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Biopsies from patients show that hepadnaviral core proteins and capsids – collectively called core – are found in the nucleus and cytoplasm of infected hepatocytes. In the majority of studies, cytoplasmic core localization is related to low viraemia while nuclear core localization is associated with high viral loads. In order to better understand the molecular interactions leading to core localization, we analysed transfected hepatoma cells using immune fluorescence microscopy. We observed that expression of core protein in the absence of other viral proteins led to nuclear localization of core protein and capsids, while expression of core in the context of the other viral proteins resulted in a predominantly cytoplasmic localization. Analysis of which viral partner was responsible for cytoplasmic retention indicated that the HBx, surface proteins and HBeAg had no impact but that the viral polymerase was the major determinant. Further analysis revealed that ε, an RNA structure to which the viral polymerase binds, was essential for cytoplasmic retention. Furthermore, we showed that core protein phosphorylation at Ser 164 was essential for the cytoplasmic core localization phenotype, which is likely to explain differences observed between individual cells.

INTRODUCTION

Hepatitis B is a major health problem responsible for 600 000 deaths per year (WHO, 2012). Infection is caused by the human hepatitis B virus (HBV) and leads to liver fibrosis, cirrhosis and primary liver cell carcinoma. Belonging to the pararetroviruses, HBV comprises a partially dsDNA genome, which is derived from reverse transcription of an RNA pregenome (PG) and incomplete second strand DNA synthesis (genome maturation). Transcription of the PG occurs in the nucleus of hepatocytes requiring transport of the DNA genome from the cell periphery into the nucleus. Similarly to other DNA viruses, the viral genome is transported within the viral capsid, which is composed of 240 copies of the core protein. Upon arrival on the nuclear side of the nuclear pore (Schmitz *et al.*, 2010), the capsid

dissociates to core protein dimers leading to release of the viral genome (Rabe *et al.*, 2003). As core proteins assemble in the absence of any other viral or cellular factor at increased concentrations (Seifer & Standring, 1995), the core dimers reassemble in the nucleus to form capsids (Rabe *et al.*, 2009).

After nuclear release, the genome is then repaired to a covalently closed circular form, which is the template for transcription of five mRNA sets. The shortest mRNA encodes HBx, which is a transactivator of multiple signalling cascades including protein kinases such as protein kinase C (Diao *et al.*, 2001). In immortalized hepatocytes, HBx is not essential for virus production (Blum *et al.*, 1992) but in highly differentiated hepatocytes, such as HepaRG cells, HBx is essential (Lucifora *et al.*, 2011).

Two further subgenomic mRNAs encode the three surface proteins (LHBs, MHBs and SHBs). The surface proteins share a common C-terminal section, termed S, but differ in their N-terminal domains, termed preS1 and preS2. The

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One supplementary figure and one supplementary table are available with the online Supplementary Material.

preS1/preS2 domains of LHBS have two possible localizations (Bruss *et al.*, 1994), in either the luminal side or cytosolic face of the endoplasmic reticulum (ER). The junction of preS1 and preS2 is essential for interaction with the viral capsid (Bruss, 2004). Interaction of the capsid with the surface proteins requires genome maturation (Gerelsaikhan *et al.*, 1996) and occurs at multi-vesicular bodies (MVB; Watanabe *et al.*, 2007). The surface proteins are overexpressed with regard to the quantity required for virion formation, and the excess forms spheres or filaments, which are secreted.

HBV also encodes two mRNAs of supergenomic length. Only the longer mRNA contains the AUG start codon for the e protein (HBeAg). The primary translation product of HBeAg comprises a signal peptide (preC), which directs the polypeptide to the secretory pathway (Garcia *et al.*, 1988) but a limited amount is also found in the cytosol (Duriez *et al.*, 2008).

The shorter of the supergenomic mRNAs is the PG and encodes the core protein (Beck & Nassal, 2007). Core proteins assemble into capsids even in the absence of other viral proteins. *In vivo*, the core proteins become phosphorylated at different serine residues (Gerlich *et al.*, 1982) by cellular protein kinases. Amongst these is PKC alpha, which was shown to be essential for genome maturation (Wittkop *et al.*, 2010). Core proteins expose a nuclear localization signal (NLS), which causes nuclear import by the nuclear transport receptors of the importin family (Rabe *et al.*, 2003). For assembled capsids at least, nuclear import requires phosphorylation (Kann *et al.*, 1999). While the nuclear transport capacity of core is well established, a recent publication also reported nuclear export of core protein, which was thought to be implicated in export of HBV specific transcripts by the TAP/NxF1 pathway (Li *et al.*, 2010).

Either by leaky scanning or by internal initiation, the PG also encodes the viral polymerase (pol) from a start codon downstream of the core AUG (Hwang & Su, 1998; Fouillot *et al.*, 1993; Chang *et al.*, 1989). Pol requires interactions with different heat-shock proteins (at least hsp40, hsp70 and hsp90; Beck & Nassal, 2003; Hu *et al.*, 2004; Stahl *et al.*, 2007) for binding to ε. Subsequently the pol-ε complex becomes encapsidated into the assembling capsids but only if core is phosphorylated at Ser 162 (Gazina *et al.*, 2000). Conversion to DNA is facilitated by pol and occurs inside the capsid requiring phosphorylation of core at further sites (Gazina *et al.*, 2000).

Both pol and core protein are overexpressed during infection. Pol can be found throughout the entire cytoplasm in HuH-7 and HepG2 cells after transfection of supergenomic HBV DNA (Cao & Tavis, 2004). Excessive core is found in the nuclei of hepatocytes in infected patients (Chu & Liaw, 1987, 1997) in the form of assembled capsids (Gerlich *et al.*, 1982) and it is thought that these capsids are derived from nuclear import of unassembled core proteins (Guidotti *et al.*, 1994). Accordingly, nuclear capsids are devoid of the viral genome (Gerlich *et al.*, 1982).

The localization of core varies between different cells and expression systems but the reasons for the differing localization remain unclear. In HepaRG cells, which can be infected with HBV, core has been detected in the cytoplasm and occasionally in the nucleus (Gripon *et al.*, 2002). The localization of core in humans is also variable: Akiba *et al.* (1987) and Sharma *et al.* (2002) observed more frequent nuclear localization while others observed a mainly cytoplasmic core and/or capsid localization (Liu *et al.*, 2009; Michalak & Nowosławski, 1982; Park *et al.*, 1999; Petit & Pillot, 1985). In terms of disease and viral load, the majority of studies associate cytoplasmic capsids with high hepatocellular injury (Chu *et al.*, 1995, 1997; Kim *et al.*, 2006; Naoumov *et al.*, 1990) and a low level viraemia (Liu *et al.*, 2009) while nuclear dominance is associated with high viral load and minor hepatitis activity (Chu *et al.*, 1997).

As core and capsid localization in different compartments is poorly understood, we analysed factors influencing core protein and capsid trafficking using two hepatoma cell lines.

