

Phosphorylation-dependent Binding of Hepatitis B Virus Core Particles to the Nuclear Pore Complex

Michael Kann,* Beate Sodeik,[†] Angelika Vlachou,* Wolfram H. Gerlich,* and Ari Helenius[‡]

*Institute of Medical Virology, Justus Liebig University, D-35392 Giessen, Germany; and [†]Yale University School of Medicine, Department of Cell Biology, New Haven, Connecticut 06520-8002

Abstract. Although many viruses replicate in the nucleus, little is known about the processes involved in the nuclear import of viral genomes. We show here that *in vitro* generated core particles of human hepatitis B virus bind to nuclear pore complexes (NPCs) in digitonin-permeabilized mammalian cells. This only occurred if the cores contained phosphorylated core proteins. Binding was inhibited by wheat germ agglutinin, by antinuclear pore complex antibodies, and by peptides corresponding either to classical nuclear localization signals (NLS) or to COOH-terminal sequences of the core protein. Binding was dependent on the nuclear trans-

port factors importins (karyopherins) α and β . The results suggested that phosphorylation induces exposure of NLS in the COOH-terminal portion of the core protein that allows core binding to the NPCs by the importin- (karyopherin-) mediated pathway. Thus, phosphorylation of the core protein emerged as an important step in the viral replication cycle necessary for transport of the viral genome to the nucleus.

Key words: core • hepatitis B virus • nuclear pore • nucleocapsid • phosphorylation

SINCE many viruses depend on the host cell machinery present in the nucleus for genome replication, transcription, and mRNA processing, they have the capacity to target their genome and accessory proteins to the nucleus. This requires multiple steps in nondividing cells: active transport or diffusion of the virus or viral capsids to the nucleus, binding to the nuclear pores, and importing of the genome and accessory proteins through the nuclear pore complex (NPC).¹ In this study, we have addressed nuclear targeting of human hepatitis B virus (HBV) cores.

HBV is an important pathogen that causes acute and chronic hepatitis and eventually hepatocellular carcinoma. HBV particles contain an envelope with surface glycopro-

teins and an icosahedral capsid (core) with a diameter of 32 or 36 nm (7, 33). Cores occur in two forms with either 180 or 240 copies of a positively charged protein, the core protein (21 kD). They also contain a viral DNA polymerase covalently attached to the viral DNA (14) and the host cell proteins, heat shock protein (hsp90; 25) and protein kinase C (PKC; 31, 32).

The pathway of HBV entry is difficult to study because the virus does not infect cultured cell lines. However, it is known that after fusion of the viral envelope with a cellular membrane, the core is delivered into the cytosol (45). Afterwards, the DNA genome enters the nucleus (53, 54). With a diameter of >25 nm, the core exceeds the size limit for nuclear localization signal (NLS)-coated colloidal gold particles that can enter through nuclear pores (9). Thus, import of DNA should involve disassembly or deformation of the core particle.

Inside the nucleus, the partially double-stranded viral DNA is converted into a covalently closed circular DNA (cccDNA) and transcribed by host RNA polymerase II (34). The resulting mRNAs are transported to the cytosol and used to translate viral proteins. A pregenomic, full length RNA is also produced as an RNA polymerase transcript. After transport to the cytosol, it associates with a protein complex that contains viral polymerase (2, 23, 28) and host cell hsp90 (25). The RNA protein complex is encapsidated together with PKC (31) into the assembling core structure made up of the core protein. Inside this par-

The present address of B. Sodeik is Institute of Biochemistry, Medical School Hannover, OE4310, D-30623 Hannover, Germany. The present address of A. Helenius is Laboratory for Biochemistry, Swiss Federal Institute of Technology Zürich, CH-8092 Zürich, Switzerland.

Address correspondence to Dr. Michael Kann, Institute of Medical Virology, Justus Liebig University, Frankfurter Street 107, D-35392 Giessen, Germany. Tel.: 49-641-99-41243. Fax: 49-641-99-41209. E-mail: michael.kann@viro.med.uni-giessen.de

1. **Abbreviations used in this paper:** cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; hHBc, human HepG2.2.15-derived core; hsp, heat shock protein; MALDI, matrix-assisted laser desorption/ionization; NBS, nuclear pore binding signal; NLS, nuclear localization signal; NPC, nuclear pore complex; PKC, protein kinase C; P-rHBc, phosphorylated *E. coli*-derived cores; rHBc, unphosphorylated *E. coli*-derived cores; RRL, rabbit reticulocyte lysate; TFA, trifluoroacetic acid.

ticle, the viral polymerase uses the RNA as a template to reverse-transcribe a negative sense DNA (51). As in retroviruses, reverse transcription is concomitant with the stepwise degradation of the RNA template. Next, a positive strand viral DNA is synthesized using the minus strand as a template. Coordinately with reverse transcription, the encapsidated PKC phosphorylates COOH-terminal serines in the core protein giving rise to mature phosphorylated progeny core particle (31).

The mature core particles assembled in the cytosol have two possible fates. They can be targeted to the nucleus for additional rounds of genome replication and for amplification of cccDNA (54). This reinfection process is particularly frequent early in infection (53). It is thought to promote establishment of the full-blown infection. Alternatively, in the presence of large and small surface proteins, cores can bud into the intermediate compartment (5, 26) between the endoplasmic reticulum and the Golgi complex, and exit the cell as enveloped virions through the secretory pathway (29).

Core proteins contain an NLS in their COOH terminus (10) overlapping with a nucleic acid-binding domain (21) and five potential serine phosphorylation sites (36). Cryoelectronmicroscopy has shown that the COOH-terminal sequence is not exposed on the surface of unphosphorylated core, but rather faces the internal cavity (60). Unassembled core proteins, where this sequence is exposed, were shown to be imported into the nucleus of HBV-infected cells (36). Inside the nucleus, the imported core proteins spontaneously assemble into core particles devoid of nucleic acids (19). There are several strategies for nuclear import of viral genomes (57). As with adenoviruses and herpes viruses, the capsids may bind to the nuclear pore and release a complex of viral DNA and associated viral proteins through the pore into the nucleoplasm. Alternatively, smaller genome-protein complexes may be generated by disassembly and targeted for import into the nucleus. Small viruses may in fact enter intact through the NPC.

For HBV the nuclear import of the genome must be a tightly regulated event. Premature release of the polymerase-DNA complex into the nucleus (30) would lead to abortion of second strand DNA synthesis (41) and abortion of the viral life cycle. To identify the strategy of nuclear binding and its regulation, we analyzed the structures and mechanisms that target HBV core particles to the nucleus. We reconstituted the nuclear binding of core particles *in vitro* and analyzed its regulation in permeabilized human hepatoma cell lines. Using bacterially expressed and authentic cores, we identified the required host factors.

Materials and Methods

Preparation of PKC-phosphorylated Core Particles

Purified *Escherichia coli*-derived core particles, as characterized by Crowther et al. (7), were obtained from Drs. Galina Borisova and Paul Pumpens (Biomedical Research and Study Centre, University of Latvia, Riga, Latvia). The particles were subjected to permeabilization and phosphorylation as described previously (31). Because of the RNA content of the particles, the incubation temperature and time for phosphorylation were increased to 37°C and 30 min, respectively. After reconstitution of

particle integrity, entire core particles were separated from free nucleotides, protein kinases, and unassembled core proteins by sedimentation through a 0.5 ml 25% (wt/vol) sucrose cushion in TNE buffer (40 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) using a rotor (TL100; Beckman Instruments) at 100,000 rpm at 15°C for 1 h. The pellet was resuspended in 50 μ l TNE, aliquoted, and stored at -80°C until further use. The core particle concentration was determined using a core ELISA (>20 ng/ μ l; ref. 47) using a standard of *E. coli*-expressed core particles (Chiron).

