

*Full Length Research Paper*

# Analysis of the nuclear localization signal of the hepatitis B virus capsid

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**Capsid protein of hepatitis B virus (HBV) consists of a single karyophilic protein species. It mediates the entry of the viral DNA into the nucleus using the cellular transport receptors importin  $\alpha$  and  $\beta$ . The nuclear localization signal (NLS) is localized within the C-terminus of the HBV capsid protein, overlapping with eight phosphorylation sites. We first investigated the NLS in more detail observing that the amino acid sequence PRRRTSPRRR is sufficient to mediate efficient nuclear import of bovine serum albumin. Phosphorylation of this sequence blocked its transport capacity. However, empty *E. coli*-expressed capsids that exposed the C-terminus on their surface failed to interact with the nuclear envelope in digitonin-permeabilized cells unless becoming phosphorylated. Consistently, treatment of cells with a protein kinase inhibitor prevented nuclear localization of a fusion protein of EGFP and capsid protein. The results show that phosphorylation is required for nuclear transport of the HBV capsid protein but that phosphorylation must occur outside the transport signal, a phosphorylation pattern that is similar to that observed for the SV40TA<sub>g</sub>. Moreover, it implies a complex regulation of phosphorylation and dephosphorylation during the hepadnaviral life cycle.**

**Keywords:** HBV capsid, NLS, EGFP core, fusion protein.

## INTRODUCTION

Hepatitis B virus (HBV) infection is one of the major causes of hepatocellular carcinoma world-wide. HBV comprises a capsid protein, also termed as core particle, which is enveloped by the viral surface proteins. Although containing a DNA genome, HBV replicates via an RNA pregenome that is synthesized within the nucleus of the cells by RNA polymerase II (Rall et al., 1983), requiring import of the viral DNA genome into the karyoplasm. In HBV infected cells the capsid is predominantly found in the nuclei of hepatocytes (Furuta et al., 1975; Gerlich et al., 1982; Gudat et al., 1975). Only in highly active infections, with high virus titers in the serum capsids are found within the cytoplasm (Akiba et al., 1987; Serinoz et

al., 2003). Apparently, nuclear localization is intrinsic to the capsid since its expression in heterologous systems such as transgenic mice, results in the same distribution pattern (Guidotti et al., 1994). Rabe et al. (2003) showed that in *in vitro* transport systems the capsids become imported into the nucleus. This import is associated with the release of the encapsidated viral genome into the karyoplasm.

The HBV capsid comprises a single protein species that consists of 183 or 185 amino acids (aa) with a molecular weight of 21.5 kDa. Core proteins assemble spontaneously to capsids after reaching a threshold concentration of 0.8  $\mu$ M, even in the absence of other viral gene products (Seifer et al., 1993). It is predominantly formed by 180 or 240 amino acids with a T=3 or T=4 symmetry and a diameter of 32 or 36 nm (Crowther et al., 1994). While the first 140 amino acids of the core protein are essential for folding and capsid formation (Zlotnick et al., 1996), the C-terminus of the

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HBV capsid protein is flexible (Crowther et al., 1994; Watts et al., 2002). This C-terminus region comprises RNA and DNA binding sites (aa 149 – 157 and 165 – 173, respectively (Hatton et al., 1992; Nassal, 1992) [numbering based on isolate 991, genotype A, EMBL accession no. X51790]). The core proteins become phosphorylated by a protein kinase of cellular origin at serine residues (Gerlich et al., 1982). Potential acceptor sites are eight residues that are found within the C-terminal domain (aa 141, 157, 164, 170, 172, 178, 180, 184). Their individual functions as well as the identity of the protein kinase are not fully understood. Phosphorylation at serine 157 was shown to be essential for pre-genome packaging (Gazina et al., 2000). The phosphorylation of serine 170 and/or 172 was established in capsids of virions as determined by phospho-specific antibodies against this domain (Machida et al., 1991). Moreover, at least serine residues 157, 164 and 172 must be phosphorylated for genome maturation (Melegari et al., 2005) during which the viral polymerase converts the RNA pregenome into a partially double stranded DNA. However, the amount of phosphates decreases with ongoing genome maturation (Perlman et al., 2005).

In the HBV life cycle the pre-genome is encapsidated into the capsid in a complex with the viral polymerase and at least one other protein, the heat shock protein or Hsp90 (Hu et al., 2004). In these capsids the C-terminus of the core protein are localized internally and are inaccessible to proteases (Rabe et al., 2003). Consistently, RNA-containing capsids that are expressed in *E. coli* consistently show an internal localization of this domain (Zlotnick et al., 1997). Genome maturation is correlated with increasing numbers of externally exposed C-terminus of HBV capsid protein (Rabe et al., 2003), presumably translocated through the 2 nm holes that are found in the capsid shell. However, not only genome maturation causes C-terminus exposure. Evaluations with *E. coli*-expressed capsids show that *in vitro* phosphorylation induces their exposure (Kann et al., 1999). In addition to the RNA- and DNA-binding sites a nuclear localization signal (NLS) was identified in the C-terminus of the core protein using different peptides that represent parts of this domain (aa 158-168 and aa 165-178, (Kann et al., 1999)). Consistently, the C-terminus interacts only with the NLS-binding nuclear transport receptors importin  $\alpha$  and  $\beta$  when being exposed on the capsid surface. Removal of the C-termini prevented importin-binding and translocation to the nucleus (Rabe et al., 2003). Remarkably, the C-terminus does not contain lysine residues as are present in the consensus sequence K(K/R)X(K/R) of NLS (4). Such a lysine residue was thought to be essential for sufficient interaction with the binding pocket of importin  $\alpha$  (Fontes et al., 2000).