RESULTS

Core localization in the context of other viral proteins

We first transfected a 1.1mer of the HBV genome (genotype A; pHBV_1.1mer) into HuH-7 and HepG2 cells, which represent hepatoma cells in different differentiation states (Su & Yee, 1992; Yaginuma *et al.*, 1987). HepG2 cells have been shown to produce infectious virions (Sells *et al.*, 1988). The transfected plasmid encodes all hepadnaviral proteins under the control of their physiological promoters. We monitored core and capsid localization using two antibodies: the monoclonal mouse antibody Fab3105, which detects core protein dimers, higher assembled forms and HBeAg (Belnap *et al.*, 2003; Watts *et al.*, 2010), and a polyclonal rabbit antibody (DAKO) reacting with core hexamers and with assembled capsids (Rabe *et al.*, 2009). As assembly of core monomers to dimers and from hexamers to entire capsids occurs rapidly (Zlotnick *et al.*, 1999), we concluded that the Fab3105 stain detects all apparent forms of core and HBeAg, while the DAKO antibody detects virtually only capsids.

Fig. 1(a) shows the merged results of five transfections demonstrating that in the vast majority of cells, capsids and core proteins localized predominantly in the cytoplasm. In HuH-7 cells stained with Fab3105, 92 % of cells exhibited predominant cytoplasmic accumulation, 3 % exhibited an equal distribution in the nucleus and cytoplasm and 5 % showed a predominantly nuclear stain (for results of individual experiments, see Table 1).

Based on the multiplicity of the experiments performed with different plasmid preparations, we calculated an SD of 13 % for the DAKO stain and 5 % for the Fab3105 stain
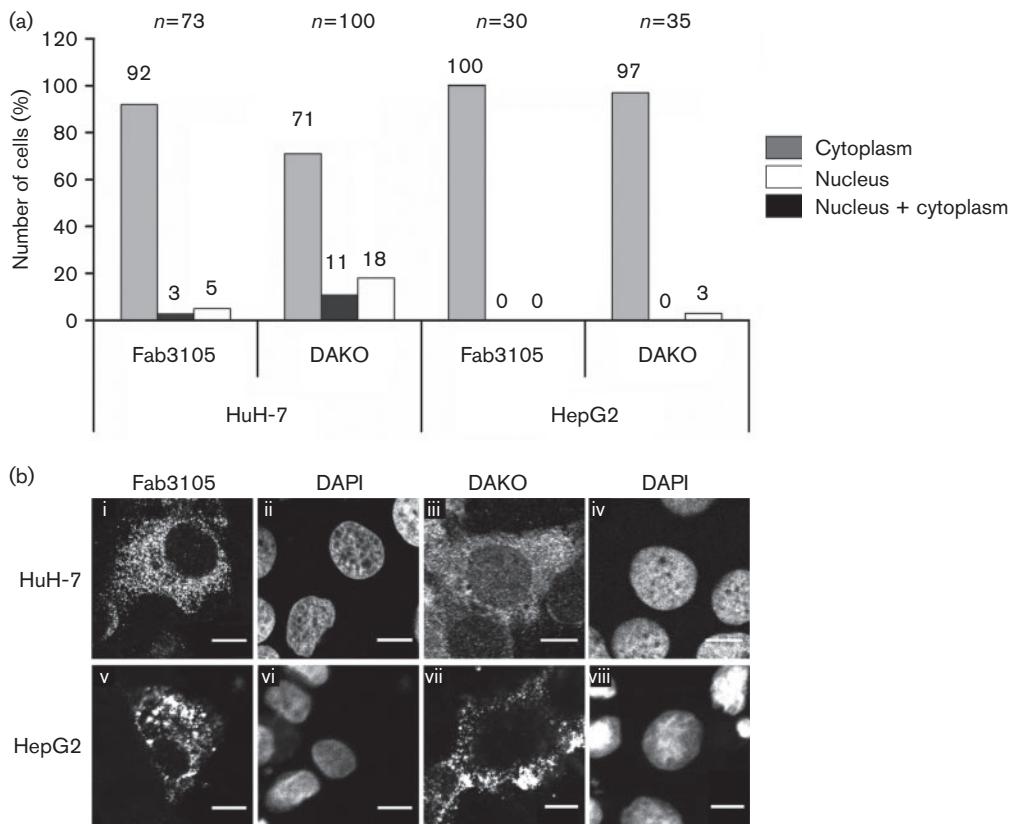
Fig. 1. Core localization in the context of other viral proteins. (a) Determination of core concentration depicted as quantitative results of three transfections for Fab3105 and five transfections for DAKO. HuH-7 and HepG2 cells were transfected with pHBV_1.1mer. Cells were immunostained with a mouse monoclonal anti-HBc antibody (Fab3105) or a polyclonal rabbit anti-HBc antibody (DAKO). The columns show the predominant localization of core. Accumulation of fluorescence in the cytoplasm or nucleus is shown in grey or white columns, respectively. Black columns indicate an equal distribution between the nucleus and cytoplasm. The numbers above each column show the percentage of core/capsid positive cells. (b) Localization of core in individual cells. i–iv: HuH-7; v–viii: HepG2; i, v: Fab3105; iii, vii: DAKO. Nuclei were visualized by DAPI staining (ii, iv, vi, viii). Bars, 10 μm.

(Table 1). Thus, we considered that the difference between a value of 92 % in HuH-7 cells with a dominant cytosolic capsid stain does not differ significantly from 100 % in HepG2 cells for Fab3105, or 71 % versus 97 % for DAKO.

Intracellular core staining with both antibodies showed a granular appearance (Fig. 1b) and in cells with a significant amount of nuclear core, no stain was observed in nucleoli.

Further experiments revealed that neither the serum concentration, the genotype (A, D), nor cell density had a significant impact on core localization (data not shown), indicating that distribution in the cytoplasm and nucleus is a stable phenomenon under various conditions.

Core protein and capsid localization in the absence of other viral proteins

Cytoplasmic core localization could have been caused by cellular factors resulting in a non-functional NLS, e.g. by

phosphorylation or dephosphorylation of the core proteins. Alternatively, viral proteins could have interfered with nuclear translocation by binding core to a larger cellular structure such as an organelle, or by sterically interfering with the NLS-bearing C terminus of core. In order to discriminate between these options, we transfected HuH-7 and HepG2 cells with a plasmid which expresses only core under the control of the CMV promoter, and investigated core and capsid localization. To exclude artefacts, which may be caused by overexpression of core protein under CMV promoter control, we determined the mean core fluorescence in individual cells showing $29 +/- 15$ (arbitrary units) in the context of other viral proteins with the BCP promoter, and $26 +/- 15$ (arbitrary units) upon expression under control of the CMV promoter (data not shown). Fig. 2(a) shows that in contrast to the localization in the context of other viral proteins, core and capsids localized predominantly in the nucleus in the majority of cells. Little difference occurred between HepG2

Table 1. Individual experimental results for transfection of pHBV_1.1mer in HuH-7 cells

Details of experiments presented in Fig. 1. HuH-7 cells were transfected with the pHBV_1.1mer. Cells were immunostained with a monoclonal mouse anti-HBc antibody (Fab3105) in three different experiments or a polyclonal rabbit anti-HBc antibody (DAKO) in five different experiments. The percentage of cells with an accumulation of fluorescence in the cytoplasm, in the nucleus or with an equal distribution between nucleus and cytoplasm are shown.

