Lipid-Mediated Introduction of Hepatitis B Virus Capsids into Nonsusceptible Cells Allows Highly Efficient Replication and Facilitates the Study of Early Infection Events

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The hepatitis B virus (HBV) is an enveloped DNA virus which is highly infectious in vivo. In vitro, only primary hepatocytes of humans and Tupaia belangeri or the novel HepaRG cell line are susceptible to HBV, but infection is inefficient and study of early infection events in single cells is unsatisfactory. Since hepatoma cells replicate the virus efficiently after transfection, this limited infection efficiency must be related to the initial entry phase. Here, we describe the lipid-based delivery of HBV capsids into nonsusceptible cells, circumventing the natural entry pathway. Successful infection was monitored by observing the emergence of the nuclear viral covalently closed circular DNA and the production of progeny virus and subviral particles. Lipid-mediated transfer initiated productive infection that was at least 100-fold more effective than infection of permissive cell cultures. High-dose capsid transfer showed that the uptake was not receptor limited and allowed the intracellular transport of capsids and genomes to be examined microscopically. The addition of inhibitors confirmed an entry pathway by fusion of the lipid with the plasma membrane. By indirect immune fluorescence and native fluorescence in situ hybridization, we followed the pathway of capsids and viral genomes in individual cells. We observed an active microtubule-dependent capsid transfer to the nucleus and a subsequent release of the viral genomes exclusively into the karyoplasm. Lipid-mediated transfer of viral capsids thus appears to allow efficient introduction of genetic information into target cells, facilitating studies of early infection events which are otherwise impeded by the small number of viruses entering the cell.

The human hepatitis B virus (HBV) is a major pathogen that causes acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Worldwide, more than 350 million people suffer from chronic HBV infection, and about 1 million people die every year from HBV-related liver failure (32). HBV is the prototype member of the Hepadnaviridae, a family of small, enveloped viruses with a partially double-stranded DNA genome. All hepadviruses are hepatotropic and highly species specific. In cell culture, hepadviruses infect only well-differentiated primary hepatocytes of their specific hosts, but their infection efficiency is limited (1, 9, 10, 42). An appropriate and effective in vitro infection system that allows study of the early steps of the hepadnaviral life cycle, such as viral uptake, capsid transport, uncoating, and delivery of the viral genome to its nuclear replication site, is still not available. While the surface proteins are responsible for entry of the virus into the host cell, the capsid directs the DNA toward the nucleus (29). After nuclear release, the partially double-stranded viral DNA is converted to a covalently closed circular DNA (cccDNA), which serves as the template for multiplication of genomic information by synthesis of an RNA pregenome (for a review, see reference 32). The pregenome encodes the capsid protein and the viral polymerase. While the capsid proteins spontaneously assemble to form a capsid, the polymerase mediates specific encapsidation of the RNA pregenome into the viral capsid. Hepadnaviral genome maturation that involves reverse transcription and second-strand synthesis takes place within the cytosolic capsids. Early in infection, the progeny capsids deliver the mature genome into the nucleus, causing amplification of the nuclear cccDNA and thus establishing persistent infection on the cellular level (20, 29, 41). The secretion of progeny HBV occurs after some days at a later stage of infection, when surface proteins are expressed (39). Surface proteins are synthesized in 100- to 1,000-fold excess of the virus and are secreted as 20-nm spheres or as filaments.

The exposure of appropriate receptors for a virus species on the cell surface and viral entry parameters that define, among other things, the host and tissue specificity of a virus and often limit the susceptibility of a cell line to a virus species. This problem is exemplified by HBV. Although HBV is highly infectious in vivo (43), hepatoma cell lines, such as HuH-7 cells and HepG2 cells, are known to be nonsusceptible to infection but allow viral replication after artificial import of the viral genome (e.g., transfection of cloned HBV DNA) (25). Even primary hepatocyte cultures from the natural host allow infection of only 10 to 20% of the cells (26, 41, 46). Furthermore, they lose their susceptibility within a few days (5, 10, 20, 42) and synthesize fewer viral genomes than were used for infection (5, 10). In addition, primary human hepatocytes show a high variation between individual infection experiments (5) and are difficult to obtain.

To circumvent the problem that primary hepatocytes of the natural host, especially those of human origin, are not readily available, heterologous cells, such as primary hepatocytes from Tupaia belangeri, were successfully tested for their susceptibility to human (7) and woolly monkey hepatitis B virus (21, 46). Furthermore, a recently established cell line, HepaRG, has
retained susceptibility to HBV similar to that of primary hepatocytes. However, both cell culture systems show the same advantages that occur when primary hepatocytes are used, such as a small proportion of infected cells and a poor synthesis of virus HBV (11, 21, 46). More-efficient delivery and higher expression of HBV gene products were observed by using mutant adenoviruses containing a greater-than-genome-length HBV genome (30, 36). However, as with the transfection of plasmid DNA, cloning of the genome is required.