The domains of nucleic acid binding interact with the DNA or RNA inside the lumen of the capsids, while function of the overlapping NLS requires exposure on the

surface. Thus localization of the C-terminus must be coordinated in the HBV life cycle. However, not only genome maturation but also phosphorylation may act as a key regulator. Thus we first narrowed the core protein NLS and subsequently evaluated the effect of phosphorylation on the capacity of the NLS for nuclear import. *In vitro* transport assays showed that phosphorylation must occur but should exclude a site within the NLS, implying a well coordinated phosphorylation. Expression of core fusion proteins in hepatoma cells verified that such a phosphorylation pattern occurred *in vivo*.

## MATERIAL AND METHODS

### Generation of FITC BSA-peptide conjugates

Peptides were linked via a cysteine residue flanking the spacer region to the BSA according to Görlich et al., (1995). Excess of peptides were removed from the conjugates by gel filtration using a PD 10 column as described previously (Görlich et al., 1995). SDS PAGE revealed an average molecular weight of the conjugates of ~100 kDa and showed that all BSA were linked to peptides (data not shown). Phosphorylation of the peptides was performed as described below in "immune precipitations".

### *In vitro* transport assays

*In vitro* transport assays were performed as described previously using HuH-7 cells that were grown on collagen-coated cover slips in a 24-well dish (Kann et al., 1999). In the transport reaction conjugates were added at a concentration of 20  $\mu\text{g/ml}$ . When transport was inhibited, 50  $\mu\text{g/ml}$  wheat germ agglutinin (WGA, ROCHE) was added to the import reaction mixture. In these experiments WGA was also present in the washing steps that occur prior to transport. To determine the transport capacity of the different capsids, they were added at a concentration of 5  $\text{ng}/\mu\text{l}$ . In all transport assays, cells were washed three times with washing buffer (2 mM Mg-acetate/20 mM Hepes 7.3/110 mM K-acetate/1 mM EGTA/5 mM Na-acetate/1 mM DTT) for 10 min at 0°C prior to the fixation for 30 min with 500  $\mu\text{l}$  3% paraformaldehyde/PBS per cover slip at room temperature (RT).

### Immune stain and microscopy

Fixed cells were washed twice with 1 ml PBS before the nuclear membrane was lysed by a 10 min incubation with 0.5 ml 0.1 % Triton-X-100/PBS. The detergent was removed by 3x washing with 1 ml PBS, followed by

incubation in 300  $\mu$ l blocking buffer (PBS/0.5 % BSA/5 % goat serum) for 15 min at 37°C. The cells were incubated with primary antibodies in blocking buffer for 60 min at 37°C in a humidified box (concentrations: rabbit anti capsid [DAKO] or mouse FAb3105 [Institute of Immunology, Tokyo, Japan] 1:200, mouse anti NPC [mAb414, COVENCE] 1:500). The cells were then transferred back into the 24-well-dish and washed 4x with 1 ml PBS. Incubations with the second antibodies were done in blocking buffer in a humidified box for 30 min at 37°C. For NPC stain a Texas Red-labelled goat anti mouse antibody (DIANOVA) at a dilution of 1:100 was used. For detection of core protein dimers by FAb3105 an Alexa 647-labelled goat anti mouse antibody (MOLECULAR PROBES) was subjected in a concentration of 1:100 while detection of the capsids using the anti capsid primary antibody was performed with an Alexa 488-labelled goat anti rabbit antibody ((MOLECULAR PROBES) in a dilution of 1:200. In these stains no mAb414 was used. Following this incubation cells were washed as described above. Propidium iodide (SIGMA) staining was performed then the first washing step was added at a concentration of 0.1  $\mu$ g/ml. Cover slips were then embedded in Moviol (HOECHST) containing 100 mg/ml DABCO (SIGMA) to prevent bleaching. After polymerization of the Moviol, the cells were examined using a LEICA DM IRBE confocal laser scan microscope using the filter settings for FITC, TRITC and Cy5.

### Generation of the different capsid preparations

Phosphorylated capsids were generated as described previously using protein kinase C (PKC) and [ $\gamma$ 32P]ATP to test for successful reactions (Kann et al., 1994). When empty phosphorylated capsids were generated 0.5 U/ $\mu$ l S7 nuclease was added during the phosphorylation step. To obtain unphosphorylated capsids no PKC was added. The reactions were stopped by addition of 5 mM EGTA to the reaction mixture. To confirm successful nuclease digestion, 5  $\mu$ g of capsids were separated under native conditions on an agarose gel (NAGE), as previously described (Kann et al., 1994). Nucleic acids were stained by ethidium bromide followed by a protein staining using Coomassie brilliant blue for 1 h at RT and a destaining overnight. Quantification was described elsewhere using a geometric standard dilution series of wild type *E. coli*-expressed capsids (Kann et al., 1994). The trypsin-digest and subsequent immune blotting was done as described previously (Rabe et al., 2003).