	Experiment number	Cells with an accumulation of fluorescence in the cytoplasm (%)	Cells with an equal distribution between nucleus and cytoplasm (%)	Cells with an accumulation of fluorescence in the nucleus (%)
pHBV_1.1mer; Fab3105	1	95	0	5
	2	85	4	12
	3	96	4	0
pHBV_1.1mer; DAKO	1	76	6	18
	2	81	10	8
	3	39	30	30
	4	83	5	12
	5	75	3	22

and Huh7 cells; the first showing a slightly increased proportion of nuclear core positive cells (HepG2: Fab3105, 92 %; DAKO, 85 %; Huh-7: Fab3105, 84 %, DAKO, 77 %).

Considering the variability between different transfections, we assumed that this difference was not significant. As shown in Fig. 2(b), we observed a granular stain which
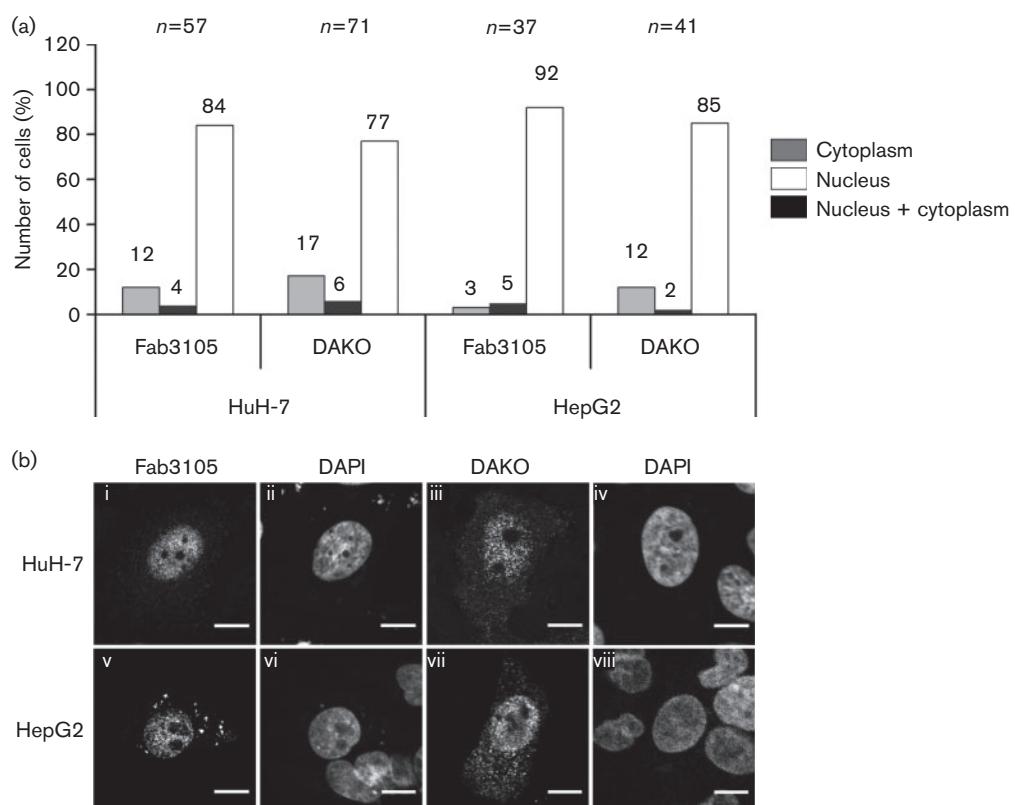

Fig. 2. Core protein and capsid localization in the absence of other viral proteins. (a) Determination of core concentration depicted as quantitative results of three transfections for Fab3105 and four transfections for DAKO. HuH-7 and HepG2 cells were transfected with the plasmid pcep21. Cells were immunostained using either the Fab3105 or DAKO antibodies. The columns show the predominant localization of core using the same colour code as in Fig. 1(a). The numbers on top of each column show the percentage of core positive cells. (b) Localization of core in individual cells. i–iv: HuH-7; v–viii: HepG2; i, v: Fab3105; iii, vii: DAKO. Nuclei were visualized by DAPI staining (ii, iv, vi, viii). Bars, 10 µm.

appeared finer than we observed for core protein and capsids expressed in the context of other viral proteins.

Nuclear entry of core depends upon active nuclear import

In hepatocytes, core proteins enter the nucleus by active nuclear import only, as cell division occurs rarely. Such transport requires nuclear transport receptors of the importin family (Kann *et al.*, 1999; Rabe *et al.*, 2003). In cell culture wherein cells divide rapidly, nuclear entry can also occur by passive trapping of core or capsids upon reconstitution of the nuclear envelope during cell division. In order to verify active nuclear import in our transfection experiments, we transfected the core gene mutant devoid of its C terminus into HuH-7 cells. Fig. 3(a) demonstrates that core remained cytoplasmic when devoid of the C terminus and identical results were obtained using Fab3105 and DAKO antibodies (Fig. 3a). Cytoplasmic localization of core protein and capsids showed fine granular staining as observed upon WT core protein expression (Fig. 3b) arguing against a fixation of core at an organelle.

Collectively, these data suggest that cytosolic retention of core is caused by a viral factor, and not by a difference between hepatoma cell lines or hepatocyte differentiation status.

Impact of viral proteins on core and capsid localization

In order to identify the viral protein affecting core localization, we transfected a 1.1mer of the HBV genome in which expression of different ORFs were suppressed either by the introduction of stop codons or by mutating the ATG start codon. We first introduced a stop codon at codon 26 of X ORF, which does not affect the amino acid sequence of the overlapping pol ORF (pHBV_X-). Fig. 4(a) shows the merge of three experiments, demonstrating that the presence of the X protein had no impact on the number of cells exhibiting a predominant cytoplasmic core or capsid stain (Fab3105, 92 %; DAKO, 70 %). Further, the intracytoplasmic staining pattern remained unchanged compared to the WT (Fig. 4b i, iii).

