capable of co-assembling into mixed capsids with HBc subunits that were free from this constraint.

On closer inspection, the sedimentation analysis revealed a distinctly lowered assembly competence of the GFP fusion,
as only about half of it migrated to the capsid position (Fig. 4d), compared with >80% of the unfused HBc protein subunits (Fig. 4c). This biochemical quantification is in apparent contrast to our prediction from the immunofluorescence data, where the fusion protein was detected mainly in the cytoplasm. A possible explanation for this difference may be the presence of lower-order mixed aggregates, which may result in lowered transport across the nuclear membrane. Furthermore, a part of the mixed capsids possibly disintegrates during the sedimentation process. Taken together, our observations thus fit the concept that capsid assembly and dimer formation are both reversible and dynamic processes. As HBc dimers form in statu nascendi (Chang et al., 1994), it is to be expected that the dissociation of wild-type HBc homo-dimers may be a prerequisite for the formation of heterodimers with monomeric 2GFP–HBc.

**Mutational elimination of three major phosphorylation sites leads to predominantly nuclear HBc distribution**

Based on mutational inactivation, three serine-phosphorylation sites at positions 155, 162 and 170 have been identified as important elements that prevent the nuclear accumulation of assembly-competent wild-type HBc (Eckhardt et al., 1991; Liao & Ou, 1995). To test whether this observation was upheld in the assembly-incompetent GFP-fusion system, plasmids encoding analogously mutated GFP fusion proteins were constructed. In addition, these constructs offered an advantage in allowing observation of the core subunits within a dynamic and reversible balance that is not altered by capsid formation. In cells expressing 2GFP–HBc,AAA, carrying exchanges of these three serines to alanine, the fluorescence was no longer found to be distributed evenly, but predominantly accumulated in the nucleus (Fig. 5b(ii)). Exchange of the serines to glutamate (in 2GFP–HBc,EEE), introducing negative charges mimicking phosphorylation, also resulted in preferential, although somewhat less pronounced, nuclear enrichment [Fig. 5b(iii)]. That these changes reflected a redistribution of a freely diffusible protein was confirmed by digitonin permeabilization of 2GFP–HBc,AAA-expressing cells. As a result of this treatment, the mutant protein diffused rapidly and completely out of the cell, as described above for wild-type HBc (data not shown).

Thus, mutational inactivation of three serine-phosphorylation sites in the C-terminal domain changed the long-term distribution balance of HBc subunits towards the nucleus. We interpret these data to indicate that the shift of HBc subunits towards the cytoplasm probably reflects the phosphorylation of these serine residues. As charge differences between the substituting amino acids had only a minor influence, the important feature created by this modification appears to be the presence of phosphoserine residues per se, rather than the negative charges introduced by phosphorylation.

Interestingly, when co-expressing 2GFP–HBc,AAA with an assembly-competent alanine mutant, HBc,AAA, the fluorescence was found predominantly in the cytoplasm [Fig. 6a(ii)]. The same was seen using glutamate mutants [Fig. 6a(iii)]. In both cases, this was accompanied by capsid formation, as revealed by sedimentation in a sucrose gradient followed by Western blotting (data not shown), analogous to the data presented for wild-type HBc in Fig. 4. The results of these experiments suggest that the assembly of core protein subunits to capsids is responsible for the observed protein shift and is the dominant factor that leads to enhanced cytoplasmic HBc accumulation, regardless of changes within the phosphorylation sites.

**The protamine-like domain serves as an export signal for HBc subunits, but has no major NLS function**

The above data suggest that the presence of the three serine-phosphorylation sites in the C-terminal tail of HBc is relevant for the nuclear export of core subunits, as their
loss leads to an intranuclear accumulation. However, they are also compatible with the previously proposed hypothesis that phosphorylation of serine residues enhances nuclear import by modulating the nuclear-import function of the surrounding arginine clusters (Kann et al., 1999; Yeh et al., 1990). To distinguish between these alternatives, we constructed two additional plasmids. The first is 2GFP–HBc,Rminus, containing the target phosphoserines, but lacking six of the 16 C-terminal arginines due to alanine replacement [Fig. 5b(iv)]. In this construct, none of the four arginine clusters was left intact and no arginine repeat remained, whereas the serine sites were not altered.