Analysis of PKC-phosphorylated Core Particles

20 ng of phosphorylated or unphosphorylated core particles was separated on a 1% agarose gel, using agarose gel loading buffer without SDS. To disintegrate the particle structure, cores in parallel samples were incubated in 0.1% trifluoroacetic acid (TFA), heated to 50°C for 10 min, neutralized by adding 0.5 vol of 0.1 M Tris-HCl, pH 8.8, before loading on the gel. Proteins were blotted on an Immobilon-P membrane overnight (Millipore; 50). The membrane was blocked for 1 h at room temperature in 5% (wt/vol) fat-free milk in PBS followed by addition of the first antibody (anti-HBc; DAKO) at a dilution of 1:4,000 in 5% milk/PBS for 2 h at room temperature. After washing (3 \times for 10 min in 0.1% milk/0.1% Tween 20/PBS at room temperature), the second antibody reaction was performed with HRP-labeled donkey anti-rabbit antibody (Dianova Co.) at a dilution of 1:5,000 in 5% milk/PBS for 1 h at room temperature. After washing, the antibodies were visualized by an enhanced chemiluminescence kit (Boehringer Mannheim GmbH). For analyzing the radioactive phosphorylation with [γ -³²P]ATP (Amersham), the membrane was washed three times for 10 min in PBS before analysis by phosphorimaging (BAS-IIS for Fuji BAS1000 Bioimager; Fuji Photo Film Co.).

For quantification of core phosphorylation, 2 μ l of the samples was spotted on GF/C filters (Whatman), air dried, washed twice for 10 min in ice-cold 10% (wt/vol) TCA and twice for 5 min in ice-cold 5% TCA. The filters were dried and protein-bound radioactivity was determined by liquid scintillation counting.

For isopycnic centrifugation a CsCl gradient was performed with a density of 1.51–1.22 g/ml PBS in an SW60 rotor (Beckman). The ³²P-labeled core particle preparation was loaded on top and centrifuged at 35,000 rpm for 16 h at 10°C. The gradient was harvested in 20 fractions of 175 μ l. 20 μ l of each aliquot was subjected to core ELISA and to liquid scintillation counting. To analyze the dephosphorylation of phosphorylated core particles, 20 ng of particles was incubated with bacterial alkaline phosphatase (200 U/ml; New England Biolabs) or with 4,000 U/ml calf intestinal phosphatase (Boehringer Mannheim GmbH) in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl or 100 mM Tris-HCl, pH 8.5, 150 mM NaCl, respectively. In the control reaction, phosphorylated core particles were disintegrated by TFA, as described above, before dephosphorylation. Samples were incubated for 30 min at 37°C. Samples containing core particles and the unphosphorylated control were separated on an agarose gel, blotted, and exposed as described above. Disintegrated core protein samples were separated on a 16% SDS-PAGE, stained with Coomassie brilliant blue, and dried before autoradiography.

Generation of Core Particles from the Culture Medium of HBV-transfected Cells

HepG2.2.15 cells were grown on 16-cm dishes in 5% CO₂ at 37°C. After reaching confluency DMEM containing 3% FCS was added for 4 d. 500 ml of medium was collected and insoluble components were sedimented by centrifugation at 3,500 g for 30 min at 4°C. HBV of the supernatant was sedimented through 3-ml cushions of 25% (wt/wt) sucrose/TNE in an SW27 rotor at 27,000 rpm for 36 h at 4°C. Each pellet was resuspended in 150 μ l TNE adjusted to a CaCl₂ concentration of 5 mM and incubated with 25 U/ml S7 nuclease (Boehringer Mannheim GmbH) for 1 h at 37°C. To remove the surface proteins from the virus, NP-40 was added to 0.1%. After incubation, insoluble components were sedimented through a 0.5-ml cushion of 25% (wt/wt) sucrose/TNE for 10 min at 10°C in a TL100 rotor (Beckman Instruments) at 100,000 rpm. The upper phase, including the interphase, contained the core particles. They were sedimented through a 0.5-ml cushion of 25% (wt/wt) sucrose/TNE for 2 h at 10°C in a TL100 rotor (Beckman) at 100,000 rpm. The pellet was resuspended in 200 μ l TNE.

Generation of FITC-BSA Conjugates

FITC-BSA (Sigma Chemical Co.) was conjugated with NLS according to Görlich et al. (18). The NLSs used were the SV-40TAG NLS

(PKKKRQVED; 18) and M9 domain of hnRNP (YNNQSSNFGPMK). Both contain a spacer (amino acid sequence CGGG; 18) at their NH₂ terminus. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Dietmar Linder and Monica Linder, Institute of Biochemistry, Department of Medicine, Giessen, Germany) of the conjugates showed, on average, 19 NLS were linked to 1 BSA molecule.

Binding and Transport Assay

Cells were grown in DMEM with 7% FCS on collagen (Sigma Chemical Co.) or Cell-Tak (Becton Dickinson) coated 12-mm coverslips in 24-well dishes in 5% CO₂ at 37°C. The cells were washed with 1 ml serum-free DMEM and permeabilized with 80 µg/ml digitonin (Sigma Chemical Co.) in DMEM for 10 min at 37°C (1). They were washed twice for 10 min at 4°C in washing buffer (transport buffer: 2 mM Mg-acetate, 20 mM Hepes, pH 7.3, 110 mM K-acetate, 1 mM EGTA, 5 mM Na-acetate/1 mM DTT) containing 1% BSA and 10% goat serum (Dianova) followed by a 10-min incubation at 37°C in a humidified box using washing buffer. Preincubation of permeabilized cells with 50 µg/ml WGA/ml transport buffer (Boehringer Mannheim GmbH) or 200 µg/ml antibodies (anti-NPC antibody, mAb414 [BABC0]/ml transport buffer, anti-NS2, or anticalnexin), was performed during this step.

Permeabilized cells were incubated with 5 µg/ml core particles in 20 µl transport buffer containing 30 mg/ml rabbit reticulocyte lysate (RRL; Promega Corp.); 10 µg/ml aprotinin (Sigma Chemical Co.), 10 µg/ml leupeptin (Sigma Chemical Co.), 10 µg/ml pepstatin (Sigma Chemical Co.) for 20 min at 37°C. If required, an ATP-generating system (1 mM ATP, 5 mM creatine phosphate, and 20 U/ml creatine phosphokinase; Sigma Chemical Co.) or ATP-depleting system (7 mM glucose, 1 U/ml hexokinase; Sigma Chemical Co.) was added (1).

In some experiments, RRL was preincubated with peptides, representing parts of the COOH terminus of the core protein (amino acids 142–155, TLPETTVVRRRDRG; amino acids 158–168, PRRRTSPRRR; amino acids 165–175, PRRRRSQSPRR; amino acids 173–183, PRRRRSQSRES); or the NLS of SV-40 T antigen (STPPKKRKRKV), or lamin B2 (RSSRGKRRRIE) at a concentration of 2 mM for 10 min on ice. The peptides were synthesized by Dr. Ursula Friedrich (Institute of Medical Virology, Giessen, Germany) on an Applied Biosystems 431A Peptide Synthesizer, purified by HPLC, and analyzed by MALDI mass spectrometry (Dietmar Linder and Monika Linder).