We next transfected a 1.1mer (pHBV_preS1-, preS2-, S-) into HuH-7 cells in which the start codons of preS1, preS2
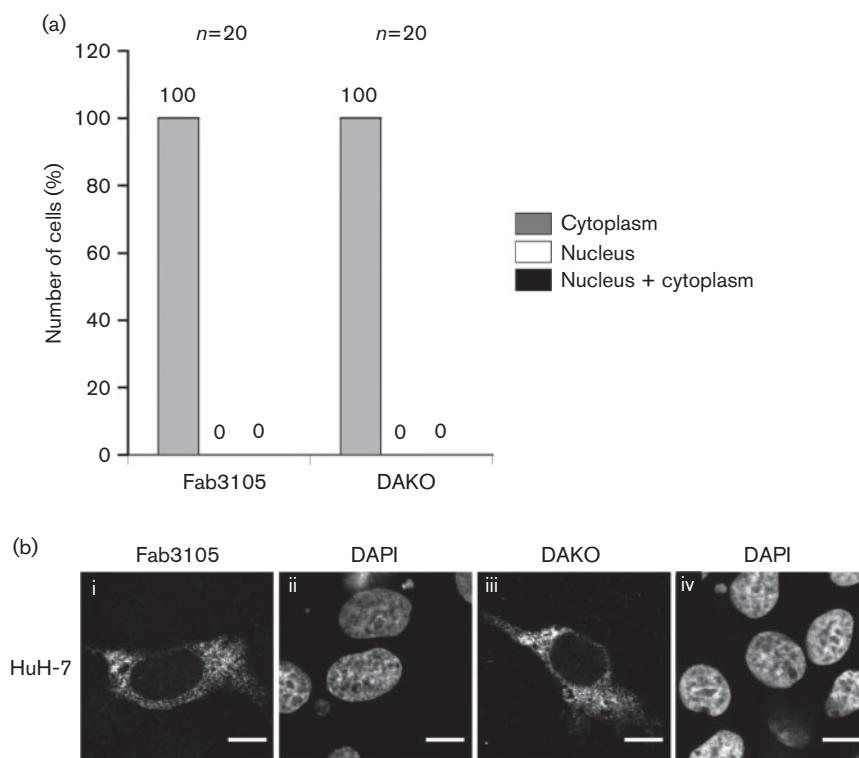

Fig. 3. Intracellular localization of core and capsids devoid of the core protein C terminus. (a) Determination of core concentration depicted as quantitative results of one transfection for Fab3105 and DAKO. HuH-7 cells were transfected with a fragment of core devoid of C terminus. Cells were immunostained either with the Fab3105 or DAKO antibodies. The columns show the predominant localization of core using the same colour code as in Fig. 1(a). The percentage of cells with a predominantly cytoplasmic localization is shown in grey. The numbers on top of each column show the percentage of core/capsid positive cells. (b) Core/capsid localization in individual HuH-7 cells. i: Fab3105; iii: DAKO. Nuclei were visualized by DAPI staining (ii, iv). Bars, 10 μm.
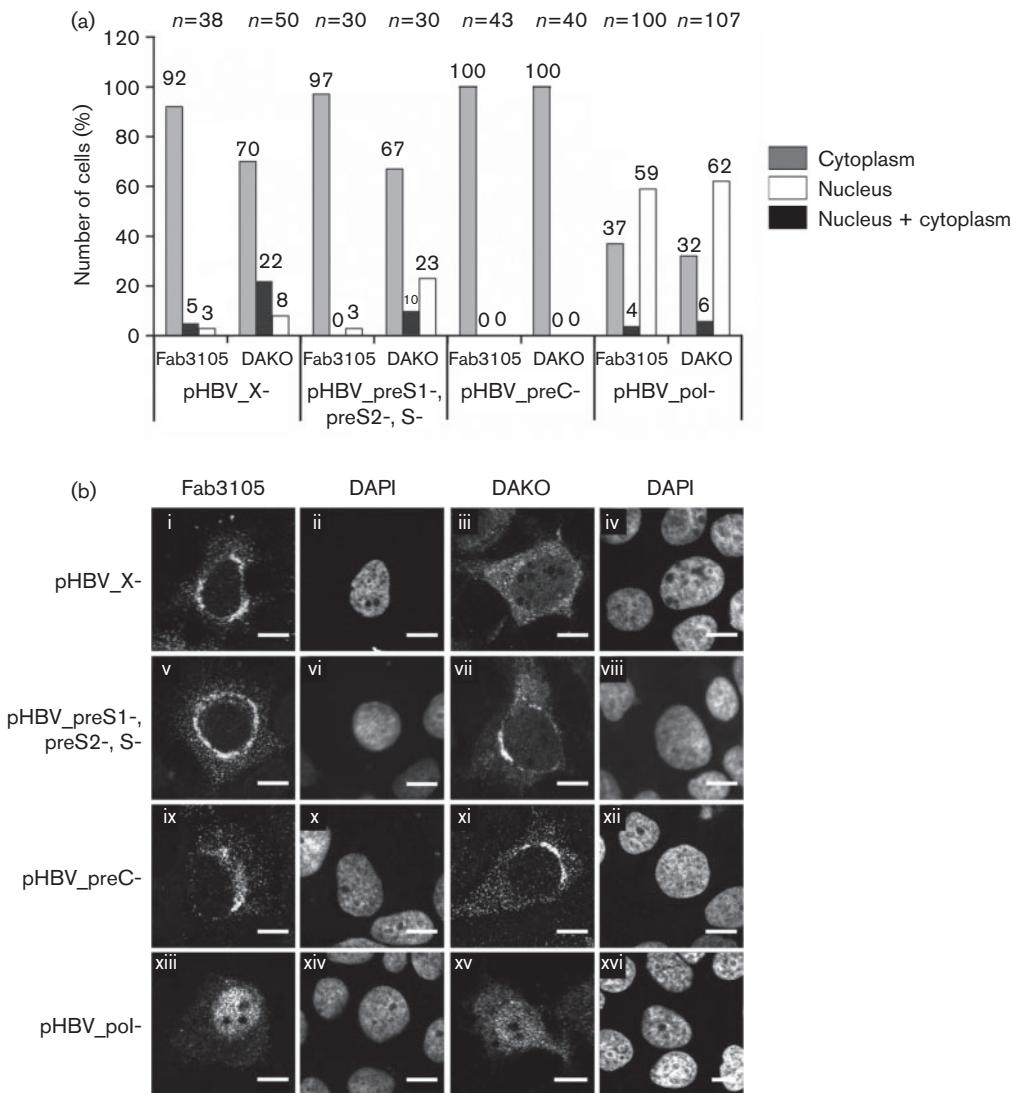
Fig. 4. The viral polymerase is essential for cytoplasmic core localization in HuH-7 cells. (a) Determination of core concentration depicted as quantitative results of two transfections for pHBV_X-, one transfection for pHBV_preS1-, preS2-, S- and pHBV_preC-, and four transfections for pHBV_pol-. Cells were immunostained with either Fab3105 or DAKO antibody. The columns show the predominant localization of core using the same colour code as in Fig. 1(a). The numbers on top of each column show the percentage of core/capsid positive cells. (b) Localization of core/capsids in individual HuH-7 cells. i, v, ix, xiii: Fab3105; iii, vii, xi, xv: DAKO. Nuclei were visualized by DAPI staining (ii, iv, vi, viii, x, xii, xiv, xvi). Bars, 10 μm.

and S were inactivated by replacing the codons with ACG. Again, no significant change in the proportion of cells with a predominantly cytoplasmic core protein or capsid stain was observed (Fab3105, 97 %; DAKO, 67 %; Fig. 4a) and no change of intracytoplasmic core protein or capsid staining pattern was observable (Fig. 4b v, vii). Mutating the preC start codon to ACG also showed dominant cytoplasmic core localization (Fab3105, 100 %; DAKO, 100 %; Fig. 4a) and the staining pattern remained unchanged (Fig. 4b ix, xi).