Overcoming these obstacles and facilitating efficient transfer and replication of hepatitis B viruses into nonsusceptible cells would allow us to address several questions, including those concerning early replication events, such as the generation of intranuclear hepadnaviral DNA.

Here, we report on the use of a lipid-mediated protein transfection system that circumvents these problems. By applying the efficient protein transfer method (48) on viral capsids, we were able to initiate productive HBV infection in nonsusceptible cells. Using virus-derived capsids from the HepG2.2.15 cell culture supernatant and from plasma of a chronically HBV-infected patient, we show that lipid-mediated capsid transfer (LMCT) was as efficient as mutant adenoviruses in the establishment of HBV infection and thus at least 100-fold more efficient than the infection of primary hepatocytes or HepaRG cells. The large number of incorporated capsids by LMCT also allowed microscopical analysis of early HBV infection events, such as the intracoplasmic transport of capsids and genomes in different cell lines.

MATERIALS AND METHODS

Generation of capsids. HBV was obtained from the supernatant of a stably HBV-transfected cell line (HepG2.2.15) that secretes infectious virus (33). For the preparation of capsids, we used 25 ml of a 4-day-old supernatant from a 15-cm dish with 3.5 x 10^6 cells. The preparation of capsids yielded 1.4 x 10^9 genome-containing particles.

To exclude the possibility that the source of virions has an effect on the results, we used HBV from the plasma of a chronically HBV-infected patient in some experiments. The HBV genotype of both virion preparations was D.

The plasma of an HBeAg-positive carrier with 2 x 10^9 HBV genomes/ml was precleared by centrifugation for 10 min at 4°C and 4,000 g. To remove antiprotease antibodies, protein A Sepharose (Sigma) that was preincubated in 500 ml phosphate-buffered saline (PBS) (136 mM NaCl, 3 mM KCl, 9 mM NaHPO_4, 2 mM KHPO_4, pH 7.4) was added to 5 ml of precleared plasma for 12 h at 4°C, followed by centrifugation for 10 min at 4°C and 4,000 g. This protein A Sepharose treatment was repeated.

HBV surface proteins were removed by treatment with the nonionic detergent Nonidet P-40 (NP-40) following a protocol modified as described by Kapoor et al. (19). First, the HBV in the supernatant was concentrated by sedimentation through a 25% (wt/vol) sucrose–0.75% (vol/vol) NP-40–TNE (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, pH 8.0) cushion for 22 h at 10°C and 140,000 g. The sediment was resuspended in 1 ml PBS–0.75% NP-40 and incubated for 1 h at 37°C. The released capsids were separated from the surface proteins by sedimentation through a cushion of 25% (wt/vol) sucrose–0.75% (vol/vol) NP-40–TNE for 2 h at 10°C and 337,000 g. The pellet was resuspended in 500 ml PBS, and cosedimentable insoluble components were removed by centrifugation for 5 min at 4°C and 13,000 g. Traces of detergent that may have contaminated the samples were washed out in a filtration device (10 K Nanosep microconcentrator; Pall Gelman) by centrifugation at 4°C and 10,000 g to a final volume of 50 ml, followed by the addition of 450 ml PBS and subsequent concentration. Dilution and concentration were performed three times.

Capsid generation from HBV of the HepG2.2.15 cell culture supernatant was performed using the protocol without protein A Sepharose treatment.

Control of the capsid preparations. To exclude contamination of the capsid preparations with free viral genomes, 2.5 x 10^9 capsids were treated with 20 U/ml S7 nuclease (Roche Diagnostics) for 40 min at 37°C in the presence of 5 mM CaCl_2. The reaction was stopped by the addition of EGTA at 15 mM. The viral DNA of nuclease-treated samples and untreated controls was isolated by a Roche High Pure viral nucleic acid kit as described below. To control the efficiency of nuclease treatment, HBV genomes isolated from the virus were subjected to S7 nuclease- and replicase purified again. Quantification of viral DNA was done by real-time PCR (see below) and showed no difference between nuclease- and untreated HBV capsids, while no viral genomes were detected when isolated HBV genomes were treated with S7 nuclease.

Quantification of the capsids and proof of capsid integrity were done according to Kann et al. (17), using the separation of HBV capsids on an agarose gel under native conditions, followed by immune blotting. Capsid detection was performed using an antibody (Dako) that binds to assembled capsids at least 250-fold more efficiently than to nonassembled capsid proteins (17). Quantification was achieved by comparing the signals of the capsid preparation with a standard dilution series of Escherichia coli-derived capsids. Capsid integrity was demonstrated by the migration of the capsids as a single sharp band, while denatured capsids showed a slower and diffuse migration pattern (17).