For incubation of cores in the absence of cytosolic proteins, BSA was added instead of reticulocyte lysate to the same protein concentration (30 mg/ml). In some experiments, importin (karyopherin) α (Rch 1) and β (gift of D. Görlich, ZMBH, Heidelberg, Germany) were added to BSA-containing buffer at a concentration of 30 ng/µl (16) each. For the transport assay, FITC-labeled BSA conjugate with SV-40TAg NLS was added to a final concentration of 20 µg/ml. After incubating for 20 min at 37°C, cells were washed in washing buffer as described above.

When the phosphorylated *E. coli*-derived core (P-rHBc) was used for blocking the import of the FITC-BSA-M9 conjugate, P-rHBc was added at a concentration of 100 ng/µl to the assay in the presence of RRL and incubated for 20 min at 37°C as described above. In the control experiment, cells were treated identically but without addition of P-rHBc. After the binding reaction, cells were washed three times in washing buffer for 10 min at 0°C. The coverslips were loaded on a new reaction mixture containing reticulocyte lysate, ATP-generating system and 20 ng/µl of the FITC-BSA-M9 conjugate. After a second incubation period of 20 min at 37°C, the coverslips were washed as described above.

To quantitate the amount of bound core particles, cells were scraped off the coverslip using a rubber policeman, counted in a Neubauer chamber, and lysed by addition of 100 µl 10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 10 mM MgCl₂, 150 mM NaCl containing 20 U DNase I, and 2 mg/ml RNase A. Cells were incubated for 15 min at 37°C and lysis was controlled microscopically before analysis by ELISA.

Immunofluorescence Microscopy

For immunofluorescence microscopy, cells were fixed onto the coverslips using 3% paraformaldehyde (Merck) in PBS, pH 7.4, for 30 min at room temperature. The coverslips were washed once in PBS and in PBS/0.1% Triton X-100 for 5 min. After two additional washings for 5 min in PBS at room temperature, cells were exposed to a mixture of anti-HBc antibody (1:200) and anti-NPC (1:1,000) in PBS/10% goat serum/1% BSA for 90 min at 37°C. Coverslips were washed in PBS three times for 5 min at room temperature and incubated with secondary antibodies (affinity-purified FITC-conjugated goat anti-rabbit IgG, H+L; Dianova; affinity-purified

Texas red-conjugated goat anti-mouse IgG, H+L; Dianova) in PBS/10% goat serum/1% BSA for 40 min at 37°C. Cells were washed as described above and embedded in 50 mg/ml DABCO/moviol (Sigma Chemical Co. and Hoechst, respectively). Microscopy was performed on a Zeiss fluorescence microscope, equipped with a 63× plan apochromat objective (Zeiss). Photographs were taken on Kodak TMAX 400 pro film. Negatives were developed at 1600 ASA.

Confocal immunofluorescence microscopy was performed on a Leica DM IRBE microscope. Analysis of core-NPC colocalization and of the nuclear localization of FITC-labeled conjugates was done by using the TRITC- and FITC-fittings at a pinhole size of 0.

Coimmunoprecipitation of Importin β and Core Particles from Reticulocyte Lysate

7 × 10⁶ sheep anti-rabbit conjugated biomagnetic beads (Dyna) were washed twice in PBS according to the vendor's manual, resuspended in 500 µl PBS containing 2 µl anti-core antibody-containing rabbit serum (DAKO), and incubated overnight at 4°C. After washing as described above, 25 ng core was added and incubated overnight at 4°C. The beads were washed three times in 500 µl PBS, resuspended in 500 µl 0.1% BSA/PBS, and incubated for 1 h at 4°C. Afterwards, the beads were added to 100 µl of RRL for 30 min at 37°C. In a control experiment, 2 mM of lamin B2 NLS (RSSRGKRRRIE) was added to the lysate. Further controls were performed by replacing lysate either with 300 ng importin β and 450 ng importin α in transport buffer, or by 300 ng importin β alone. The beads were washed three times in 500 µl PBS, 1 × 500 µl PBS/0.1% Tween 20, and transferred to a new tube. After three cycles of washing in 500 µl PBS, the pellet was resuspended in 30 µl Laemmli buffer (66 mM Tris-HCl, pH 6.8, 10% [vol/vol] glycerol, 5% [vol/vol] β-mercaptoethanol, 2% [wt/vol] SDS, 0.015% [wt/vol] bromophenol blue), and denatured for 5 min at 96°C. The samples were separated on an 12% SDS-PAGE and blotted overnight to an Immobilon-P membrane (Millipore) by wet transfer. The membrane was blocked for 1 h at room temperature in 5% fat-free milk in PBS and the first antibody (anti-importin β, gift from D. Görlich) was added at a dilution of 1:4,000 in 5% milk/PBS for 2 h at room temperature. Washing, incubation with the second antibody, and detection were performed as described above.

Results

Generation of Core Particles

The *in vitro* generated cores (rHBc) used in this study were obtained by expressing the core protein in *E. coli* in the absence of other viral proteins. In the bacterial cytosol, the core proteins assemble into particles (43) that have the same size and symmetry as authentic cores (33). Instead of viral DNA, they contain unspecific *E. coli* RNA (3, 13, 39). When analyzed by isopycnic centrifugation in CsCl, the cores produced banded at 1.355 g/ml in CsCl (Fig. 1 A), the same density as authentic cores (1.355–1.36 g/ml; ref. 22). In contrast to cores expressed in eukaryotic cells, *E. coli*-derived cores are not phosphorylated.

To generate P-rHBc gradient purified cores were permeabilized using low salt, incubated in the presence of PKC and ATP, reconstituted in physiological salt solution, and repurified by gradient centrifugation (31). Using [³²P]ATP for labeling, the extent of phosphorylation was ~12–24 phosphate groups per particle. In native agarose gel electrophoresis, both P-rHBc and rHBc comigrated as a homogeneous band (Fig. 1 B). Alkaline phosphatase did not remove the phosphorus-32 label of intact core particles (Fig. 1 C, lanes 2 and 3) but after disruption of the particle structure (Fig. 1 D, lanes 2 and 3). Thus, it was apparent that the phosphorylation sites were inaccessible to enzymatic hydrolysis as in authentic cores (15). The phosphorylation sites are located close to the COOH terminus of the core protein overlapping with an arginine-rich region