In contrast, mutation of the pol AUG to ACG which does not affect the overlapping core ORF caused a major change

of core and capsid localization with the majority of cells exhibiting a predominantly nuclear localization (Fab3105, 59 %; DAKO, 62 %; Fig. 4a). Intranuclear core staining revealed that the nucleoli remained excluded (Fig. 4b xiii, xv), thus showing the same pattern as nuclear staining upon expression from the WT genome or from core expression without other viral proteins.

To confirm that pol prevents core translocation into the nucleus, we next trans-complemented HuH-7 cells transfected with the pol-negative plasmid with a plasmid expressing the viral polymerase. In order to detect pol-expressing cells despite the limited specificity of available

anti-pol-antibodies, we used a vector expressing pol fused to the C terminus of eGFP. Analysing core protein and capsid localization in GFP positive cells showed that both the Fab3105 and DAKO stains were found predominantly in the cytoplasm in most cells (Fig. 5a; Fab3105, 92 %; DAKO, 70 %) as it was found in WT HBV. eGFP-pol remained cytoplasmic irrespectively of the presence of core, but appeared to be more diffuse than the core stain (Fig. 5b).

The NLS of core is exposed on unassembled core proteins and on mature capsids but mature capsids leave the cell rapidly via virus secretion (Rabe et al., 2003). However, assembly depends upon core protein concentration (Seifer & Standring, 1995) and high expression levels could subsequently lead to rapid cytoplasmic assembly resulting in a dominant cytoplasmic capsid stain. We verified the intensity and the total signal derived from both antibodies in individual cells with cytoplasmic and nuclear phenotypes

after expression of core in the absence of other viral proteins or in the genomic context. As shown in Fig. S1 (available in the online Supplementary Material), the cells showed cytoplasmic (Fig. S1a) or nuclear accumulation (Fig. S1b) with high variation observed in fluorescence intensity. Thus there appears to be no correlation between the amount of capsid/core protein and the dominant localization, irrespective of the presence of viral proteins. This observation is supported by a kinetic analysis performed in HuH-7 cells transfected with WT HBV 1.1mer, in which we did not observe a significant change in the predominant core localization using Fab3105 between days 1, 3 and 5 (data not shown). However, we observed a slight increase in cytoplasmic capsid using DAKO (cytosolic Fab3105 stain: day 1 92 %, day 2 88 %, day 5 100 %; cytosolic DAKO stain: day 1 60 %, day 2 75 %, day 5 87 %). Such accumulation was expected for capsids, as their half-life (>24 h) in hepatoma
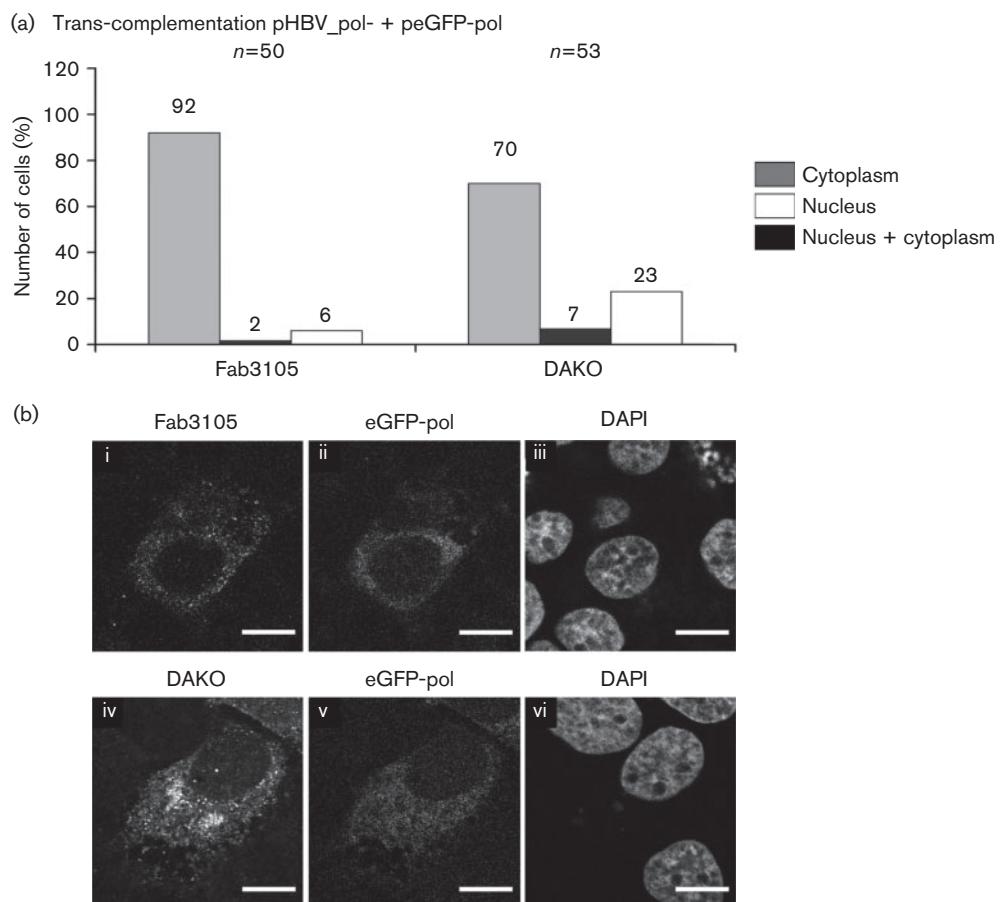

Fig. 5. Core localization after expression from a polymerase-negative HBV genome upon eGFP-pol trans-complementation. (a) Determination of core concentration depicted as quantitative results of two transfections for Fab3105 and for DAKO. HuH-7 cells were co-transfected by pHBV_pol- (mutated HBV genome which lacks the expression of polymerase) and peGFP-pol. Cells were immunostained with either the Fab3105 or DAKO antibodies. The columns show the predominant localization of core/capsids using the same colour code as in Fig. 1(a). The numbers on top of each column show the percentage of core positive cells. (b) Localization of core in individual HuH-7 cells co-transfected with pHBV_pol- and peGFP-pol. i: Fab3105; iv: DAKO. Nuclei were visualized by DAPI staining (iii, vi). Polymerase was visualized by its eGFP domain (ii, v). Bars, 10 μm.

cells is longer than that of monomeric core protein (Deres et al., 2003; Xu et al., 2010).