### Fig. 5. Intracellular distribution of 2GFP–HBc proteins carrying mutations affecting the C-terminal basic domain, altering the essential three serines (red) and/or the four arginine-rich clusters (blue).

(a) Sequences and (b) fluorescence micrographs of the intracellular distribution of cells transfected with wild type and different mutants. (i) The non-mutated 2GFP–HBc as control shows an equal distribution, as already shown in Fig. 1. (ii, iii) 2GFP–HBc mutants with exchange of three phosphoserine residues to alanine (ii; 2GFP–HBc,AAA) or to glutamate (iii; 2GFP–HBc,EEE). HBc is located predominantly in the nucleus (AAA > EEE). (iv, v) Modifying the C-terminal domain, either by replacing six of 16 arginines with alanine (orange) (iv; 2GFP–HBc,Rminus) or by complete removal of the terminal 34 aa (v; 2GFP–HBc149stop). The last two mutations did not result in a change of intracellular distribution compared with the wild type. All images were obtained at day 4 post-transfection.

### Fig. 6. The state of the cell influences HBc distribution.

Cells containing preformed mixed capsids [a(i–iii) as shown in Figs 4 and 5] were treated at day 4 post-transfection with the cell-cycle inhibitor nocodazole [b(i–iii)]. (a) (i) Cotransfection of unmutated 2GFP–HBc and HBc; (ii) triple-alanine mutants; (iii) triple-glutamate mutants. In all three images, the fluorescence is seen predominantly in the cytoplasm. (b) After treatment with nocodazole, the fluorescence was distributed more comparably to that of cells transfected with the assembly-incompetent fusion protein only: the wild type was distributed equally (i), whereas the mutants showed their predominantly nuclear accumulation (ii, iii).
Secondly, we generated a construct, 2GFP-HBc149stop, that lacked the complete C-terminal tail, including the presumed NLS and the phosphorylation sites [Fig. 5b(v)]. Contrary to the expectation that a lack of the presumed NLS would lead to predominantly cytoplasmic localization, the GFP fluorescence was found to be distributed homogeneously throughout the cell after transfection with either plasmid [Fig. 5b(iv, v)], similar to what was observed for the wild-type 2GFP–HBc [Fig. 5b(i)]. These results show that the arginine clusters are not part of a strong nuclear-import signal affecting the long-term distribution balance of HBc subunits analysed in our assay. Thus, nuclear import of HBc subunits is probably a diffusion-dependent process.

The state of the cell influences the balance of HBc assembly and disassembly

Based on subcellular fractionation, the phosphorylation of HBc has previously been suspected to interfere with nuclear import (Yeh et al., 1993). These authors used an HBc-expressing Vero cell line, either synchronized by serum starvation or arrested by using the cell-cycle inhibitor aphidicolin, to demonstrate that the intracellular distribution of wild-type HBc changes during the progress of the cell cycle. It was observed that HBc was preferentially cytoplasmic during the S phase, but mainly intranuclear during the G1 phase of mitosis. To check whether these changes also occur at the subunit level, we tested the effect of another cell-cycle inhibitor, nocodazole, which arrests cells in the G2 or M phase. Nocodazole treatment of cells expressing the fusion proteins alone led to no change in the distribution of GFP fluorescence (data not shown). However, when the inhibitor was tested on the mixed capsids at day 4 post-transfection, when a stable, predominantly cytoplasmic distribution was attained [Fig. 6a(i–iii)], we found that, about 24 h after treatment, the wild type was distributed equally [Fig. 6b(i)], whereas the mutants showed nuclear accumulation [Fig. 6b(ii, iii)] (also see Supplementary Table S1, available in JGV Online). In other words, the fluorescence was distributed in a fashion comparable to that in cells transfected with the assembly-incompetent fusion protein only. These results suggest that the state of the cell could influence the balance of HBc assembly and disassembly.

The here-presented data confirm that the intracellular distribution of HBc subunits is regulated strongly by cytoplasmic capsid formation. They further suggest that HBc assembly itself is modulated by changes in cellular activity, e.g. during cell proliferation. Phosphorylation at serine residues within the tail of HBc appears to be part of this system by upregulating nuclear export of HBc subunits. A candidate kinase with a requirement for neighbouring basic arginines is SR domain protein kinase (SRPK), which has been shown to phosphorylate the serines within this region (Daub et al., 2002; Enomoto et al., 2006).