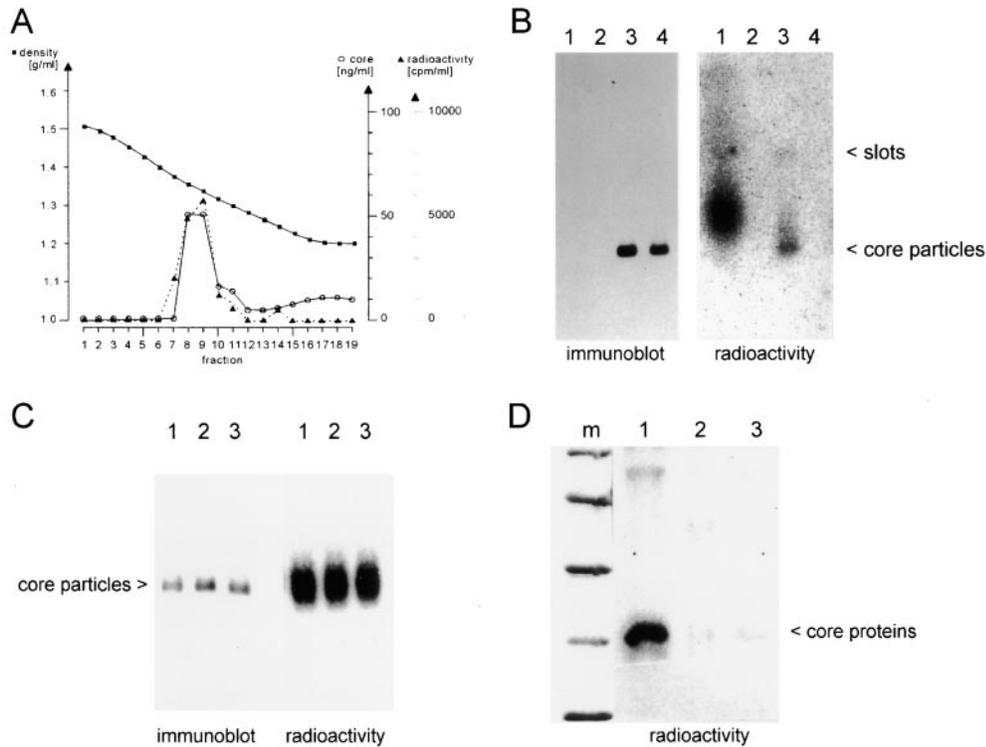


Figure 1. Characterization of in vitro phosphorylated core particles. (A) Centrifugation of PKC-phosphorylated core particles through a continuous CsCl/PBS-gradient with a density from 1.51 to 1.20 g/ml. The different fractions were analyzed for their core content by ELISA and for their radioactivity by liquid scintillation counting. Densities are drawn as black squares; core concentrations as triangles, and radioactivity as open circles. Radioactivity and core particles comigrated at a density of 1.355 g/ml. (B) Native agarose gel of unphosphorylated and PKC-phosphorylated core particles and proteins. Phosphorylation was performed in the presence of [γ - 32 P]ATP. (Left) Immunoblot with anti-core antibody is shown. Detection was performed by enhanced chemiluminescence. (Right; radioactivity) Bioimager scan of the

same membrane is shown: (lane 1) TFA-denatured phosphorylated core protein; (lane 2) TFA-denatured core protein; (lane 3) phosphorylated core particles; and (lane 4) unphosphorylated core particles. The immunoblot shows the reactivity of the anti-core antibody exclusively with the core particles (lanes 3 and 4) but not with the denatured core subunits (lanes 1 and 2). Phosphorylated (lane 3) and unphosphorylated core particles (lane 4) reacted equally well. (C) Phosphatase resistance of in vitro phosphorylated core particles. Phosphorus-32-labeled core particles were treated with phosphatase and separated on a 1% native agarose gel. Particles were detected by immunoblotting (left) or by autoradiography (right): (lane 1) untreated core particles; (lane 2) core particles treated with bacterial alkaline phosphatase; or (lane 3) calf intestinal phosphatase. The phosphatases had no effect on phosphorylation of core particles. (D) Dephosphorylation of TFA-denatured PKC-phosphorylated core proteins. Phosphorus-32-labeled core protein was separated on an 16% SDS-PAGE and autoradiographed. Lanes 1–3 are as described in C; lane m shows the molecular mass marker (66, 46, 30, 21.5, and 14.3 kD). Phosphorylated core protein migrated with an apparent molecular mass of 20.5 kD (lane 1). Treatment of the core subunits with phosphatases removed radioactive phosphate residues (lanes 2 and 3).

(15, 37) containing several putative NLS (59). Immunoprecipitation of core protein after disruption of particle structure using an antibody specific to a phosphorylated peptide derived from amino acids 166–177 (mAb 2212; ref. 37) demonstrated that serine 172 was phosphorylated by PKC (data not shown). Identical results were obtained for core proteins derived from human liver (37) or HBV DNA-transfected hepatoma cell lines (36).

In addition, infectious HBV particles (48) from the stable HBV DNA-transfected hepatoma cell line HepG2.2.15 were purified from the culture medium. In contrast to in vitro generated cores, these cores are from mature viruses and contain the full complement of viral DNA, polymerase, and host cell components. Treatment of the virus with the detergent NP-40 released the cores from the surface proteins (P-hHBc; 24). These cores migrated in CsCl gradients with a density of 1.36 g/ml and did not show any reactivity to antisurface glycoprotein antibodies in ELISA or immunoblot after separation on a native agarose gel (data not shown). Thus, regardless of the source of the cores, P-rHBc and P-hHBc behaved similarly by several biochemical and immunological criteria.

In Vitro Phosphorylated Core Particles Bind to Nuclei

To determine whether the viral cores can associate with nuclei, we used the human hepatoma cell line, HuH-7, grown on coverslips. The plasma membrane was permeabilized using digitonin (1), the cytosol was washed out, and the *E. coli*-generated HBV cores (rHBc or P-rHBc) were added in the presence or absence of RRL, which is a source of cytosolic factors. In some samples, the lysates were complemented with either an ATP generating (+ATP) or depleting system (–ATP). After fixation, cores and NPCs were localized by double indirect immunofluorescence microscopy using core specific antibodies and antibodies to NPC. Immunoblotting of cores electrophoresed in agarose gels (Fig. 1 B) and quantitative ELISA assays (not shown) confirmed that both rHBc and P-rHBc reacted equally well with the anti-core antibody. The antibodies were selective for intact cores, since they bound to heat-denatured particles with ~100-fold lower efficiency.

The P-rHBc gave a strong rimlike fluorescence pattern characteristic of ligand association with the nuclear enve-

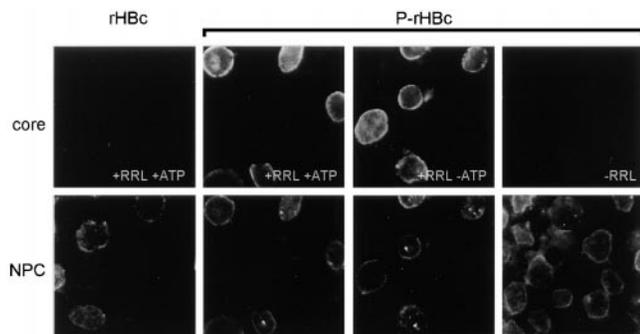


Figure 2. In vitro binding of rHBc and P-rHBc to digitonin-permeabilized HuH-7 cells. (Top) Core-specific immunofluorescence and (bottom) immunofluorescence of NPCs are shown. Binding was performed in the presence (+RRL) or absence (–RRL) of RRL and an ATP-generating (+ATP) or depleting system (–ATP). Only P-rHBc bound to the nuclei. Binding was dependent on RRL but independent of ATP.

lope (Fig. 2, P-rHBc, +RRL, top row). The labeling colocalized extensively with the one for NPCs (Fig. 2, lower row). In contrast, core particles that had not been phosphorylated (not shown) or had been mock phosphorylated in the absence of PKC (rHBc) did not bind to the permeabilized cells (Fig. 2, rHBc, top row), to the nucleus, nor to other cellular structures. Thus, the lack of association of the rHBc with the nucleus cannot be explained by competition with nonnuclear structures. Additionally, a differential entry of the different types of cores into the permeabilized cells seemed unlikely since they looked identical in electron microscopy (31) and behaved identically in biochemical analysis (Fig. 1). The binding of the P-rHBc to the nucleus indicated that phosphorylated core proteins were essential for nuclear association.