Cytoplasmic core localization requires pol and ε

In vivo, the pol–core protein interaction occurs upon envelopment of the viral PG, which in turn requires interaction of pol with ε (Bartenschlager & Schaller, 1992; Jones et al., 2012). To investigate the impact of ε, which was present in the trans-complementation experiments (Fig. 5), we expressed core under the control of the CMV promoter and eGFP-pol, resulting in expression from mRNA devoid of ε. Fig. 6(a) shows that in the vast majority of cells, core localized predominantly in the nucleus (Fab3105, 94%; DAKO, 73%) similarly to the expression of core in the absence of other viral proteins. Aside from providing evidence that pol by itself is not sufficient for cytoplasmic core arrest, the observation further demonstrates that GFP – as part of the fusion protein – had no impact on core localization.

To further search for the missing partner causing cytoplasmic core retention by pol, we next co-transfected eGFP-pol with core; the latter was expressed from a pol/preC negative vector comprising ε (plasmid ppreC-/C). We observed that the cytoplasmic core distribution was restored (Fab3105, 69%; DAKO, 72%; Fig. 6b) and that the intracytosolic distribution of core showed the same granular stain upon WT HBV expression (Fig. 6d). Recent studies have shown that mutation of the lower bulge of the ε structure (1858–1863) prevents the pol–ε interaction (Abraham & Loeb, 2006; Oropeza & McLachlan, 2006). We co-transfected eGFP-pol and core with a vector expressing ε mutated in this fashion (ppreC-/C ε mutant) and found that core localized in the nucleus (Fig. 6c). Taken together, these results support a major role of ε in cytoplasmic core distribution.

Impact of Ser 164 on core localization

As shown by others, encapsidation of the viral PG depends upon phosphorylation of the core protein at Ser 164,
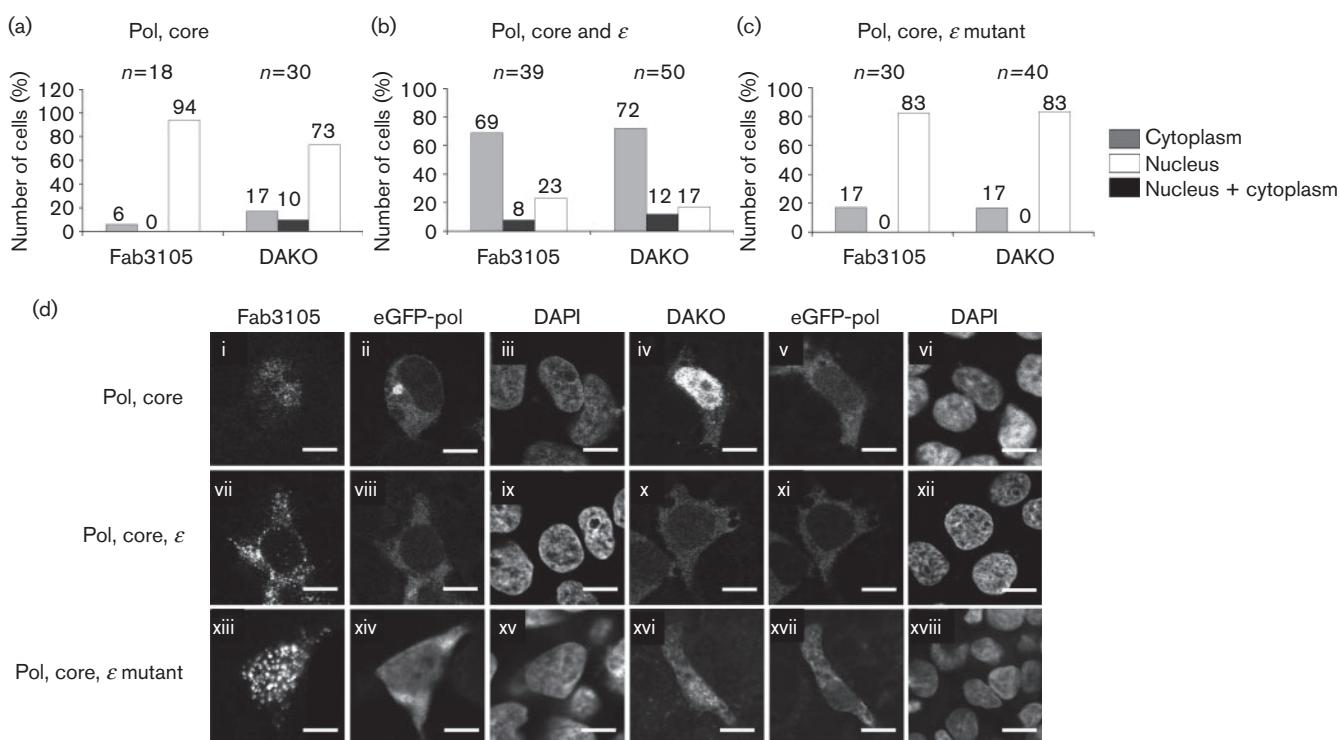

Fig. 6. Cytoplasmic core localization requires pol and ε. (a, b, c) Determination of core concentrations depicted as quantitative results of two transfections for Fab3105 and DAKO. HuH-7 cells were co-transfected with plasmid pcep21 containing the core gene genotype D, without ε, expressed under control of the CMV promoter, and peGFP-pol (a: pol, core) or ppreC-/C and peGFP-pol (b: pol, core and ε) or ppreC-/C ε mutant and peGFP-pol (c: pol, core and ε mutant). Cells were immunostained with either Fab3105 or DAKO. The columns show the predominant localization of core/capsids using the same colour code as in Fig. 1(a). The numbers on top of each column show the percentage of core/capsid positive cells. (d) Localization of core/capsids in individual cells co-transfected with pcep21 and peGFP-pol (pol, core; i–vi) or ppreC-/C and peGFP-pol (pol, core, ε; vii–xii) or ppreC-/C ε mutant and peGFP-pol (pol, core and ε mutant; xiii–xviii). i, vii, xiii: Fab3105; IV, X, XVI: DAKO. Nuclei were visualized by DAPI staining (iii, vi, ix, xii, xv, xviii). Polymerase was visualized by eGFP fluorescence (ii, v, viii, xi, xiv, xvii). Bars, 10 μm.

implying that the pol–core interaction requires core phosphorylation at this residue (Gazina *et al.*, 2000). We thus expressed the preC/core ORFs comprising ε, in which Ser 164 has been mutated to Ala in HuH-7 cells. In a further experiment, we co-transfected eGFP-pol and determined core localization by using the DAKO antibody. Fig. 7 shows that mutated core exhibited a predominantly cytoplasmic localization (81 %) and that co-transfection of pol did not significantly change the capsid accumulation (71 %). These findings do not allow conclusions to be drawn about the importance of Ser 164 for the pol–core interaction, but provide evidence that this phosphorylation site affects nuclear import of core proteins *in vivo*.