Lipid coating and transfer into cells. Lipid-coated capsids (LCC) were generated by adding capsids to the lipids of the BioPorter system (BioCarta). One vial of lyophilized lipids was dissolved in 250 ml methanol according to the instructions of the vendor. For recording the parameters of HBV infection at a low multiplicity of infection, 30 ml of dissolved lipids was air dried and 250 ml of capsid–PBS solution containing 2.5 x 10^9 capsids was used to rehydrate the lipids, followed by 5 min of incubation at room temperature (RT). After gentle mixing for 5 s, the resulting LCC were added to 5 ml of serum-free Dulbecco modified essential medium (DMEM). The mixture was subjected for 4 h at RT to a 10-cm dish with 1 x 10^5 human hepatoma cells (line HuH-7) that were washed three times with DMEM. The cells were then washed again three times with DMEM, incubated (15%) in fetal calf serum (FCS) for 24 h ( inserted into the BioPorter system. To avoid contamination by LCC adhering to the dish, the cells were removed by trypsin and seeded onto a collagen-coated 10-cm dish in 5 ml DMEM–2.5% FCS. At various intervals after incubation at 37°C, 5% CO_2, and 90% humidity, the cells were washed two times with PBS and harvested with a rubber policeman on ice. In those samples with longer incubation periods, the medium was replaced by DMEM after 17 h and every 3 days thereafter. Collected supernatants were cleared by centrifugation for 10 min at 10,000 g.

For immune fluorescence done with a high multiplicity of infection, 10 ml of PBS containing 2.5 x 10^9 capsids was used to rehydrate 2.5 ml of dried lipid. LCC were diluted to 50 ml in serum-free DMEM. Twelve-millimeter coverslips with ~1 x 10^4 HeLa or HuH-7 cells were washed twice with DMEM before they were loaded onto the LCC mixture and incubated for 1 h at RT in a humidified box. The coverslips were then transferred to a 24-well dish, washed three times with DMEM–2.5% FCS, and incubated in DMEM–2.5% FCS at 37°C.

Immune staining and NFISH. The immune staining of HBV capsids and nuclear pore complexes (NPC) and native fluorescence in situ hybridization (NFISH) for released HBV DNA, including microscopy, have been described previously (29). Immune staining was performed with the rabbit antiscaprid antigen (1:200; Dako) that was used for quantification of the capsids and with a monoclonal mouse antitubulin antibody (monoclonal antibody (Dako) served as secondary antibodies at a concentration of 1:400. Immune staining was performed using a monoclonal mouse antitubulin antibody (T9026, 1:100; Sigma), and staining of microtubules was carried out with a monoclonal mouse anti-NPC antibody (monoclonal antibody 414, 1:400; BAbCo) as primary antibody. Actin microfilaments were stained with a monoclonal mouse monoclonal monoclonal antibody (A4700, 1:100; Sigma), and staining of microtubules was performed using a monoclonal mouse antibacterial antibody (T9026, 1:100; Sigma). Cy5-labeled antirabbit antibody (Dianova) and a Cy3-labeled antitubulin antibody (Dianova) served as secondary antibodies at a concentration of 1:800.

Free viral DNA was detected by a fluorescein isothiocyanate-labeled RNA probe that was complementary to the single-stranded region of the viral HBV DNA minus strand. It was added in parallel to the incubation with the primary antibodies. The size of this probe (1 kb) did not allow diffusion into the HBV capsid, so it detected only released viral genomes. The components were visualized by confocal laser scan microscopy. Further details of this protocol, including specificity controls, are given elsewhere (29).

Determination of a capsid-microtubule association that is independent of vesicles was done in digitation-permeabilized cells. Permeabilization and subsequent capsid binding are described elsewhere (17). Staining of microtubules and capsids was done as described above.

Inhibitor treatment. To examine the import pathway of LCC, we studied their transport in the presence of different inhibitors. Cells were preincubated with 10 μM chlorpromazine, 10 μM nocodazole, 10 μM cytochalasin D, or 5 μM latrunculin B (Sigma) in DMEM–2.5% FCS for 30 min. The inhibitors were present during LCC absorption, washing steps, and subsequent incubation periods. Inhibitors and subsequent capsid binding are described elsewhere (17).

The functionality of chlorpromazine was confirmed by infecting HBV cells with parvovirus H-1, as this virus depends upon endocytosis for productive infection (27, 31). After preincubation with chlorpromazine, 1 x 10^7 H-1 viruses per cell were added to HBV cells in DMEM–2.5% FCS and incubated for 2 h at
RT. The virus-containing medium was removed, and the cells were washed two times with DMEM–2.5% FCS before they were incubated for 2 days at 37°C, 5% CO₂, and 90% humidity in DMEM–2.5% FCS. During all incubations, chlorpromazine was present. As a positive control, NBK cells were infected in the absence of the inhibitor. Successful H1 infection was monitored by cell lysis and by testing of the viability of the cells using trypan blue staining. Chlorpromazine prevented cell lysis. The number of nonviable cells was similar to the number in the noninfected control. Toxicity of the inhibitor was excluded by trypan blue staining of chlorpromazine-treated and untreated NBK cells.