P-rHBc bound to nuclei only if a cell lysate was present (Fig. 2, P-rHBc, +RRL). Inclusion of ATP-generating or -depleting systems had no detectable effect (Fig. 2, +RRL +ATP vs. +RRL –ATP). When the reticulocyte lysate was replaced by buffer containing BSA, no binding was observed (Fig. 2, –RRL). Taken together, these findings showed that HBV cores interacted with the nucleus of permeabilized cells, and that binding depended on core phosphorylation and cytosolic factors but not on metabolic energy. Identical results were obtained with the human hepatoma cell line HepG2 and with the mouse fibroblast cell line LTK (data not shown).

Confocal immunofluorescence microscopy showed that the bound cores were localized at the nuclear envelope

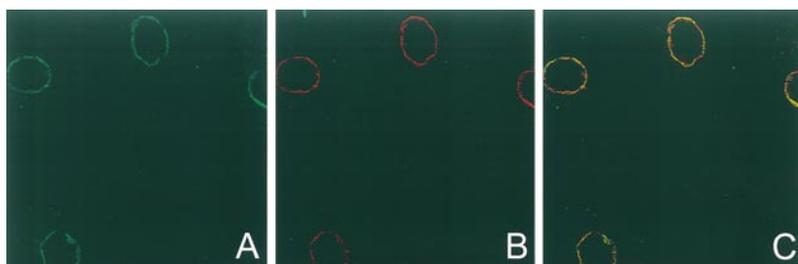


Figure 3. Confocal immunofluorescence microscopy of phosphorylated core particles bound to the NPCs in the presence of reticulocyte lysate and ATP. (A) The fluorescence of the core particles is shown in green. (B) The fluorescence of the NPC is shown in red. (C) The merge of the fluorescence is shown in yellow. The overlap of the two stains show an extensive colocalization of core particles and nuclear pores.

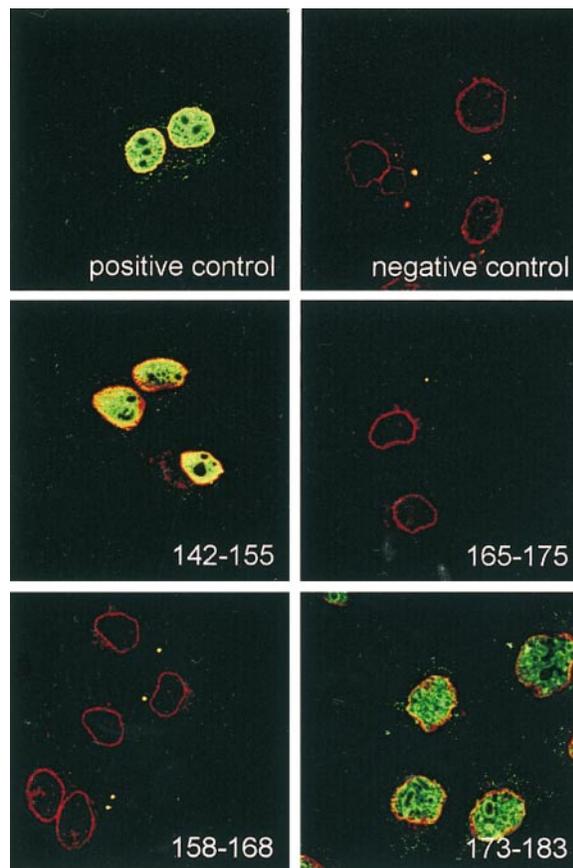


Figure 4. Confocal immunofluorescence microscopy of FITC-labeled BSA conjugated with SV-40TAg NLS after nuclear import into the nuclei of digitonin-permeabilized HuH-7 cells. (Top) The following was performed for the controls: transport in the presence of RRL and an ATP-generating system (left); transport as described above, but after pretreatment of the permeabilized cells with WGA (50 μ g/ml; right). (Middle and bottom) Transport in the presence of RRL and an ATP-generating system but with addition of the different core peptides 142–155, 158–168, 165–175, and 173–183 (all 2 mg/ml). Two of the COOH terminus-derived peptides (158–168 and 165–175) blocked the import of the karyophilic FITC-labeled conjugate, whereas the two other peptides (142–155 and 173–183) did not.

and not imported deeper into the nucleoplasm (Fig. 3), at least not in a form detected by the antibodies used. The nuclei were import-competent as shown by the efficient uptake of FITC-labeled SV-40TAg NLS-linked BSA (Fig. 4, positive control). Preincubation of the cells with WGA, a lectin that binds to *N*-acetylglucosamine containing pro-

Table I. Quantification of the Binding of Phosphorylated and Unphosphorylated Core Particles to Permeabilized HuH-7 Cells by ELISA

Sample	Input	Bound to un-permeabilized cells	Bound to permeabilized cells
	ng cores/assay	ng cores/assay	ng cores/assay
Unphosphorylated cores	92	3.6	4.0
Phosphorylated cores	96	3.2	16.0

All samples were tested in duplicates and were compared with a standard dilution series of *E. coli*-derived core particles. The depicted numbers show the mean value of the tests. The cut off was set as the mean value of four negative samples plus three SDs and was determined as 1.6 ng core particles per sample. The detection limit was <1.6 ng.

teins of the NPC (11), prevented detectable nuclear import of the substrate (Fig. 4, negative control).

Phosphorylation-dependent binding of cores to nuclei was quantitated by an ELISA (Table I). As already mentioned, the ELISA detected both types of cores equally well (Fig. 1 A). 17% of the P-rHBc were bound to permeabilized cells. This corresponds to ~3,400 core particles per nucleus. Of the rHBc, only 4% associated with the

permeabilized cells. If the cores were added to cells without prior digitonin permeabilization, a background association of <4% was observed. Analysis of the supernatants after binding verified the presence of intact core particles (data not shown). Thus, unbound core particles were neither disassembled nor degraded during the assay.

Cores Bind to NPCs

To characterize the site of association, binding assays were performed in the presence of agents known to block binding of karyophilic proteins to NPCs. Preincubation with WGA prevented detectable binding of cores and also reduced the signal of the anti-NPC labeling (Fig. 5, WGA). Binding was also blocked by pretreating the permeabilized cells with an excess of anti-NPC antibodies (Fig. 5, anti-NPC). Antibodies against irrelevant proteins, e.g., anti-NS2 (Fig. 2, anti-NS2) or against the cytoplasmic tail of calnexin (20), an integral membrane protein of the ER (data not shown), had no effect. These observations strongly suggested that cores bound to components of the NPC. Note, in Fig. 2, anti-NS2, no labeling for NPC was performed since the competing NS2-antibody was generated in the same species.