DISCUSSION

We used GFP-tagged HBV core proteins for in vivo studies of the parameters, factors and signals that determine the proteins’ intercellular distributions. The N-terminal GFP fusions used displayed several properties that turned out to be particularly suitable for this purpose, in particular for studies with and without the protein’s assembly function: (i) the bulky GFP domain sterically blocks the formation of stable homodimers and higher aggregates, as the protein subunits remained in the monomeric state in non-reducing SDS-PAGE. In the case of a parental unfused HBc, homodimer formation proceeds very rapidly, probably already in statu nascendi (Chang et al., 1994). (ii) The protein equilibrated evenly across the nuclear membrane despite its large size of 84 kDa, probably by diffusion. (iii) GFP-tagged HBc subunits maintained the capability to co-assemble with assembly-competent, sterically uncompromised HBc subunits. Such assembly into mixed capsids provided a second assay giving insight into the effects of capsid assembly. (iv) Finally, the protein remained freely diffusible without a detectable bias from stable attachment to cellular structures, as demonstrated by its release from digitonin-permeabilized cells. This is in contrast to results from earlier studies with the parental HBc in fixed cells, or of assembly-competent internal GFP insertions (Kratz et al., 1999) or of a GFP-tagged DHBc protein, which all resulted in heterogeneous intranuclear localization core protein patterns. It should be emphasized that our data reflect the end points of long-term equilibration, as all steps appear to be fully reversible, and therefore do not permit the evaluation of nucleocytoplasmic-transport rates.

Surprisingly, our experiments indicate that the basic arginine clusters present within the C terminus of HBc have no dominant NLS function in the import of core subunits. Neither removing the whole C-terminal domain nor exchanging six of the 16 arginines in these clusters to alanine led to a change in the cellular distribution of assembly-incompetent HBc-fusion proteins. Therefore, active NLS-mediated nuclear import of HBc protein subunits seems unlikely. Conceivably, the protamine-like, nucleic acid-binding domain may participate in the reversible intranuclear retention of HBc protein subunits, which we observed after mutational inactivation of three serine-phosphorylation sites in arginine-rich regions (Fig. 5).

This interpretation does not necessarily exclude the presence of a weak NLS function that may only become apparent in the multivalent HBV capsid. For example, the C-terminal domain seems to direct viral capsids to the NPC (Kann et al., 1999, 2007).

Extending earlier data (Liao & Ou, 1995), the inactivation of the three major serine-phosphorylation sites within this region by exchanging serine to alanine led to intranuclear HBc accumulation in a system preventing assembly. Exchange to glutamate also resulted in predominantly
nuclear localization of the core protein, indicating that a negative charge was not sufficient to increase the cytoplasmic HBC fraction. Thus, the presence of the multiple phosphorylation sites could either enhance nuclear export or reduce import. As the deletion of the complete C terminus of the core protein did not result in predominantly nuclear localization, we conclude that the formation of the phosphoserines is an essential component for active nuclear export of HBC subunits. This assumption is supported by data of Daub et al. (2002), which demonstrate that SRPK1 and SRPK2 phosphorylate HBC C-terminally. In addition, preliminary experiments in which we supertransfected cells expressing 2GFP–HBC with an SRPK1-producing plasmid showed that the equal intracellular distribution of 2GFP–HBC was changed to a preferentially cytoplasmic one (Supplementary Fig. S2, available in JGV Online). These observations further support the assumption that phosphorylation of HBV core subunits enhances their export.

Based on these observations, we further suggest that the phosphoserine structure itself is recognized by an adaptor protein similar to that observed for other phosphoproteins (Gama-Carvalho & Carmo-Fonseca, 2001). For example, the 14-3-3 family of proteins mediates signal transduction by binding to phosphoserine-containing proteins, in turn leading to interaction with a nuclear-export signal sequence identified in helix z1 of 14-3-3 (Rittinger et al., 1999; Yaffe et al., 1997). The sequence RRXXpSPRX, identified as refining substrate specificity for 14-3-3, is very similar to the RRXXpSPRRR motif repeatedly present in the basic clusters of Hbc.