Successful treatment with nocodazole (a microtubule inhibitor), cytochalasin D, and latrunculin B (both actin filament inhibitors) was confirmed by indirect immunofluorescence of the respective filaments in both treated and untreated HuH-7 cells. The staining of the filaments is described above. The viability of the inhibitor-treated cells was tested by trypan blue staining.

Unspecific disintegration of capsids caused by the inhibitors that would have prevented successful LCC infection was excluded by the incubation of capsids with the inhibitors for 4 h at RT, followed by native agarose gel electrophoresis and immune blotting as described above.

Quantification of HBV genomes and surface proteins. Quantification of genomes was done by real-time PCR performed in two runs with a LightCycler (Roche Diagnostics), using duplicates (total HBV DNA) or triplicates (HBV cccDNA) in each run. Amplification of the correct product was confirmed by the melting curve of the LightCycler amplification product and by determination of the correct size (735 bp for cccDNA; 189 bp total HBV DNA).

Before we extracted intracellular viral DNA, the harvested cells were resuspended in 1 ml PBS and counted. Cells were then washed twice by sedimentation for 5 min at 1,000 × g and 4°C and re suspension in PBS.

To determine total viral DNA, 1 × 10⁶ cells were lysed by three freezing and thawing cycles at −70°C and 37°C, followed by the addition of 0.1% Triton X-100 and 0.5% sodium dodecyl sulfate (SDS) for 4 h at 65°C. Proteins were removed by phenol-chloroform extraction and the DNA was concentrated by ethanol precipitation in the presence of 1.5 M NaCl (Roche Diagnostics). The pellet was resuspended in 50 μl H₂O. Ten microliters H₂O containing the DNA of 2 × 10⁹ cells was subjected to PCR as described by Jursch et al. (16).

For analysis of cell culture medium, supernatants, or plasma, 200 μl was subjected to DNA extraction using the Roche High Pure viral nucleic acid kit. From the 50-μl eluate, 10 μl was subjected to PCR as described by Jursch et al. (16). As a standard, a geometric dilution series of extracted DNA from human HBV-containing plasma comprising 2.5 × 10⁹ to 2.5 × 10¹ HBV genomes was used.

Extraction of intracellular cccDNA was done as described by Hirt (14). This method precipitates chromosomal DNA while cccDNA remains in the supernatant. A geometric dilution series of extracted DNA from human liver was used as a standard, a geometric dilution series of extracted DNA from human liver was used. The standard ranged from 6.67 × 10¹ to 6.67 × 10⁹ copies.

The detection of secreted HBV surface proteins (HBsAg) was done by enzyme-linked immunosorbent assay (ELISA; Abbott) using a dilution series of an HBV-positive reference plasma, which was calibrated as described by Gerlich and Thomssen (6).

Results

Lipid-Mediated Entry of HBV Capsids

Comimmune precipitations. To demonstrate an interaction between capsids and tubulin by immune precipitation, confluent HuH-7 cells in a 15-cm dish were lysed. The cells were washed two times with PBS, harvested with a rubber policeman, resuspended in 1 ml PBS, and counted. The majority of tubulin molecules in a cell are arranged in filaments. To increase the amount of soluble tubulin, cells were lysed by sonication (Sonopuls UW70/HD70) for 30 s on ice. Lysis buffer was added to a final concentration of 0.1% NP-40, 2 mM dithiothreitol, 5 mM CaCl₂, 1.5 mM GTP, 1.5 mM ATP, 1.5 μM s7 nuclease, and 5 μg/ml of each antipain, aprotinin, leupeptin, and pepstatin A (Sigma). After an incubation of 15 min at 37°C, the lysate was sonicated again. Lysis was checked microscopically, and insoluble components were removed by centrifugation (1 min, 13,000 × g, RT). The protein concentration in the supernatant was determined by comparing a dilution series of the supernatant with a standard of bovine serum albumin, using Coomassie brilliant blue staining on a polyvinylidene difluoride membrane.