DISCUSSION

The cellular and viral factors determining core localization are not known despite its correlation to viral load and disease progression. In an attempt to understand the molecular interactions linked to core localization, we analysed the impact of hepadnaviral factors.

Our observation of a predominantly cytoplasmic core phenotype in hepatoma cells after expression in the context of other viral proteins corresponds to histological data derived from HBV infected patients with low viral loads (Liu *et al.*, 2009). The similarity between hepatoma cell lines and *in vivo* infection indicates that the level of cell differentiation has no major impact on core localization, and we conclude that viral factors are decisive. In agreement with the similarity to this histological pattern, we showed that nuclear localization was linked with active nuclear import. This active nuclear transport must also be concluded for hepatocytes *in vivo*, as cell division allowing passive entry rarely occurs (lifespan 4.8–284 days; Whalley

et al., 2001). Our observation that core localization was independent of time after transfection and of genotype further confirms the robustness of the results.

In all cells with a detectable cytoplasmic core stain, we observed granular staining but no characteristic pattern in relation to MVB or the ER. This indicates that the proportion of capsids interacting with the viral surface proteins is low, which is in agreement with the overexpression of core relative to the amount needed for virion formation. This finding further indicates that the number of capsids with a mature genome, which could attach to preS1, is limited and also suggests that nuclear import of capsids with a mature genome, as was shown *in vitro* in permeabilized cells (Rabe *et al.*, 2003), cannot account for a significant nuclear stain. This idea is further supported by the absence of a rim-like stain at the nuclear envelope, where capsids with an immature DNA maturation accumulate.

Despite a dominant phenotype, we observed individual cells with different core localization, and most cells showed some staining in both compartments. These differences between individual cells indicate inter-individual differences, but similar observations were made *in vivo* (Chu *et al.*, 1997; Kim *et al.*, 2006). Mechanistically, such differences could be caused by factors such as phosphorylation (Liao & Ou, 1995; Kann *et al.*, 1999; Kang *et al.* 2006) or the expression of intracellular transport factors needed for core translocation to the nucleus. However, nuclear import factor expression variations are only described in embryogenesis and in breast cancer, making phosphorylation a more likely candidate. Accordingly, we observed that mutating the phosphorylation site 164 from Ser to Ala prevented nuclear import of core in the absence of other viral proteins. This finding is in agreement that
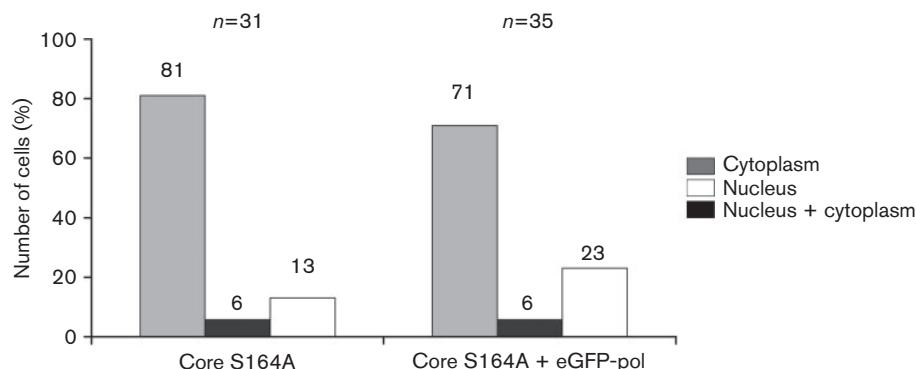

Fig. 7. Influence of Ser 164 on capsid localization. Determination of capsid concentration depicted as quantitative results of one transfection for DAKO. HuH-7 cells were transfected with DNA fragments allowing the expression of core alone (core S164A) - transfection of pUC991_S164A where Ser 164 has been mutated into Ala, digested in order to have only core region and its promoter. The second experiment shows the result of co-transfection of DNA fragments allowing the expression of core with the S164A mutation and peGFP-pol (core S164A + eGFP-pol). The columns show the predominant localization of core/capsids using the same colour code as in Fig. 1(a). The numbers on top of each column show the percentage of core/capsid positive cells.

phosphorylation is essential for interaction of at least the capsid with importin α and β (Kann et al., 1999) but shows that phosphorylation is also crucial in vivo and in unassembled core proteins. The finding that not all core stain was cytoplasmic in transfections with the S164A mutant further implies that the non-mutated phosphorylation sites could play a minor role.

In aiming to identify the viral protein which had a dominant impact on core and capsid localization, we observed that neither preC/HBeAg nor the surface proteins affected core/capsid localization significantly. The first finding is in agreement with a missing impact of HBeAg, which could eventually cause staining, in our fluorescence results. The latter observation is consistent with an absence of capsid accumulation at MVB. In contrast, suppression of the pol start codon changed capsid localization significantly, becoming predominantly nuclear in the majority of cells. However, the nuclear pattern was not so pronounced as upon expression of the core ORF alone.

In keeping with our observation that pol is the major determinant of core and capsid localization, trans-complementation by eGFP-pol restored core and capsid localization but only in those cells showing eGFP fluorescence. However, trans-complementation did not fully restore core retention. This finding is in agreement with observations of others showing that expression of core and pol from different RNAs is less efficient regarding PG packaging than translation of pol and core from the same RNA (Von Weizsäcker et al., 2002). Although we do not have evidence for polymerase activity of the fusion protein after encapsidation into capsids, the fusion protein localized like WT pol, as was shown recently (Cao & Tavis, 2004).

In summary, our data indicate that core and capsid localization are based on at least four partners: core protein, a protein kinase phosphorylating core, pol and ε. This conclusion explains several observations of others who have analysed different aspects of the viral life cycle, as follows. (1) The encapsidation of PG mediated by pol requires ε on the PG (Bartenschlager & Schaller, 1992). (2) This finding supports that the hypothesis that the pol–core interaction requires ε binding by pol, and is in agreement with a pol-dependent cytoplasmic core retention. It further suggests that ε binding changes the structure of pol aside from the already documented changes caused by pol–hsp70 and 90 interaction (Stahl et al., 2007). (3) The observation that pol becomes activated by binding to its RNA (Tavis & Ganem, 1996) supports the hypothesis that the supposed structural change of pol is essential for the viral life cycle. (4) The observation that in the absence of core, HBV pol is only poorly active, allowing priming and synthesis of a few nucleotides of the minus DNA strand (Chen et al., 2003; Wang et al., 2013) is also in agreement with the requirement of ε-induced structural pol changes allowing core interactions. (5) The observation of Gazina et al. (2000) showing that core protein phosphorylation is required for PG packaging can also be explained by our findings that

phosphorylation is the main requirement for the pol–core interaction.