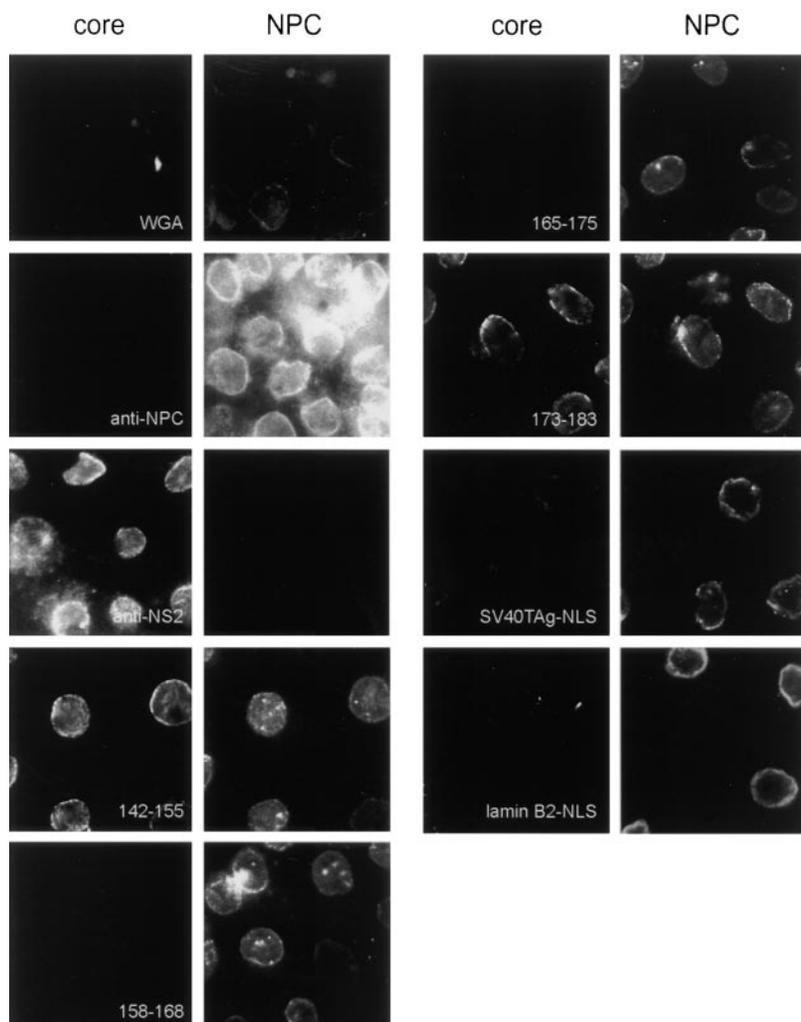


Figure 5. In vitro binding of P-rHBc to digitonin-permeabilized HuH-7 cells in the presence of RRL. (Core) Core-specific immunofluorescence and (NPC) immunofluorescence of NPC (not done in anti-NS2) are shown. Transport was performed after pretreatment of the permeabilized cells with WGA (50 μ g/ml), anti-NPC and anti-NS2 antibodies (influenza virus; 200 μ g/ml each), or in the presence of various peptides (2 mg/ml) representing potential NLS of the core (142–155, 158–168, 165–175, 173–183) or classical basic NLS (SV-40TAg-NLS, lamin B2-NLS).

Putative NLS on the Phosphorylated Core Particles

To map NLS on the cores, various synthetic peptides were added to the RRL before incubation with cores and cells. Four of the peptides corresponded to positively charged sequences in the COOH-terminal domain of the core protein. They were chosen because they resemble classical nuclear localization sequences found in karyophilic proteins and because they are close to the core phosphorylation sites. They had the following positions within the core protein and amino acid sequences: 142–155 (TLPETTVVR-RRDRG), 158–168 (PRRRTPSPRRR), 165–175 (PRRR-RSQSPRR), and 173–183 (PRRRRSQSRES). As controls, two peptides corresponding to the known NLS sequences of SV-40 T antigen (STPPKKKRKV) and lamin B2 were used (27).

As shown by immunofluorescence microscopy, four of the peptides blocked binding of P-rHBc to the NPCs. These were peptides 158–168 and 165–175, and both control peptides (Fig. 5, 158–168, 165–175, SV-40TAg-NLS, lamin B2-NLS). The other two peptides 142–155 and 173–183 did not block binding (Fig. 5, 142–155 and 173–183), although peptide 173–183 differed only by two amino acids from peptide 165–175. The similarity between these two peptides with regard to their basic amino acids strongly argues against an unspecific blocking caused by their positive charges. Peptides 158–168 and 165–175 also inhibited nuclear import of SV-40TAg-NLS linked FITC-BSA (Fig. 4). These results suggested that the binding of HBV cores to the NPCs was mediated by factors involved in the nuclear import of proteins containing classical NLS. Furthermore, the data implied that the COOH-terminal domain of the core protein contained sequences that served as a nuclear pore binding signal (NBS).

Nuclear Binding Is Mediated by Importins

To determine whether binding of cores was mediated by importins, the experiment was performed in the presence of a buffer complemented with recombinant importins α and β . No other cytosolic components were present. The added importins conferred full binding activity to the P-rHBc (Fig. 6, importin α + β), indicating that core-NPC association was mediated by importins. To confirm that the binding of the cores was mediated by the classical nuclear import pathway that involves both importin α and β , the assay was performed using either importin α or β separately. Neither component alone (Fig. 6, importin α , importin β) could promote binding of P-rHBc to the NPCs. The requirement of both importins suggested that an interaction of importin α to the NBS signal on the core particle surface was required for binding of importin β .

Core Particles from Infectious HBV Coimmunoprecipitate Importin β out of Reticulocyte Lysate

To confirm the binding of importin β , authentic core particles were purified from the supernatant of HepG2.2.15 cells. As P-rHBc and cores from human liver (37), these cores are phosphorylated at serine residues between amino acids 166 and 177, which is a prerequisite for secretion of enveloped virus (58). For our immunofluorescence experiments $\sim 10^{10}$ particles were used, an amount which cannot be purified from HBV of culture medium ($\sim 10^6$ /ml). Therefore, the binding of P-rHBc to importins was studied by coimmunoprecipitation performed with smaller amounts of cores. P-rHBc immobilized to biomagnetic beads by the particle-specific anti-core antibody reaction

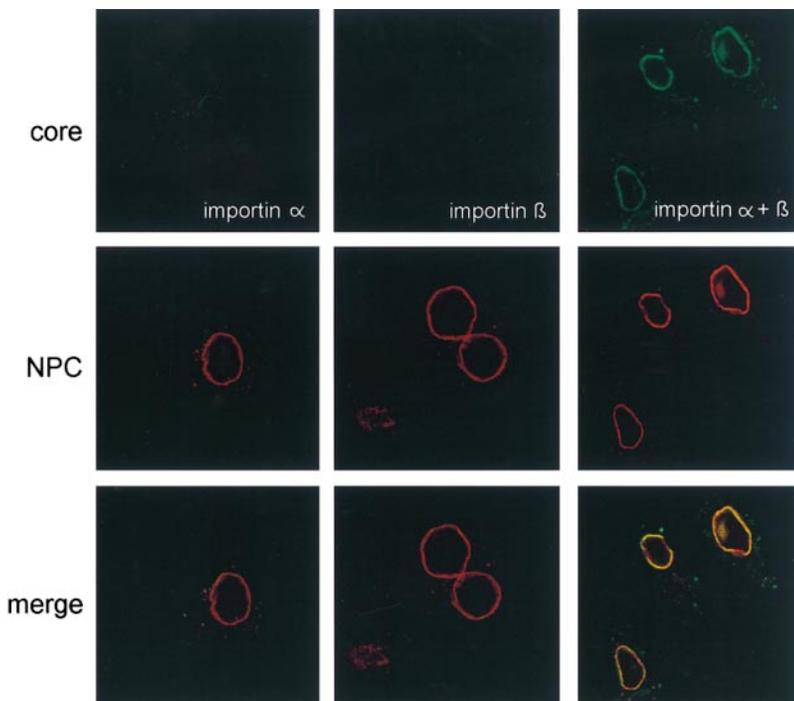


Figure 6. Confocal immunofluorescence microscopy of in vitro phosphorylated core particles bound to HuH-7 nuclei in the presence or absence of importin α and β . The following amounts were used: (importin α) 5 ng/ μ l core and 30 ng/ μ l importin α ; (importin β) 5 ng/ μ l core and 30 ng/ μ l importin β ; and (importin α + β) 5 ng/ μ l core and 30 ng/ μ l importin α and 30 ng/ μ l importin β . All assays were performed in the presence of buffer and BSA. (Top) Core-labeling is shown in green. (Middle) NPC-labeling is shown in red. The bottom row shows a merge of core- and NPC-labeling. Neither importin α nor β alone reconstituted binding of P-rHBc to the NPCs. Addition of both importin α and β mediated the binding of P-rHBc to the NPCs.