For comimmune precipitation, 3.5 × 10⁹ sheep antirabbit- or antimouse-conjugated biomagnetic beads (Dynal) were washed twice in PBS according to the vendor’s manual, using magnetic absorption of the beads. The beads were incubated in 100 μl PBS containing 22 μg rabbit anticaspid antibody (Dako) or mouse antitubulin antibody (Sigma) overnight at 4°C on a rotator. The beads were washed three times in PBS, incubated in 0.1% bovine serum albumin–PBS for 1 h at RT, and washed twice in PBS. Fifty nanograms of capsids (4–10⁹ capsids) derived from the supernatant of HepG2.2.15 cells was mixed with 25 μg HuH-7 cell lysate and incubated for 1 h at 37°C before the antitubulin antibody- or anticaspid antibody-saturated beads were added overnight at 37°C on a rotator. The precipitate was washed twice with PBS, resuspended in 0.1% NP-40, PBS, and transferred to a new cup. After three further washes with PBS, the precipitate was loaded onto an SDS-polyacrylamide gel electrophoresis (PAGE) gel (NuPAGE 4–12% Bis-Tris gradient gel; Invitrogen) and blotted onto a polyvinyldene difluoride membrane in a wet blot. For immune detection, the membrane was blocked with 5% milk buffer–PBS for 1 h at RT, and the mouse antitubulin antibody (1:250) was added in 5% milk buffer–PBS for 3 h at RT. After the membrane was washed in 0.5% milk buffer-0.1% Tween 20–PBS, peroxidase-labeled antimouse antibody (Dianova) was added (1:5,000 in 5% milk buffer–PBS for 1 h at RT), and after being washed, the bands were visualized by enhanced chemiluminescence (Perkin-Elmer).

Results

LMCT induces HBV synthesis. To measure the efficiency of LMCT-induced HBV infection, we subjected 2.5 × 10⁶ genome-containing LCC, derived from the plasma of a chronic HBV carrier, to 1 × 10⁶ cultured cells (line HuH-7; 2.5 LCC/ cell). Cells and supernatants were harvested at different time points postlipofection (p.l.) and were analyzed for the synthesis of different viral markers, such as viral DNA, cccDNA, and surface proteins (Fig. 1). The observation period of 12 days was limited only by the natural viability of the cells.

LCC were preincubated with the cells for 4 h at RT. At this temperature, active intracellular transport processes do not occur (23). Quantitative PCR of HBV genomes after preincubation revealed that 2.37 × 10² of the genome-containing capsids were absorbed to or incorporated into the cells (0.25 genomes per cell; ~10% of the subjected LCC). Due to the trypan treatment of the cells after LMCT, this finding most likely reflects incorporated genome-containing capsids instead of capsids that are bound to the surface of the cell or tissue culture dish. At this time, neither cccDNA nor HBsAg was detectable.

At 17 h p.l., HBV infection was established as indicated by the occurrence of 1.41 × 10³ cccDNA molecules, reflecting a conversion of ~60% of input HBV DNA into cccDNA. The amount of cccDNA was shown to be 250-fold greater than the detection limit (5.65 × 10² copies). The specificity of cccDNA detection was confirmed using different samples. As a positive control, we analyzed the cccDNA extracted from 1 × 10⁹ and
1.5 × 10^5 HepG2.2.15 cells and observed 10 cccDNA copies per cell in the different extractions. These amounts of cccDNA are in accordance with those described by Sells et al. (33). In contrast, no amplification product was found when extracted DNA from 2.5 × 10^5 plasma-derived viruses or 2.5 × 10^5 extracted capsids from the supernatant of HepG2.2.15 cells were subjected to this PCR that showed a detection limit of 5.6 × 10^6 cccDNA molecules. Thus, the cccDNA PCR amplified cccDNA at least ~450-fold more effectively than the partially double-stranded DNA, as is found in virions.

During the following 3 days, cccDNA was amplified 2.4-fold to approximately 3.39 × 10^7 copies. Amplification of cccDNA molecules continued to 5.18 × 10^7 (day 6), 7.88 × 10^7 (day 9), and 1.33 × 10^8 (day 12) copies. On average, 1.3 cccDNA molecules were found per cell at the end of the observation period.

Amplification of cccDNA was combined with viral genome multiplication. At 17 h p.l., total intracellular HBV DNA molecules increased from 2.37 × 10^5 to 3.43 × 10^5. After 12 days, total intracellular DNA accumulated to 1.93 × 10^6 copies, thus showing an ~8-fold multiplication.

The onset of surface antigen secretion occurred between 17 h and day 3 postlipofection and increased over time, with an average secretion rate of ~10 ng per 3 days. Secretion of nuclease-resistant viral genome equivalents into the supernatant became detectable between days 3 and 6, with a delay compared to the secretion of the surface antigen. Antisurface antibodies precipitated 97% of the viral genomes (data not shown), indicating that the genomes were encapsidated into viruses.

Virus production slowed down over time: between days 3 and 6, 1.4 × 10^7 viruses were secreted, and between days 6 and 9, 7.1 × 10^6 (accumulation from 1.39 × 10^7 to 2.1 × 10^7) viruses were secreted, while between days 9 and 12, only 4 × 10^6 (accumulation from 2.1 × 10^7 to 2.5 × 10^7) viruses were secreted per 10^6 cells. According to the amount of surface protein detected, it could be estimated that 280 to 500 times more subviral surface protein particles were secreted than HBV particles. The ratio between cccDNA and secreted virus significantly decreased from 27 virions per cccDNA molecule (day 6, 1.39 × 10^7 viruses/5.18 × 10^6 cccDNA molecules) to 3 virions per cccDNA molecule (day 12, 4 × 10^6 viruses/1.33 × 10^7 cccDNA molecules).