### Plasmid construction

Plasmid pRcCMV core comprises the core open reading frame (ORF) of HBV genome (isolate 991, genotype A,

EMBL accession no. X51790) in the vector pRcCMV (INVITROGEN). In this plasmid the ATG start codon is fused to a *Hind* III restriction site. pRcCMV core was digested with *Hind* III and *Apa* I that cleaves 6 bp downstream of the core ORF stop codon. For generation of pEGFP core 1C, plasmid pEGFP C3 (CLONTECH) was digested with *Hind* III and *Apa* I. The large 4702 bp fragment was purified and ligated to the purified insert comprising the core ORF. The plasmid was then transformed into *E. coli* XL1 blue. Correct ligation was confirmed by *Hind* III/*Apa* I and *Bgl* II digestions. pEGFP core 2C was generated by restriction digest of pEGFP core 1C with *Ava* I and *Apa* I. *Ava* I cleaves at nt 539 of the core ORF. The larger restriction fragment of 5234 bp was purified and ligated to a double-stranded oligo nucleotide (MWG BIOSCIENCES) harbouring ends of the same restriction enzymes (sense 5'-CTC GGG AAT CTC AAT GTC CTA GAA GAA CTC CCT CGC TCG CAG ACG ATCTCA ATC GCC GCC GCG TCG CTA GGG CC-3', antisense 5'-CTA GCG ACG CGG CGA TTG AGA TCT GCG TCT GCG AGG CGA GGG AGT TCT TCT TCT GGA CAT TGA GAT TC-3'). This sequence encodes the amino acid sequence SQSPRRRTPSPRRRRSQSPRR. Positive clones showed a 610 bp fragment after *Hind* III/*Apa* I digestion. Plasmid pEGFP core 3C was generated in a similar way. Instead of the oligonucleotides shown above a double-stranded oligo nucleotide (sense: 5'- CTC GGG AAT CTC AAC CTA GAA GAA GAA CTC CCT CGC CTC GCA GAC GCA GAT CTC AAT CGC CGC GTC GCC CTA GAA GAA GAA CTC CCT CGC CTC GCA GAC GCA GAT CTC AAT CGC CGC GTC GCT AGG GCC-3', antisense: 5'- CTA GCG ACG CGG CGA TTG AGA TCT GCG TCT GCG AGG CGA GGG AGT TCT TCT TCT AGG GCG ACG CGG CGA TTG AGA TCT GCG TCT GCG AGG CGA GGG AGT CTT CTT CTA GGA CAT TGA GAT TC-3') encoding amino acid sequence SPRRRTPSPRRRRSQSPRRRRSPRRRTPSPRRRRSQSPRRRR was inserted. Here positive clones showed a 661 bp fragment after *Hind* III/*Apa* I digestion. Preparation of the plasmids for transfection was done with a Maxi Preparation kit (QIAGEN).

### Transfection

For localization of the fusion proteins HuH-7 cells were grown on collagen-coated cover slips as for the *in vitro* transport assays. The cells were transfected with the vectors using the lipofection agent Tfx-20 for 1 h in serum-free DMEM according to the manual of the manufacturer (PROMEGA). The medium was then replaced by DMEM/2.5 % FCS and cells were incubated for 24 h at 37°C, 5 % CO<sub>2</sub> in an incubator. For inhibition of protein kinases, staurosporine (SIGMA) was added during this incubation period at a concentration of 40 nM.

## Immune precipitation

For immune precipitation of capsids and EGFP core fusion proteins, HepG2.2.15 cells were expanded onto twenty 16 cm dishes. Cells were transfected with pEGFP core 1C and cultivated as described above. Cells were washed with PBS before harvesting by trypsin-treatment and centrifugation at 4°C 600 x g. The cells were resuspended in 1 ml PBS and lysed by five times of freezing and thawing. The lysate was sonified three times for 60 sec on ice (70% power/70% impulse cycles, Bandelin UW70 with microtip). Insoluble components were removed by centrifugation for 20 min 4°C at 13000 x g. To obtain more sensitive detection the core proteins and capsids present in the supernatant were labelled by radioactive phosphorylation. Thirty microcurie [ $\gamma$ 32P]ATP and a protease inhibitor mixture (complete, EDTA-free tablet; ROCHE) was added to the lysate, which was adjusted to pH 7.0, 10 mM MgCl<sub>2</sub>, 0.2 mM CaCl<sub>2</sub> and then incubated for 3 h at 37°C. For immune precipitation 10  $\mu$ l anti rabbit-coated biomagnetic beads (3.5 x 10<sup>6</sup> beads, DYNAL) were incubated overnight with 10  $\mu$ l of anti capsid antibody (DAKO (38)) in 100  $\mu$ l PBS/0.1% BSA at 4°C. Unbound antibodies were removed using magnetic absorption to the wall of the tube. The beads were dissolved in 150  $\mu$ l PBS/0.1% BSA and absorbed again. The washing was done three times. When precipitation was performed with the anti EGFP antibody, anti mouse coated biomagnetic beads (DYNAL) and a monoclonal anti EGFP antibody (CLONTECH) were used instead. The beads were added to 300  $\mu$ l of lysate and incubated overnight at 4°C on a rotating wheel. The beads were washed two times with 1 ml PBS/0.1% BSA and 1 x with PBS/0.1% NP-40. The cups were changed for every washing step. Finally, the beads were resuspended in 20  $\mu$ l 1 x loading buffer, 5 min denaturated at 95°C and loaded onto a 4-12 % SDS-PAGE (INVITROGEN). The gel was dried under vacuum at 80°C followed by exposure of a phosphoimager screen. After 3 days the screen was developed on a phosphoimager (Typhoon 9200, MOLECULAR DYNAMICS).