Finally, our data show that capsid assembly leads to a reversible cytoplasmic retention of HBC subunits, counter-balanced by a dissociation of capsids, and is influenced by the state of the cell. Whilst cell-cycle inhibitors had no effect on the intracellular distribution of assembly-incompetent 2GFP–HBC subunits, we found a change in their distribution balance when the cell-cycle inhibitor nocodazole was added to cells containing predominantly cytoplasmic mixed HBC capsids, achieved by the co-transfection of GFP–HBC with unfused core proteins. This treatment, independently of the presence or absence of the arginine clusters and the three major phosphorylation sites, led to a distribution known from the assembly-incompetent fusion proteins. These data suggest that cytoplasmic capsid assembly is the dominant pathway for HBC subunits in HBV-infected cells and that this mechanism can be modulated in different ways, e.g. by destabilization of genome-free HBC capsids. Conceivably, HBV uses the formation of such empty capsids to reduce the amount of diffusible HBC subunits capable of nuclear entry.

In conclusion, the data obtained in this study demonstrate clearly that the fate of HBC subunits is dominated by two major reactions that are controlled by separate segments of the core protein polypeptide. Cytoplasmic capsid assembly is determined by the globular assembly domain encompassing the N-terminal 80% of the protein, whereas active nuclear export is regulated by serine phosphorylation in the unstructured C-terminal domain. It should be noted that the assembly process proceeds much faster in the infected cell, as it becomes irreversible by nucleocapsid formation in the presence of pregenomic RNA and viral polymerase (Bartenschlager et al., 1990; Seifer & Standring, 1995).

We can only speculate about a potential function of intranuclear hepadnavirus core protein. Its accumulation in specific dots, co-localizing with genomic RNA and splicing factors, has been taken to indicate that it may be part of the factories involved in the synthesis and maturation of nascent HBV transcripts (Bock et al., 1994; Mabit et al., 2003). Another possible reason for intranuclear HBC may be the necessity of a nuclear passage to acquire protein modifications, e.g. phosphorylation, that are subsequently required for the cytoplasmic assembly of genome-containing nucleocapsids of HBV.

**METHODS**

**Plasmids.** HBC cDNA was amplified by PCR from plasmid pCH-3142 (Frotzer et al., 1999) and inserted via HindIII and BstEII restriction sites into plasmid pCD16-S-GFP (Mabit et al., 2001). This resulted in pGFP-HBC, a plasmid containing the HBC cDNA linked N-terminally at aa 3 to GFP under the control of the cytomegalovirus (CMV) immediate-early promoter. Digestion of a tandem-repeat GFP dimer (Mabit et al., 2001) via BstBI and insertion of the thus-obtained fragment into pGFP-HBC resulted in a plasmid that expressed two GFP molecules linked C-terminally to HBC, designated p2GFP-HBC. Mutants of this construct with amino acid exchanges of certain serine residues (underlined) to alanine or glutamate in the C-terminal domain of HBC (RRGRSPRRTSPRRRSQSPRRRSQSQRESQ) were obtained via PCR alteration (Gazina et al., 2000). This resulted in plasmids designated p2GFP-HBC,AAA and p2GFP-HBC,EEE, respectively. p2GFP-HBC149stop was constructed by inserting a stop codon at position 150 using mutational PCR amplification. The resulting protein lacked the complete C-terminal domain of 38 aa.

Using the four oligodeoxynucleotides (MWG-Biotech) Fw1, Fw2, Bw1 and Bw2, we exchanged six of the 16 arginines within the protamine-like domain of HBC (underlined) with alanine (RRGRSPRRTSPRRRSQSPRRRSQSQRESQ), without altering the phosphorylation sites, in order to disrupt the basic arginine cluster (see Supplementary Fig. S3, available in JGV Online). Fw2 and Bw1 (100 pmol each) were treated with T4-polynucleotide kinase (New England Biolabs) for 30 min at 37 °C in the presence of T4 buffer containing 1 mM ATP. Five minutes at 95 °C inactivated the kinase. Then, 100 pmol each of Fw1 and Bw2 was added. The total volume was adjusted to 30 μl and to a final concentration of 0.2 M NaCl, 0.1 mM EDTA and 10 mM Tris(HCl). After 5 min at 95 °C and slow recooling to room temperature, the two double-stranded DNA fragments were ligated by adding T4 ligase (New England Biolabs). The resulting product was inserted via BspEI and XbaI sites into plasmid p2GFP-HBC, producing in turn p2GFP-HBC, Rminus.

pHBC was constructed by the digestion of p2GFP-HBC with KpnI and HindIII and by religation via a linker sequence. pHBC is therefore a plasmid expressing the original core protein under the control of the CMV promoter. The same was done for pHBC,AAA and pHBC,EEE (see Supplementary Fig. S4, available in JGV Online).
All final constructs were checked by sequencing the areas of interest (Supplementary Table S1 lists the distribution patterns and sedimentation positions of the different GFP–HBc fusion proteins).