**Visualization of the HBV capsid transport.** To follow the LCC-mediated infection in individual cells, a large excess of LCC had to be loaded into the cells. We added 2.5 × 10^5 capsids derived from HBV of the HepG2.2.15 cell supernatant per HuH-7 cell and determined their fate by confocal laser scan microscopy after indirect immune staining. For localization within the cell, a control stain of the NPC was performed. As shown in Fig. 2, at low magnification, all cells were loaded with similar amounts of capsids, indicating that all cells were susceptible to LCC uptake. The capsids were distributed in the cytoplasm and accumulated within the nucleus.

Uptake was dependent upon the lipid coat of the capsids. When no lipids were added, some capsids bound to the plasma membrane but did not enter the cell (data not shown).

To study the different steps of HBV entry in more detail, the fate of the capsids and the fate of free genomes were determined in a time course study (Fig. 3). Viral genomes released from the capsids were detected by NFISH with a probe against...
the single-stranded region of the viral genome. The detection specificity of the viral components was confirmed by a negative control to which no capsids were added.

Capsids were detected at filamentous intracytoplasmic structures within 15 min p.l. In addition, the capsids accumulated at the nuclear envelope, as previously observed in digitonin-permeabilized cells (29). NFISH demonstrated released viral genomes only in the nucleus, accumulating in some defined areas. This staining pattern persisted for at least 1 h p.l., showing intranuclear accumulation of HBV DNA. The absence of intranuclear capsids at that time does not mean that there were no intranuclear capsid proteins, since the anticapsid antibody only poorly recognizes capsid protein in the unassembled form. Intranuclear capsids became detectable at 17 h p.l. Although it remains unknown whether these capsids were derived from input capsids or from newly synthesized capsids, this staining pattern is identical to that observed in liver biopsy samples from infected patients (4, 12).

The human cervix carcinoma cell line (HeLa cells) (Fig. 3E) showed the same binding pattern of capsids to intracytoplasmic structures and to the nuclear membrane, and the viral genomes also became released into the karyoplasm. Additionally, intranuclear capsids were detectable, but as with the genomes, to a lesser extent, implying that liver cell-specific factors support their appearance. However, this finding does not necessarily mean that this factor(s) is involved in genome release. HuH-7 cells already multiplied viral genomes (Fig. 1) and capsids (Fig. 3B, C, and D) at that time point. As the core promoter is liver specific (40), HeLa cells may produce fewer progeny capsids and genomes.

**Mode of uptake and transport.** To examine the import pathway of LCC, we analyzed transport in the presence of different inhibitors. We used chlorpromazine, an inhibitor of clathrin-mediated endocytosis (37), and cytochalasin D and latrunculin B (45), which depolymerize actin filaments. Actin is responsible for vesicle release from the plasma membrane. Thus, these inhibitors block particle uptake via phagocytosis, macroinocytosis, or caveosomes (2, 24, 28, 34).

For analysis of an active intracellular transport, we added the microtubule-mediated transport inhibitor nocodazole, which depolymerizes microtubules (15, 44).

First, we excluded an unspecific disintegration of capsids caused by the inhibitors that would have prevented successful
LCC infection. Therefore, we incubated the capsids with the inhibitors for 4 h at RT, followed by analysis of the samples by immune blotting after native agarose gel electrophoresis. In contrast to disintegrated capsids that show a slower migration (17), inhibitor-treated capsids showed an unchanged migration pattern compared to untreated controls (data not shown).

We performed quantitative analysis of the HBV infection markers, first by adding 2.5 LCC derived from HBV of the HepG2.2.15 cell supernatant per HuH-7 cell in the presence of transport inhibitors (Fig. 4). The cells were treated with the respective inhibitor for 30 min prior to lipofection, and the inhibitors were present during all incubation steps. Due to the toxicity of the drugs, the analysis was restricted to 17 h p.l. using these cells. At this time, there was no difference in viability of the cells between the different samples as determined by trypan blue staining (data not shown).

The addition of chlorpromazine had no effect on the entry process of LCC. In both the positive control without inhibitor and chlorpromazine-treated cells, total intranuclear DNA accumulated ~1.7-fold (Fig. 4) and cccDNA was generated to the same extent (~95% of input genomes). The functionality of chlorpromazine was confirmed by infecting NBK cells with parvovirus H-1. Parvoviruses are known to be dependent upon endocytosis for productive infection (27, 31). While the untreated control showed lysis of all cells, chlorpromazine prevented cell lysis.

To investigate whether LMCT used the pathways requiring actin-mediated vesicle release from the plasma membrane, we added one of the actin-depolymerizing drugs, cytochalasin D or latrunculin B. Successful inhibitor treatment was verified by immune fluorescence showing the depolymerization of actin filaments (Fig. 5A).