## RESULTS AND DISCUSSION

### Characterization of the nuclear localization signal

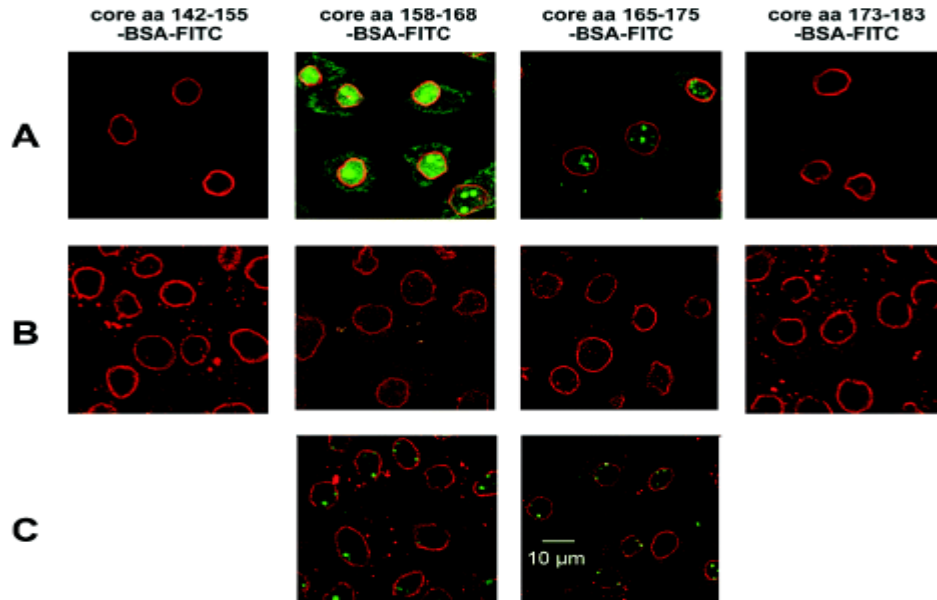
Previous results indicated that two domains of the HBV core protein C-terminus are able to compete with the importin  $\alpha/\beta$  in the importin-mediated pathway (Kann et al., 1999). However, such competition experiments require high concentrations of peptides and are therefore not suitable to determine the effectiveness of importin  $\alpha$ -binding. To identify the minimal sequence of NLS in the core protein, we fused four peptides representing different parts of the C-terminus (aa no. 142-155, 158-

168, 165-175 and 173-183) to FITC-labelled BSA. All peptides comprised a linker of three glycine residues that served as a spacer between the potential NLS and the BSA backbone. MALDI-TOF MS analysis revealed that all BSA conjugates were linked equally well with in average of 19 peptides. Subjecting the conjugates to digitonin permeabilized cells showed that only one conjugate exhibiting the core protein sequence aa 158 - 168 (PRRRTPSPRRR) mediated efficient nuclear transport (Figure 1A). Blocking the nuclear pores by the lectin wheat germ agglutinin (WGA, (Dabauvalle et al., 1988)) inhibited nuclear translocation (Figure. 1B) indicating that nuclear entry occurred through the nuclear pores and was not caused by disruption of the nuclear envelope. Based on crystallization of importin  $\alpha$  with NLS peptides (Fontes et al., 2000) it is assumed that the C-terminal RRR cluster interacts with the binding pockets of the importing  $\alpha$  molecule.

Peptides aa 142 – 155 (TLPETTVVRRRDRG) and 173 – 183 (SPRRRRSQSRE) did not mediate an observable nuclear import (Figure 1A). Since peptide 173 – 183 possessed the same PRRR motif as peptide 158 – 168, it is suggested that this motif is not sufficient for effective importin  $\alpha$  binding. However, NLS may consist of two basic clusters forming a bipartite NLS with Lamin B2 as a prototype (RSSRGKRRRIE, (Hannekes et al., 1993)) and peptide 158 – 168 comprises PRRR motifs at the N- and the C-terminus. Surprisingly the peptide representing aa 165 – 175 (PRRRRSQSPRR) showed only a poor import reaction in the context of the conjugate (Figure.1A). Apparently, the presence of two clusters of basic amino acids is not sufficient but the spacer between the clusters is involved in nuclear import capacity. Both peptides aa 158 – 168 and aa 165 – 175 contain serine residues as potential phosphorylation sites of different protein kinases including protein kinase C (PKC). To evaluate the effect of phosphorylation the conjugates were phosphorylated with PKC. PKC was chosen because this kinase was found in capsids derived from human liver (Kann et al., 1993). When the phosphorylated conjugates were subjected to the permeabilized cells, no nuclear localization could be observed, indicating that the changed charge between the arginine clusters inhibited their binding to importin  $\alpha$  (Figure 1C).

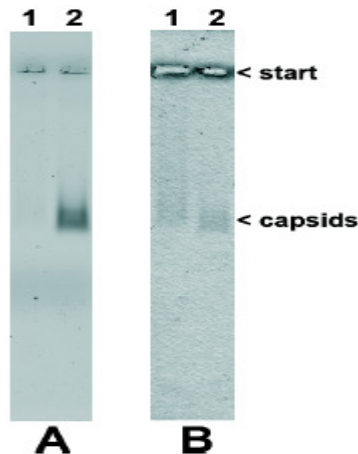
### Effect of phosphorylation on nuclear binding of RNA-containing capsids and of empty capsids

It was shown that phosphorylation of *E. coli*-expressed HBV capsids allows their interaction with importin  $\alpha$  and  $\beta$  resulting in transport through nuclear pores and the phosphorylation caused an external localization of the core protein C-terminus (Rabe et al., 2003). This finding was in accordance with another observation that phosphorylation reduces the RNA packaging affinity of



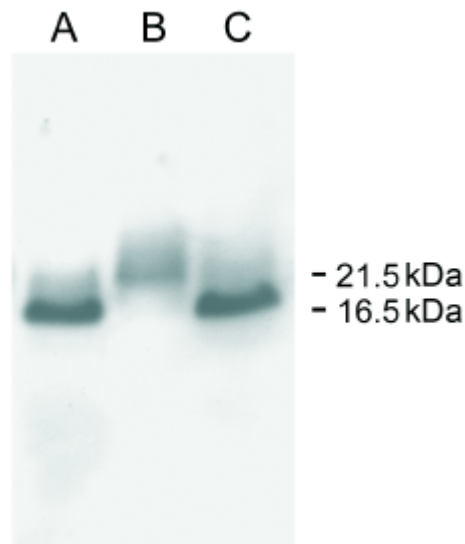
**Figure 1.** *In vitro* transport of FITC BSA-peptide conjugates into the nuclei of digitonin-permeabilized HuH-7 cells.