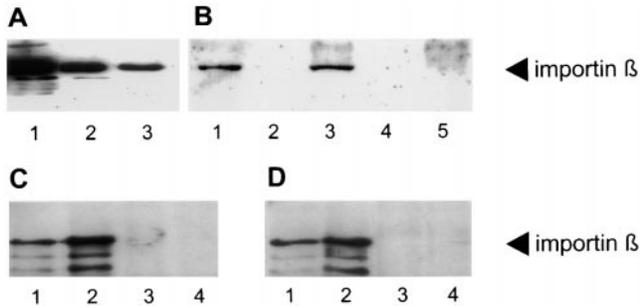


Figure 7. Coimmunoprecipitation of importin β by solid-phase bound core particles. After coimmunoprecipitation bound proteins were denatured and separated on 12% SDS-PAGE. Importin β was detected by immunoblotting. (A) Dilution series of importin β is shown: (lane 1) 50 ng, (lane 2) 10 ng, and (lane 3) 2 ng. (B) Immunoblot of the coimmunoprecipitated importin β from RRL is shown: (lane 1) core particles from HBV; (lane 2) bio-magnetic beads with core and anti-core antibodies; (lane 3) in vitro P-rHBc; (lane 4) bio-magnetic beads with anti-core antibodies but without core; and (lane 5) rHBc. (C and D) Requirement of importin α for the coimmunoprecipitation of importin β with HBV-derived cores (C) or with in vitro P-rHBc (D). Lane 1 shows the addition of recombinant importin β (300 ng/sample) and recombinant importin α (450 ng/sample) into the coimmunoprecipitation reaction and lane 2 shows the addition of RRL. Lane 3 shows the addition of recombinant importin β (300 ng/sample) alone and lane 4 shows the addition of RRL and 2 mM of competitor NLS from lamin B2 (RSSRGKRRRIE). HBV-derived core particles (B, lane 1) and in vitro P-rHBc (B, lane 3) precipitated importin β from RRL, whereas unphosphorylated core particles (B, lane 5) did not. Bio-magnetic beads without anti-core antibodies (B, lane 2) or without core (B, lane 4) did not show any precipitation. Neither HBV-derived cores (C, lane 3) nor in vitro-P-rHBc particles (D, lane 3) precipitated importin β directly, but after addition of importin α (C and D, first lanes). This finding was confirmed by the inhibition of importin β coimmunoprecipitation by these cores from reticulocyte lysate when the importin α binding lamin B2 NLS peptide was added to the lysate (C and D, fourth lanes).

that precipitated importin β from reticulocyte lysate (Fig. 7 B, lane 1). Comparison with a standard dilution series of importin β (Fig. 7 A) showed that about four importin molecules were associated with one core particle. In vitro phosphorylated P-rHBc also coimmunoprecipitated importin β (Fig. 7 B, lane 3) but not the unphosphorylated rHBc (Fig. 7 B, lane 5).

To confirm that P-rHBc and HBV-derived cores do not interact with importin β directly without importin α , as shown for P-rHBc (Fig. 6), a lamin B2 NLS that binds importin α was added to RRL. The loss of importin β precipitation by P-rHBc and HBV-derived cores (Fig. 7, C and D, fourth lanes) showed the involvement of importin α in the importin β binding to these cores. To further confirm this finding, RRL was replaced by importin β containing buffer. The results documented in Fig. 7, C and D (third lanes), showed that regardless of the origin or phosphorylation status of the core particles no coimmunoprecipitation of importin β with core occurred. In the control reaction, where importin α and β were added, importin β was

precipitated by the P-rHBc and HBV-derived cores (Fig. 7, C and D, first lanes).

Nuclear Binding of Core Particles Blocks Nuclear Import

The above data showed an importin-mediated binding of the core particles to the NPC. To test whether the binding of cores to the NPC occurred so close to the nuclear pore that nuclear import of other substrates was inhibited, nuclei of digitonin-permeabilized cells were pretreated with an excess of P-rHBc. Next, the cells were used in a transport assay with FITC-BSA linked to the M9-domain transport signal of hnRNPs. This substrate was chosen because its import does not require importin α and β involved in P-rHBc binding. A control without pretreatment of the cells with P-rHBc showed nuclear import of this substrate (Fig. 8, control). In contrast, preincubation with P-rHBc abolished the nuclear import of the M9-FITC-BSA (Fig. 8, preincubation with P-rHBc). The bound cores thus prevented uptake of an independently targeted karyophilic substrate.

Discussion

The target and transport of viral capsids to the nucleus constitutes a key step in the replication cycle of most DNA viruses and some RNA viruses. With the exception of influenza and retroviruses, little information exists about these processes (57). We studied the targeting of HBV cores by applying concepts and in vitro assays originally developed to analyze the cell biology of nuclear import of karyophilic cell proteins. These included the use of digitonin permeabilized cells and binding and uptake experiments with isolated ligands (16, 40).

We found that only phosphorylated HBV cores were specifically targeted to the nucleus by mechanisms and host factors involved in the NLS-mediated uptake of cellular proteins. Attachment occurred to NPCs and blocked the import of other karyophilic substrates, thereby giving evidence for association with the nuclear pore. The binding required the presence of importins α and β . The involvement of importins was confirmed by the coimmunoprecipitation of importin β from reticulocyte lysate using HBV-derived core particles. Thus, the contents of the authentic core particles, e.g., polymerase, hsp90, and viral DNA, had apparently no influence on nuclear binding. However, phosphorylation of the core subunits was necessary for exposure of NBS. The inhibition by specific peptides strongly implied that nuclear binding of HBV cores involved sequences present in the COOH-terminal portion of the core protein.

During core assembly the pregenomic RNA, the viral polymerase (2, 23), hsp90 (25), and PKC (32) are first encapsidated into a particle containing multiple core protein subunits. The COOH-terminal domain of the core protein is highly positively charged. In unphosphorylated cores, cryoelectronmicroscopy has shown that it is located inside the cores close to small holes in the capsid wall (60). It binds to nucleic acid (21, 41) and also provides the phosphorylation site(s) for the protein kinase trapped in the central cavity (21, 31, 32, 36, 37). Since the phosphoryla-