As in patients' liver biopsies, we did not observe homogeneous localization of core and capsids in all cells. Based on our results, there are two major pathways to explain these intercellular differences, as follows. (1) Phosphorylation can differ between individual cells thus altering core–pol interactions and also nuclear import capacity. (2) Our data further suggest that core localization could also depend upon the stoichiometry of core and pol. An altered balance appears possible, as the translation of core and pol may follow different pathways (such as leaky scanning or reinitiation). In fact, reinitiation depends upon factors such as the eukaryotic translation elongation factor 2 (eEF2; Skabkin et al., 2013), which is regulated by phosphorylation and dephosphorylation (Kaul et al., 2011).

The consequences of a modified equilibrium between pol and phosphorylated core could lead to two scenarios, which require further investigation. If pol is overexpressed with regard to phosphorylated core, core is retained in the cytoplasm. This assumption is in agreement with the observation that the phosphorylation site is closed to the NLS thus suggesting competition between pol and import receptors. An excess of pol would further interfere with capsid formation as each mature capsid/virion contains just one pol molecule (Bartenschlager & Schaller, 1992). Even if this assumption is still controversial (Zhang & Tavis, 2006), it is consistent with the observation that transfected cell lines produce fewer viruses than infected hepatocytes in vivo, and is in agreement with data from patient biopsies showing that cytoplasmic capsids are correlated with low viraemia (Liu et al., 2009).

METHODS

Plasmids. Plasmid pcep21 contains the core gene genotype D expressed under control of the CMV promoter. pHBV_1.1mer harbours the genotype A HBV genome 1.1mer (nt 1580–1984). Using the primers listed in Table S1 in PCR-based site-directed mutagenesis (Pfu-Turbo DNA polymerase; Agilent Technologies), this plasmid was the template for generating the plasmids in which the different HBV ORFs were inactivated. After treatment of the PCR product by DpnI, nucleotide substitutions were introduced. All mutations were confirmed by DNA sequencing. In pHBV_X- a stop codon has been introduced at codon 26 of X ORF (G1443T). pHBV_preS1-, preS2-, S- contains mutations of the preS1, preS2 and S start codons to ACG (T2854C, T3211C and T155C, respectively). In pHBV_preC- the precore start codon was mutated to ACG (T1814C) and in pHBV_pol- the polymerase start codon was changed to ACG (T2307C). As pHBV contains the 1.1mer genome, a second stop codon was introduced in the 3′ copy of core (T1918G) and precore (T1815C). ppreC-/C plasmid was obtained by amplification of the HBV core and precore ORFs by PCR using ApaI site containing primers followed by insertion of the ApaI-cleaved PCR product in pGemT-easy. The precore ATG was then mutated to ACG by mutagenesis as described before. Plasmid ppreC-/C ε mutant, which consists in a deletion of the UGUUCA sequence (Δ1858–1863) in the lower bulge of the ε structure. The plasmid for expression of eGFP-pol construct comprises the enhanced GFP gene (eGFP) fused at the N terminal to the HBV polymerase ORF using Gateway technology.

In brief, the polymerase gene was amplified by PCR from plasmid pSM2, which contains a head-to-tail HBV genome dimer of genotype D linearized by *Eco*RI. Using the primers specified in Table S1 the fragment was amplified and inserted into the entry vector, pDONR221. LR Clonase then catalysed recombination between the entry- and destination vectors (pBS_L30_eGFP_RFC) to generate peGFP-pol. In this vector, eGFP-pol expression is controlled by the mouse ribosomal protein L30 promoter. pUC991_Ser164Pro contains the genotype D HBV genome linearized by *Eco*RI, in which the codon Ser 164 of core was mutated to Pro.

Cell culture and transfections. All experiments for immunostaining were performed in HuH-7 cells and repeated in HepG2 cells. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (v/v) FCS, penicillin (100 U ml^{-1}) – streptomycin (100 µg ml^{-1}) (complete medium) unless otherwise indicated. One day before transfection, 1.5×10^5 HuH-7 cells were propagated on coverslips in 12-well dishes, in complete medium. Cells were incubated for 24 h at 37 °C, 5% CO$_2$. Cells were then transiently transfected with 800 ng DNA in Opti-MEM, using 1.5 µl Lipofectamine 2000. One day post-transfection, cells were washed with $1\times$ PBS and incubated in starved serum medium (DMEM 1% (v/v) serum, 1% (v/v) penicillin, 1% (v/v) streptomycin) for 3 days at 37 °C, 5% CO$_2$. For immunostaining, cells were fixed with 3.7% paraformaldehyde in $1\times$ PBS for 30 min at room temperature.

Immunofluorescence analysis. Cells were washed in $1\times$ PBS, permeabilized for 10 min using 0.2% (v/v) Triton X–100/$1\times$ PBS for 10 min and washed with $1\times$ PBS. Then, cells were blocked for 5 min in blocking buffer (PBS, 10% FCS, 0.1% (v/v) saponin). The rabbit polyclonal (DAKO) and the mouse monoclonal Fab3105 (Institute of Immunology) antibodies were diluted $1:1000$ and $1:500$, respectively, in blocking buffer for 1.5 h at 37 °C. After three washes with $1\times$ PBS, blocking buffer was added for 5 min at room temperature. Anti-rabbit ($1:1000$) and/or anti-mouse antibodies ($1:500$) Alexa 488- or Alexa 543 labelled donkey secondary antibodies (Invitrogen) were added for 90 min at 37 °C in $1:1000$ dilution. DNA was stained with DAPI (1 mg ml^{-1}, $1:1000$, Sigma) for 10 min. Cells were mounted using fluorescence mounting medium (DAKO). Microscopy was performed using a Leica SP5 confocal microscope, equipped with a $63\times$ objective and Leica image analysis software. Images were taken in 12 bits, with a sequential bidirectional acquisition, averaged on three images. Quantification of fluorescence was performed using ImageJ software. We determined the mean of fluorescence in the nucleus and in the cytoplasm of each transfected cell, which reflects the concentration. The mean values of fluorescence for nuclei and cytoplasm of the non-transfected cells were obtained by subtracting the background signal. The fluorescence ratio was determined using the mean values in the nucleus and in the cytoplasm. The cells were classified as a function of their ratio of fluorescence. If the nuclear/cytoplasmic ratio was below 1, there was an accumulation of fluorescence in the cytoplasm. If the ratio was above 1, there was an accumulation of fluorescence in the nucleus. The cells were then classified in a graph showing the percentage of cells with accumulation in the cytoplasm (grey columns), in the nucleus (white columns) and with an equal distribution between nucleus and cytoplasm (black columns) for *n* cells.

ACKNOWLEDGEMENTS

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