The conjugates are shown in green; the nuclear pore complexes were stained by indirect immune fluorescence and are shown in red. A: Nuclear import in the presence of cytosolic factors and ATP. In these samples only the conjugate with peptide 158 - 168 was significantly imported. B: Import reaction as in A but with addition of WGA that blocks nuclear pores. In these samples no nuclear import or nuclear binding of conjugate occurred. C: Nuclear import as in A but with *in vitro* phosphorylated conjugates showed that phosphorylation inhibits the nuclear transport capacity of the HBV core NLS.



**Figure 2.** Analysis of empty *E. coli*-expressed capsids.

1: Empty capsids, 2: RNA-containing capsids. Five µg of each capsid preparation were separated under native conditions on a 0.7% agarose gel. A: The encapsidated RNA was visualized by ethidium bromide staining. B: The presence of capsids was then determined by staining with Coomassie brilliant blue. The positions of the loading pockets on the gel (start) and the capsids are indicated.



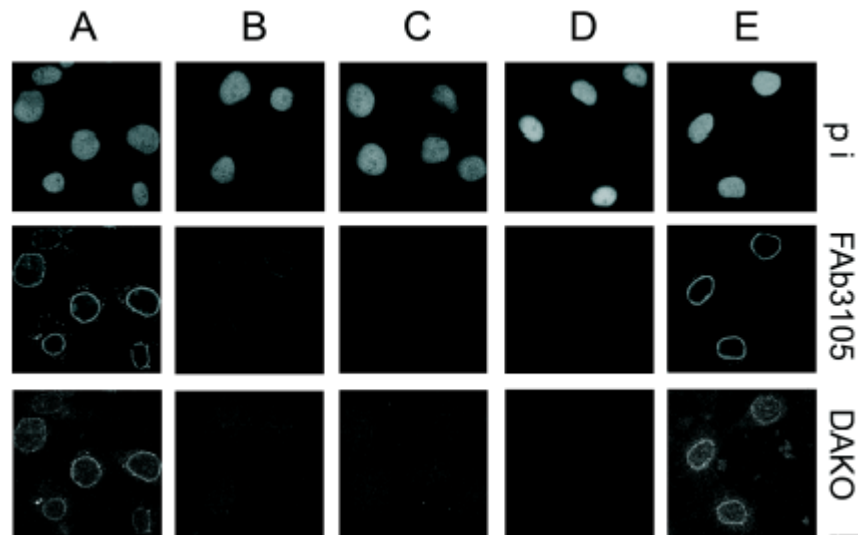
**Figure 3.** Immune blot of trypsin-treated capsids.

After treatment of the capsids with trypsin absorbed to 40 nm-colloidal gold the samples were separated on an SDS, blotted and detected with an antibody against denaturated core protein. A: Empty capsids that were digested with trypsin. B: Trypsin-treated RNA-containing capsids. C: Trypsin-treated RNA-containing capsids that were denaturated with acid prior to the digest (positive control). While the core proteins of RNA-containing capsid remained undigested by trypsin treatment (21.5 kDa) the core proteins of empty capsids and denaturated core proteins were truncated to 16.5 kDa.

the capsids *in vitro* (Kann et al., 1994), implying that a reduced affinity between the C-terminus and RNA allows translocation of the C-terminus to the surface. Despite of the necessity of an external NLS exposure to allow interaction with the transport receptors, whether the phosphorylation is directly required for NLS function is still not known. Based on the hypothesis that the luminal RNA arrests the C-terminus within the cavity of the capsid, we generated *E. coli*-derived capsids devoid of RNA. Figure 2 depicts that after disintegration of the capsid, nuclease digestion and reconstitution of the capsid, no significant amounts of encapsidated RNA were found within the capsids. Immune blot with an anti capsid antibody showed the same reactivity (data not shown).

The empty capsids migrated somewhat slower, indicating that they exhibit more positive surface charges than the untreated capsids. Such additional positive charges could be explained by the exposure of arginine clusters on the surface. To further evaluate the exposure of the C-terminus we treated the capsids with colloidal gold-bound trypsin as described elsewhere (Rabe et al.,

2003). Degradation was determined by immune blot with an antibody against denaturated core proteins (antibody 89a) after separation of the samples on SDS PAGE. As indicated by the shift of the band from 21.5 to 16.6 kDa, all C-termini were removed from the empty capsids (Figure 3). As observed in previous studies, RNA containing capsids were not degraded, whereas acid-denaturated core protein was degraded. The empty capsids were subjected to digitonin permeabilized cells. After washing of the capsid-loaded cells immune staining was performed. A capsid-specific antibody was poorly recognized core protein subunits (Figure 4, lower row, (Kann et al., 1999). To allow detection of disintegrated capsids, a second primary antibody that binds to core protein dimers (FAb 3105) as it was determined previously by others (Belnap et al., 2003) was used (Figure 4, middle row). To ensure proper section level in confocal laser scan microscopy, staining of the nuclei by Propidium iodide was performed (Figure 4, upper row). As a positive control *in vitro* phosphorylated RNA-containing capsids were used that have shown to be imported through the nuclear pores (Figure 4A, (Kann et