Intracellular HBV genome amplification was slightly reduced by latrunculin B or cytochalasin D to 1.4-fold in comparison to 1.7-fold in the untreated control (Fig. 4). This reduction was not significant, as indicated by the error bars. Generation of cccDNA was slightly reduced in comparison to the untreated control (~80% of the input DNA in cells treated with either inhibitor in comparison to ~95% in the control).

In contrast, when the microtubule-destabilizing inhibitor nocodazole was added, the total number of intracellular genomes

![FIG. 4. Markers of LMCT-initiated HBV infection in the presence of different inhibitors. The columns show the total mean numbers of intracellular HBV DNA and cccDNA per 10-cm dish at 0 h p.l. and 17 h p.l. The HuH-7 cells were pretreated with the respective inhibitor for 30 min before 2.5 LCC per cell were added. The inhibitors were present during all incubation steps. pos. control, lipofected cells without inhibitor treatment. Detection limits were as follows: total HBV DNA, 1.4 × 10^6; cccDNA, 6.6 × 10^3.

![FIG. 5. Cytoskeletons of untreated and inhibitor-treated HuH-7 cells. Cells were treated either with the actin-depolymerizing drug cytochalasin D or latrunculin B (A) or with the microtubule-destabilizing drug nocodazole (B) for 30 min. Cells were then stained with an antiactin antibody (A) or an antitubulin antibody (B). To allow localization within the cell, a control antibody against the NPC was added. Both primary antibodies were visualized by a Cy3 secondary antibody. All samples showed a confocal section on the equatorial level of the nuclei, as indicated by the rim-like anti-NPC stain of the nuclei. (A) The untreated control showed the actin cortex at the plasma membrane, the actin network, and some dotty stain in the cytoplasm. Cells treated with cytochalasin D or latrunculin B were devoid of an actin network and the actin cortex. (B) The untreated control showed the filamentous structure of microtubules, while the nocodazole-treated cells exhibited a granular and diffuse pattern of tubulin, indicating a disruption of microtubules.](http://jvi.asm.org/.../fig5a.png)
remained constant, showing no multiplication, and cccDNA was not generated. Successful disruption of the microtubules after nocodazole treatment was visualized by immune fluorescence (Fig. 5B).

The same results were obtained when we used capsids derived from viruses of a chronically HBV-infected individual, indicating similar replication competences of HBV from both sources.

To confirm the data obtained from the inhibition experiments in single cells and to exclude the possibility that the microtubule inhibitor prevented the internalization of the capsids into the cytoplasm, we studied the fate of the capsids and genomes by fluorescence techniques as described above. Figure 6 shows that after 15 min, similar amounts of intracytoplasmic capsids were found in all samples, both with and without inhibitors. This observation confirmed that the inhibitors did not interfere with the uptake of LCC into the cells but indicated that microtubules were most likely involved in the intracytoplasmic capsid transport to the nucleus. This assumption was confirmed by the observation of differences in intracytoplasmic capsid distribution. While the capsids in the positive control and the chlorpromazine- or actin inhibitor-treated samples showed binding to filamentous cytosolic structures and to the nuclear envelope, nocodazole prevented nuclear accumulation.

NFISH demonstrated the importance of capsid accumulation at the nuclear envelope and confirmed the quantitative data on replication shown before. While released intranuclear genomes were found in the positive control and in chlorpromazine- or actin inhibitor-treated cells, the microtubule inhibitor completely abolished the appearance of released intranuclear genomes.

**Interaction of HBV capsids with the microtubule network.** If microtubules are responsible for the intracellular transport of HBV capsids toward the nucleus, capsids should colocalize with the microtubule network. The intracellular localization of capsids derived from HBV of the HepG2.2.15 cell supernatant was therefore compared to that of tubulin, the major protein of the microtubule network, at 1 h p.l. by indirect immune staining. Figure 7, which depicts the intracytoplasmic distribution of tubulin (Fig. 7A) and capsids (Fig. 7B), shows a very close correspondence of both stains.

Since all known active pathways for particle uptake into the cells have been excluded, internalization of LCC should have occurred via fusion of the lipid coat with the plasma membrane. This hypothesis is supported by the large load of capsids observed in the cells, excluding a receptor restriction. Consequently, intracytoplasmic transport of the capsids to the nucleus must not be mediated by vesicles but must be based on an association of capsids and microtubules. To confirm this, we added capsids to digitonin-permeabilized cells in which the capsids have access to the cytoplasmic compartment without passing the plasma membrane, thus not requiring lipid coating. Figure 7C and D show that in fact tubulin and capsids colocalized.

Further confirmation that vesicles were not essential for capsid binding to microtubules was achieved by common precipitation using a mixture of capsids and the HuH-7 cell lysate (Fig. 7E). In this experiment, capsids that were bound to biomagnetic beads via anticapsid antibody were able to precipitate tubulin out of the cell lysate. The negative control, in which no capsids were added to the assay, confirmed the specificity of the precipitation.