**GFP fluorescence and immunofluorescence analysis.** All experiments were performed in HeLa cells cultivated in Dulbecco’s modified Eagle’s medium [10 % (v/v) fetal calf serum, penicillin and streptomycin (1000 U 1^{-1} each), 1 % non-essential amino acids, 20 mM glutamine] in six-well plates or on coverglass slides. Single experiments were repeated in HepG2, Huh7 and 293 cells, essentially confirming our data. Transfection of cell lines was performed by a standard calcium phosphate protocol (Kingston et al., 2001) (transfection efficacy was approx. 75–80 %). GFP was analysed with an inverted fluorescence microscope (Leica). To allow the proteins to equilibrate within the cell, the following experiments were performed and all images were obtained at day 4 post-transfection, if not mentioned otherwise.

For immunofluorescence analysis, the cells were fixed with 4 % formalin in PBS for 30 min and then permeabilized for 60 min with 0.25 % Triton X-100 in PBS. Immunostaining was performed using a polyclonal rabbit serum recognizing HBc (H801), as described by Nassal & Rieger (1993). A fluorescein-stained secondary antibody (Alexa green; Eugene Oregon) was used in the second step for detection.

**Cell-cycle inhibition.** To investigate the influence of cell-cycle inhibitors, aphidicolin or nocodazole (Sigma-Aldrich) was added to the medium at day 4 post-transfection and fluorescence microscopy was performed up to 72 h afterwards. As aphidicolin as used by Yeh et al. (1993) was too cytotoxic for HeLa cells, our results were obtained after nocodazole treatment (Supplementary Table S1).

**Treatment with digitonin.** Cells were permeabilized with digitonin in order to rule out stable binding of the 2GFP–HBc proteins to intracellular structures. Transfected cells were changed to a serum-free medium at day 4 post-transfection and were treated with digitonin at a final maximum concentration of 80 µg ml^{-1} added under the control of fluorescence microscopy. Digitonin removes cholesterol from the cellular membrane, thereby permeabilizing it (Kutay et al., 1997). Soluble cytoplasmic compounds are released, whereas the nuclear envelope is not affected (Kutay et al., 1997).

**Sedimentation analysis.** Sucrose-gradient centrifugation, which serves to separate HBc capsids from non-assembled core proteins, was first used and described by Zhou & Standring (1992), but modified several times thereafter. Total protein lysate from transfected cells was added to an ultracentrifuge tube containing a sucrose gradient; layers of 60, 50, 40, 30, 20 and 10 % (w/v) sucrose in PBS were used. Sedimentation was performed by ultracentrifugation at 55 000 r.p.m. for 40 min at 20 °C in a TLS55 rotor (Optima TLX). Fourteen fractions were collected from top to bottom. In these gradients, HBc capsids are known to sediment in fractions 07–09, whereas the nuclear envelope is not affected (Kutay et al., 1997). Aliquots of each fraction were analysed by SDS-PAGE and Western blotting.

**Western blot analysis.** The Laemmli system was used for the SDS-PAGE analysis (12 % polyacrylamide, 0.1 % SDS). Then, Western blot analysis was performed by standard methods (Sambrook et al., 1989). The primary antibody was the above-mentioned anti-HBc polyclonal rabbit serum (H801) or a monoclonal mouse anti-GFP antibody (Boehringer), followed by appropriate secondary antibodies conjugated to peroxidase. Peroxidase was detected with the chemiluminescent substrate ECLplus (Amersham Pharmacia) visualized on X-ray films. For technical reasons, the anti-HBc antibody did not recognize the fusion protein reproducibly in higher fractions at 80 kDa as well as the anti-GFP antibody.

**Non-reducing SDS-PAGE.** Aliquots from individual sucrose-gradient fractions were analysed as described above, except that the loading buffer did not contain dithiothreitol or mercaptoethanol. A Rainbow molecular mass marker (Amersham) was used as a protein marker.

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