**Figure 4.** *In vitro* binding of different capsid species to nuclei of digitonin-permeabilized cells.

In all assays a cytosolic extract containing the nuclear transport receptors and ATP was present. The figure shows that phosphorylation is a requirement for the interaction of the capsids with the nucleus irrespective of the exposure of the C-termini. A: *In vitro*-phosphorylated RNA-containing capsids. B: RNA-containing unphosphorylated capsids. C: Negative control to which no capsids were added. D: Empty unphosphorylated capsids. E: Empty *in vitro*-phosphorylated capsids. P.i.: propidium iodid, FAB3105: monoclonal antibody against core protein dimers, DAKO: polyclonal antibody against HBV capsids.

al., 1999)). A negative control was performed with unphosphorylated RNA-containing capsids (Figure 4B). Specificity of the stain was

determined in a sample without capsids (Figure 4C).

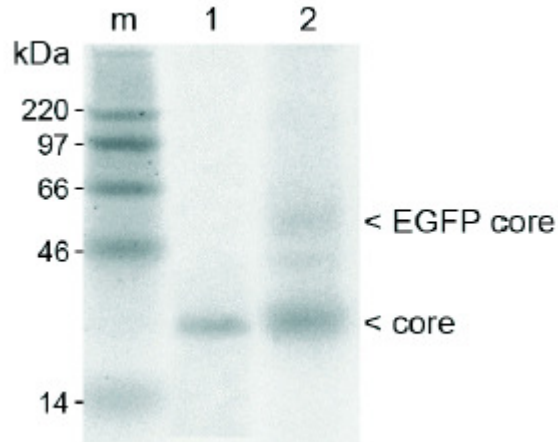
As in previous experiments unphosphorylated RNA-containing capsids did not bind to the nuclear envelope while their phosphorylated equivalents did (Kann et al., 1999) (Figure 4A, B). Surprisingly, empty capsids did not interact with the nuclei despite their exposed C-termini (Figure 4D). However, when empty capsids were phosphorylated the capacity to interact with the nuclei was restored (Figure 4E). Intranuclear staining of capsids or core protein dimers was not observed in any of the samples. This result confirms that in spite of the import through the nuclear pores the capsids became arrested at the inner face of the nuclear pore complex, as was previously shown for phosphorylated RNA-containing capsids (Pante and Kann, 2002).

#### Effect of core protein phosphorylation in living cells

Based on the *in vitro* transport assays, core protein phosphorylation is required for interaction with the nucleus but the phosphorylation must not occur within the NLS. To evaluate whether such a differentiated phosphorylation occurs in living cells, we transiently expressed the core protein that was fused with its N-terminus to the enhanced green fluorescent protein

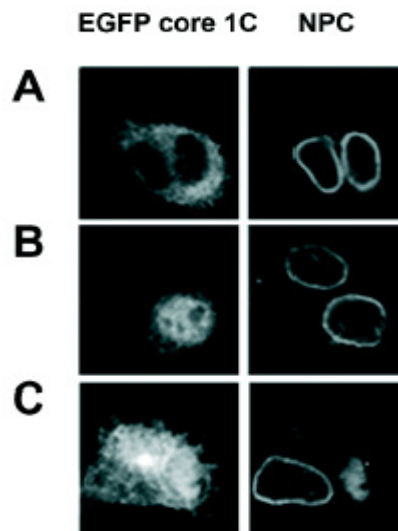
(EGFP core 1C) in the hepatoma cell line HuH-7. This fusion protein was chosen for study instead of capsids since the protein should not assemble to capsids thus exposing their C-terminus permanently to phosphorylation.

N-terminal extensions of the core protein for capsid assembly are tolerated up to 40 aa as shown for the *Puumala hantavirus* nucleocapsid protein (Koletzki et al., 1999). However as EGFP may have other properties we first evaluated the potential capsid and dimer formation of the fusion protein. Therefore, the EGFP core 1C fusion protein was expressed in HepG2.2.15 cells that stably express all hepadnaviral proteins including the wild type core protein (Sells et al., 1987). Immune precipitation of capsids with a capsid antibody from the lysate of transfected cells showed that no fusion protein was precipitated (Figure 5, lane 1) indicating that it was not incorporated into capsids. When the precipitation was performed with an anti EGFP antibody, wild type core protein was co-precipitated (Figure 5, lane 2). This indicates that the EGFP core fusion protein has retained its ability to dimerize, confirming the data of Koletzki et al. (1999). However the 21.5 kDa band of the co-precipitated wild type core protein appears stronger than that of the EGFP fusion protein. As capsid assembly occurs via core protein dimers that trimerize (Endres and Zlotnick, 2002), this observation may indicate that EGFP fusion protein has retained its ability to form these "trimers of dimers". For localization of EGFP core 1C, we first evaluated the



**Figure 5.** Immune precipitations of wild type core protein and EGFP core 1C fusion protein from a lysate of transfected HepG2.2.15 cells.

For radioactive label the core proteins and capsids in the lysate were preincubated with [ $\gamma$ - $^{32}$ P]ATP prior to precipitation and subsequent separation on an SDS PAGE. While the anti-capsid antibody failed to precipitate the EGFP core 1C fusion protein (lane 1) the anti EGFP antibody precipitated EGFP core – showing a slightly retarded migration compared to the calculated MW of 49 kDa - and wild type core protein (lane 2). m:  $^{14}$ C-labelled molecular weight marker.