**DISCUSSION**

Virus replication is possible in many cultured cells. There are, however, several examples in which cells do not provide the machinery for efficient import of some viruses, leading to a partial or total block of infection. Hepadnaviruses serve as an example of this phenomenon, as they are fully capable of replicating in a variety of hepatoma cells but are incapable of infecting the same cells. As determined previously, hepadnaviruses efficiently bind to primary hepatocytes, followed by viral uptake (3, 7, 21). Productive infection, however, is poor, indicating a block in virus release after entry.
The method of LMCT allowed us to circumvent the entry steps of endocytosis and efficiently initiate productive infection. The time course of infection was shown to be in accordance with the data known from infection of primary cells with duck hepatitis B virus (DHBV) (3, 22, 42) and HBV (5, 13, 21, 26). First, the partially double-stranded viral DNA was converted to cccDNA, followed by amplification of intracellular DNA. Surface protein secretion started at a time when intracellular viral DNA had already accumulated, and consequently, virus secretion was delayed. After the onset of surface antigen and virus secretion, the number of intracellular HBV genomes remained at a level similar to that observed upon infection of primary duck hepatocytes by DHBV (39), which shows the same replication strategy as HBV. As in the in vivo situation, more surface proteins than virus particles were expressed.

Total virus production and genome multiplication were strikingly higher than in primary cells (5, 10) or than reported for the susceptible cell line HepaRG (11). While these systems secreted only a few viruses, generally fewer than those to which the cells were subjected, LMCT led to a significant secretion of progeny HBV and multiplied the number of initially incorporated HBV genomes ~110-fold. The efficiency and quantity of the hepadnaviral infection markers by LCC-mediated transfer were very similar to those from experiments using chimeric adenoviruses with an HBV genome (36). Therefore, LMCT may serve as a system for analyzing the replication of HBV directly from patients’ sera without cloning and use of a vector. Amplification and cloning may lead to the loss of minor populations within a pool. In addition, LMCT is easier to handle and acts much faster, allowing, e.g., application for phenotype drug resistance assays.

The transfer by lipids was not significantly inhibited by actin-depolymerizing drugs. Thus, the entry pathway of macrophagyosis, phagocytosis, or caveolae-mediated endocytosis did not significantly participate in LCC uptake. These observations are in accordance with infections of primary duck hepatocytes in which actin inhibitors were shown not to block DHBV infection (3).

The addition of chlorpromazine excluded an entry via clathrin-mediated endocytosis that usually targets viral cargos to the late endosome where acidification occurs. Apparently, LMCT circumvented this passage by direct fusion of the lipid with the plasma membrane, which has been described to be the physiological entry mechanism for the human immunodeficiency virus and herpesviruses (herpes simplex virus and cytomegalovirus) (47). The observation that HBV capsids, after this mode of entry, are still capable of initiating infection is in accordance with the finding that hepatitis B viruses, in contrast to influenza and parvo- and adenoviruses (8, 31, 47), do not require acidification for infection (13, 19).

A receptor-independent uptake by fusion may also explain why LMCT is so efficient in induction of infection. First, it allows virtually every cell to take up the capsids. In addition, it circumvents the release from the endosome that was shown to be the limiting step for paroviruses (38). Receptor-independent uptake makes it possible to load high numbers of capsids per cell irrespective of the cell type. This allows the analysis of early infection events by microscopy.

We showed that the capsids were rapidly transported to the nucleus and that generation of intranuclear viral DNA occurred as early as 15 min p.i. Such a short time span can be explained only by an active transport, since passive diffusion was calculated to take 1 h (35). Accordingly, inhibitor experiments showed that the microtubule transport system is required for detectable capsid accumulation at the nuclear envelope and for generation of intranuclear capsid-released virus DNA. Capsids were found to be localized with the microtubule network that is the dominant intracytoplasmic long-distance cellular transporter (35). Binding assays with digitonin-permeabilized cells and coimmune precipitation assays showed that this binding does not require vesicles.

These studies indicate that the capsids are actively transported toward the nucleus using the cellular microtubule transport system, as was previously shown for herpesviruses (35). Such a capsid-mediated transport seems to reflect the physiological intracellular infection pathway. During infection, DHBV capsids undergo intracytoplasmic transport toward the nucleus, as suggested by Köck et al. (20) and for HBV by Rabe et al. (29), followed by a rapid release of the genome into the karyoplasm (29). The occurrence of released viral DNA exclusively inside the nucleus indicates that disassembly of HBV capsids is not random but is subject to a tight regulation. Apparently, this regulation is a well-conserved phenomenon throughout mammalian cells, as is indicated by the same observations for both hepatoma and cervix carcinoma cell lines.

As shown by our results, LMCT may provide a potent tool in viral research. LMCT is suitable to address questions about early
infection events for a variety of viruses, regardless of whether they replicate in the cytoplasm or in the nucleus of nondividing cells.

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