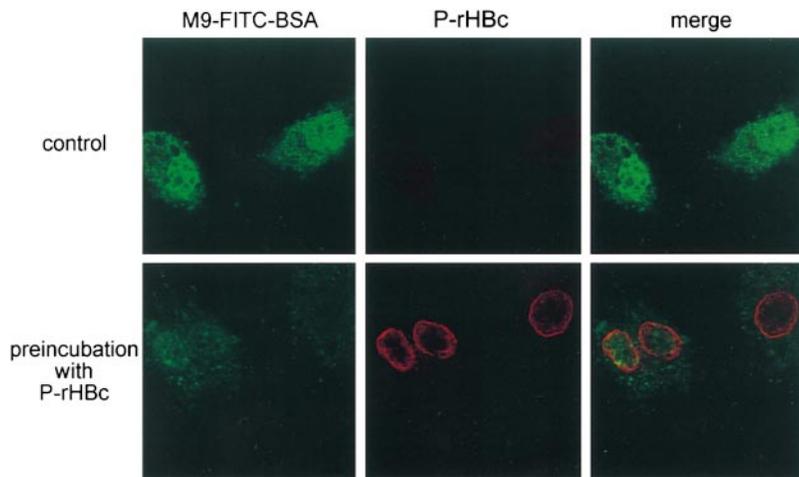


Figure 8. Confocal immunofluorescence of the nuclear transport of M9-domain linked FITC-BSA (green) into permeabilized HuH-7 cells with and without previous P-rHBc binding (core stain in red). The binding reaction was performed in the presence of RRL and an ATP-generating system without addition of P-rHBc (top, control) or in the presence of P-rHBc (bottom, 100 ng/ μ l). After washing the cells, the nuclear transport of the FITC-BSA-M9 conjugate was performed. The M9 conjugate of FITC-labeled BSA was efficiently imported in the mock-treated cells (top). Binding of P-rHBc to the NPC blocks the import of the substrate (bottom).

tion sites on core proteins compete with the encapsidated RNA (31), phosphorylation of the core protein is most likely linked with DNA synthesis.

Peptide inhibition studies suggested that the region exposed as an NBS may include the sequence between amino acids 158 and 175. As in authentic cores (15, 37), the phosphate groups were not removed by alkaline phosphatase unless the particles were destroyed. Thus, the phosphates most likely remained hidden. This suggests that only the nonphosphorylated part of the COOH terminus was exposed on the particle surface.

Many cases have been described in which phosphorylation either up- or downregulates nuclear transport of proteins. This is known for lamins (35), SV-40 T antigen (46), PKC (4), v-Jun (52), and NF-AT (49). The export of influenza viral RNPs also requires phosphorylation (56). Moreover, it has been suggested that phosphorylation of a small number of M proteins of HIV-1 by kinases trapped in the virion triggers M protein release from the viral membrane and exposure of NLSs close to the phosphorylation sites (12). Core proteins contain an NLS in their COOH terminus (10) overlapping with a nucleic acid binding domain (21) and serine phosphorylation sites (36). Unassembled core proteins, where this sequence is exposed, are imported into the nucleus of HBV-infected cells (36). Inside the nucleus, these imported core proteins spontaneously assemble into core particles (47) devoid of nucleic acids (19).

Our results suggest that phosphorylation of core subunits induces a conformational change that exposes the COOH-terminal NLSs in such a way that the phosphoserine residues remain inaccessible to added phosphatases. The positively charged COOH-terminal domain may dissociate from the nucleic acids inside the core. They may protrude through the neighboring holes in the capsid wall and become partially exposed on the surface to serve as a nuclear targeting signal. This structural change may involve only a fraction of proteins in a core. In the P-rHBc used in this study, on average 12–24 of the 240 core proteins per core were phosphorylated. This was evidently sufficient to target the cores to the nucleus.

Both importin α and β were necessary and sufficient for core binding to NPCs. Thus, HBV cores followed the classical nuclear targeting pathway and not alternative path-

ways used by protein A1 of U snRNPs (38), M9-like domains of hnRNPs (44), influenza virus nucleoprotein (55), and glycoconjugates (8). Importin α binds to positively charged, T antigen-like NLSs, whereas importin β is thought to bind to this complex and mediate attachment of the complex to the fibrils that extend from the NPCs into the cytosol. This binding reaction is energy independent (for review see 6, 17, 42). With a diameter of >25 nm, the core exceeds the size limit for NLS-coated colloidal gold particles that can enter through nuclear pores (9). Thus, import of DNA should involve disassembly or deformation of the core particle.

We postulate that phosphorylation of core protein is an important control element in the viral life cycle. During encapsidation, the interaction with the COOH-terminal arginine-rich sequences of the core proteins helps to condense the pregenomic RNA into the small space offered by the central cavity (Fig. 9, step 1). The binding of RNA prevents the encapsidated PKC from phosphorylating this core domain (Fig. 9, step 2). During reverse transcription, the RNA is degraded. This allows the PKC to phosphorylate the COOH-terminal domain of some core protein subunits (Fig. 9, step 3). Phosphorylation induces a conformational alteration in the core proteins. As a consequence, the COOH terminus is exposed on the particle surface (Fig. 9, step 4) that serves as a nuclear pore targeting signal.

By association with importins α and β (Fig. 9, step 5), the cores are targeted to the nuclear membrane and bind to the nuclear pores (Fig. 9, step 6). There, they release their DNA/polymerase complex by unknown mechanisms (Fig. 9, step 7), which is transported into the nucleoplasm (Fig. 9, step 8). Alternatively, the viral cores can undergo budding at the intermediate compartment between the ER and the Golgi apparatus leading to virus formation (Fig. 9, step 9). When the viral cores enter new host cells (Fig. 9, step 10), they can again make use of the importins and the classical pathway of nuclear import. One of the potential advantages of this strategy is that as long as the viral DNA is packaged it can be moved through the cytosol to reach its defined targets. Therefore, the release of the bulky DNA molecule occurs only upon reaching the nuclear envelope. The molecular mechanisms of this pathway can now be elucidated experimentally.

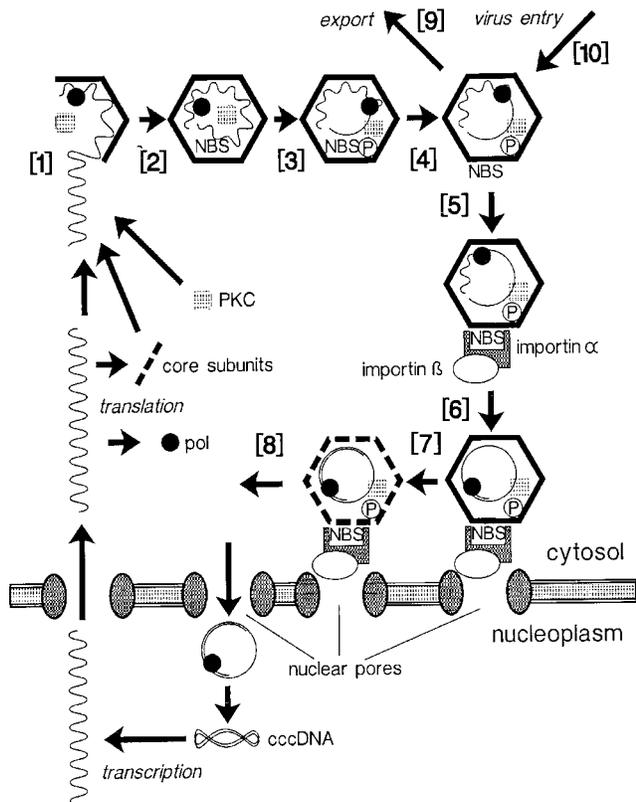


Figure 9. Schematic diagram of the intracellular life cycle of the HBV core particle and the HBV genome. (1 and 2) After assembly of RNA (waved line), pol, PKC, and core subunits, (3) pol synthesizes the minus DNA strand (circle), and phosphorylation of the core subunits by PKC. (4) Phosphorylation induces a structural change of the core so that the hidden NBS becomes exposed on particle surfaces. (5) The NBS is bound by importins (importin α and importin β) that mediate (6) binding of the cores to the NPC, where (7) they disintegrate. (8) The released pol/DNA complex is imported into the nucleus where the DNA is converted to cccDNA. From the cccDNA the pregenomic RNA is transcribed which encodes for pol and core subunits.

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