**Figure 6.** Localization of EGFP and EGFP core 1C fusion protein in transiently transfected HuH-7 cells. The figures are representative for the intracellular distributions in different cells and transfections. The nuclear envelope was visualized by indirect immune stain with an anti-nuclear pore complex antibody. The fluorescence of EGFP and EGFP core 1C fusion protein were directly recorded using a laser scan microscope. A: Cytosolic distribution. B: Nuclear localization. C: Localization in both compartments.



**Table 1.** Reliability of EGFP and EGFP-core 1 wt NLS protein expression. The transfections were done on HuH-7 cells from different cell stocks at different time points but using the same plasmid stock for transfection.

no.	expressed protein	cytoplasm	nucleus	both	total
1.	EGFP core 1C	43 45%	51 54%	1 1%	95 100%
2.	EGFP core 1C	14 35%	25 64%	1 1%	40 100%
3.	EGFP core 1C	40 38%	64 61%	1 1%	105 100%
4.	EGFP core 1C	33 48%	36 52%	0 0%	69 100%
5.	EGFP core 1C	5 25%	15 75%	0 0%	20 100%
1.	EGFP	100 71%	39 28%	1 1%	140 100%
2.	EGFP	18 78%	5 22%	0 0%	23 100%

reproducibility of the system by expression in several experiments. In control transfections EGFP without the core protein was expressed. Twenty four hours after transfection, the localizations of the proteins were determined in hundreds of cells. A representative example of intracellular EGFP core 1C and EGFP localization is shown in Figure 6. To distinguish between cytoplasmic (Figure 6A) and nuclear (Figure 6B) localization a control stain against the NPC was performed.

Table 1 shows the distribution of EGFP and fusion protein localization in hundreds of cells. While EGFP was found predominantly in the cytoplasm (78%-71%) the fusion protein was mainly localized within the nucleus (52%-75%). Only in a minority of cells (0-1%) were EGFP or the EGFP core fusion proteins found in both compartments (Figure 6C), evidence against a passive diffusion through the nuclear pores. The appearance of cytosolic EGFP core fusion protein is in accordance with observations of Yeh *et al.* (1993) who showed that HBV core proteins are excluded from the nucleus during cell division.

The co-precipitation experiments indicated that the fusion proteins may have been transported as a dimer instead of a monomer. This may have enhanced nuclear import due to the presence of two NLS by increasing the probability of an NLS-importin  $\alpha$  contact. To determine the effect of NLS redundancy, we expressed EGFP core fusion protein mutants in which the NLS comprising C-

terminus was exposed as doublet or triplet (EGFP core 2C and 3C) in HuH-7 cells. These fusion proteins localized as EGFP core 1C (data not shown) but showed a higher frequency of nuclear localization (Table 2). To study the effect of core protein phosphorylation on nuclear localization, we treated cells expressing the EGFP core 3C protein with the broad-range protein kinase inhibitor staurosporine as previously described (Wolf and Baggioni, 1988). This protein was chosen because of the higher nuclear import capacity compared to EGFP core 1C allowing a better analysis of an inhibitory effect on nuclear import. In a control transfection EGFP was expressed. Localization was compared to the transfected but untreated control cells. In both inhibitor-treated and untreated cells EGFP localized to a similar amount in cytoplasm and nucleus (Table 3). Apparently, the nuclear membrane was not disintegrated as may have occurred due to an apoptotic effect of staurosporine (Sanchez *et al.*, 1992). Although the intracellular localization of the fusion protein did not change in individual cells (data not shown) the overall distribution was dramatically changed. While 89% of the untreated fusion protein-expressing cells showed a predominantly nuclear localization, staurosporine-treatment resulted in a predominantly cytosolic distribution similar to that of EGFP, confirming that phosphorylation is required for nuclear transport of the core protein. Staurosporine prevents the nuclear localization of fusion protein-expressing cells through an

**Table 2.** Distribution of EGFP-core fusion proteins with redundant NLS.

The different vectors encoding EGFP or EGFP-core fusion proteins were transfected into HuH-7 cells that were grown in parallel. EGFP served as a control. The fusion proteins contain the natural HBV capsid NLS as a single copy or in redundancy (EGFP-core 1C, 2C, 3C).

expressed protein	cytoplasm	nucleus	both	total
EGFP	18 78%	5 22%	0 0%	23 100%
EGFP-core 1C	5 25%	15 75%	0 0%	20 100%
EGFP-core 2C	2 18%	9 82%	0 0%	11 100%
EGFP-core 3C	2 6%	33 94%	0 0%	35 100%

**Table 3.** Distribution of EGFP and EGFP-core 3C in untreated and Staurosporine-treated HuH-7 cells.

Without Staurosporin EGFP-core 3C predominantly localized within the nucleus. Staurosporin treatment changed the distribution pattern of the EGFP core fusion protein to a predominantly cytoplasmic localization but had no effect on EGFP distribution.

protein	staurosporine	cytoplasm	nucleus	both	total
EGFP core 3C	no	2	16	0	18
		11%	89%	0%	100%
	yes	10	2	0	12
		84%	16%	0%	100%
EGFP	no	18	5	0	23
		78%	28%	0%	100%
	yes	19	6	0	25
		76%	24%	0%	100%

inhibition of the phosphoprotein of core protein in hepatocyte cell line (HepG2 cell). Staurosporin also prevents the cell division, so that passive trapping of core protein is inhibited (Haryanto et al., 2007).

As hepatocytes in human livers divide only rarely the nuclear localization of HBV capsids implies that the entire particles or the unassembled core proteins are actively imported into the nucleus. Consistent with this observation, cytosolic capsids are rarely found in those cells that have recently undergone cell division. Having a molecular weight of less than 70 kDa the core protein is small enough to pass the nuclear pore by diffusion (Breeuwer and Goldfarb, 1990; Paine et al., 1975). However, there are several examples of small proteins that are actively imported using cellular transport receptors as e.g. histones (Breeuwer and Goldfarb, 1990). Accordingly, we found that EGFP core fusion proteins having a molecular weight of ~49 kDa did not diffuse between cytoplasm and nucleus but were

predominantly found within the karyoplasm.

As was determined previously by Kann et al. (1999), the core protein comprises a NSL within the C-terminus, the NLS interacts with importing  $\beta$  that serves as an adapter molecule to the transport receptor importin  $\beta$ . In the present study we found that a short sequence (PRRRTPSPRRR) mediated efficient nuclear import when being fused to non-karyophilic BSA. Comparison to other peptide conjugates revealed that apparently both N- and C-terminal clusters of basic amino acids are required implying the presence of a bipartite NLS as has been shown for Lamin B2 (Hannekes et al., 1993). However, the amino acids spacing the two clusters affected the transport capacity as peptide 165 – 175 (PRRRRSQSPRR) showed only a poor import reaction in the context of the conjugate. Nonetheless, the high import capacity of the NLS was surprising because this sequence does not contain a lysine residue as should be required within the C-terminal cluster according to

crystallization studies (Fontes et al., 2000). This finding can be explained by additional interactions between the backbones of importin  $\alpha$  and cargo outside the NLS and the corresponding binding pocket as it was described for the nuclear transport of other proteins as e.g. the Borna disease virus p10 protein (Wolff et al., 2002) and the SV40 TAG (Harreman et al., 2004).

As we have shown, phosphorylation within the NLS abolishes its nuclear transport capacity. Obviously, a negative charge in the spacer that separates the basic clusters assumed to interact with importin  $\alpha$  prevents sufficient interaction. This inhibition correlates with findings on the SV40 TAG in which phosphorylation at aa 124, adjacent to the NLS (aa 126 -132) inhibits nuclear import (Jans et al., 1991).

The identified core protein NLS overlaps with the RNA binding domain. Consistently, the NLS is located in the lumen in unphosphorylated *E. coli* expressed capsids that contain RNA (Rabe et al., 2003). However, when the C-terminus becomes phosphorylated the NLS becomes accessible at the capsid surface leading to importin  $\alpha/\beta$  binding and subsequent transport of the capsid through the nuclear pores (Kann et al., 1999). It is thought that phosphorylation inhibits interaction between the RNA and the C-terminus (Kann et al., 1994) allowing the C-termini to translocate to the capsid surface through the 2 nm holes of the capsid. This assumption would explain why capsids with a mature partially double-stranded DNA genome expose the C-terminus (Rabe et al., 2003) as the affinity of the C-terminus to double-stranded DNA is weaker than to RNA (Melegari et al., 1991). Consistently with that hypothesis capsids devoid of nucleic acids expose the NLS-containing C-termini on the surface. However, exposure of the NLS did not lead to significant interaction with the nuclear envelope. Only phosphorylated capsids bound implying a complex interaction or folding of the C-terminus to allow importin  $\alpha$ -interaction similar to the phosphorylation of the SV40TAG at aa 111/112 (Hubner et al., 1997).

To evaluate whether the differential phosphorylations have significance *in vivo* we analyzed the localization of EGFP core protein fusion proteins in HuH-7 cells. The proteins showed a nuclear transport, however in a significant number of cells they were found predominantly in the cytoplasm. It must thus be concluded that nuclear import is dependent upon the individual cell. Based on the observations of Yeh et al. (1993) who showed that the core protein is excluded from the nucleus during S phase we assume that these cells have recently undergone cell division. Thus the HBV core protein behaves like the transcription factor Swi6p of *Saccharomyces cerevisiae* that becomes cell cycle-dependently imported into the nucleus due to its phosphorylation-dependent affinity for importin  $\alpha$  (Harreman et al., 2004).

Inhibition of phosphorylation revealed that nuclear import of the EGFP core fusion proteins was strongly

inhibited. This observation is in accordance with the *in vitro* finding that phosphorylation is required for binding of the HBV capsids to the nuclear pores. We assume that in those cells in which the EGFP core fusion proteins were excluded from the nucleus the phosphorylation pattern is different. However, based on the results obtained here we cannot conclude if the NLS is phosphorylated or if no NLS function-supporting phosphorylation occurs.

In conclusion, we propose complex phosphorylation kinetics during the viral life cycle that still must be investigated. The core proteins are already phosphorylated at serine residue 157 to allow pregenome packaging. Residues 164 and 172 may be already phosphorylated at this stage or shortly afterwards as they are required for genome maturation (Melegari et al., 2005). Obviously, this phosphorylation pattern is not sufficient to cause translocation of the C-terminus to the capsid surface since replication-inhibited capsids expose the C-termini only rarely (Rabe et al., 2003). DNA synthesis within the lumen of the capsid reduces the affinity of the C-termini to the encapsidated nucleic acid allowing that the NLS becomes exposed. Serine 164 has to become dephosphorylated in order to allow importin  $\alpha$  binding. Other residues as e.g. 170/172 that are phosphorylated in capsids of virions (Machida et al., 1991) stay phosphorylated. Phosphorylation kinetics would establish a coordinated C-termini location for pregenome packaging, genome maturation and NLS function.

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