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In Vitro Model for the Nuclear Transport of the Hepadnavirus Genome

MICHAEL KANN,* ANDREAS BISCHOF, AND WOLFRAM H. GERLICH

Institute of Medical Virology, Justus-Liebig-University, D-35392 Giessen, Federal Republic of Germany

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Hepadnaviruses contain a DNA genome, but they replicate via an RNA intermediate, synthesized by the cellular RNA polymerase II in the nucleus of the infected cell. Thus, nuclear transport of the viral DNA is required in the viral life cycle. Protein-free DNA is only poorly imported into the nucleus, so one or more of the viral proteins must be involved in the transport of the viral genome. In order to identify these viral proteins, we purified woodchuck hepadnavirus (WHV) core particles from infected woodchuck liver, isolated WHV DNA, and extracted the covalent complex of viral polymerase from the particles using urea. Intact core particles, the polymerase-DNA complex, or protein-free WHV DNA from core particles was added to digitonin-permeabilized HuH-7 cells, in which the cytosol was substituted by rabbit reticulocyte lysate (RRL) and an ATP-generating system. The distribution of the viral genome was analyzed by semiquantitative PCR or by hybridization in total nuclei, RRL, nuclear membranes, and nucleoplasm. The polymerase-DNA complex was efficiently transported into the nucleus, as indicated by the resistance of the nucleus-associated DNA to a short-term treatment with DNase I of the intact nuclei. The DNA within core particles stayed mainly in the cytosol and remained protected against DNase I. A minor part of the encapsidated DNA was bound to nuclei. It was protected against DNase I but became accessible after disruption of the nuclei. Deproteinized viral DNA completely remained in the cytosol. These data show that the viral polymerase is probably sufficient for mediating the transport of a hepadnavirus genome into the nucleus and that the viral core particles may release the genome at the nuclear membrane.

The family of the *Hepadnaviridae* is composed of hepatotropic species-specific viruses which infect certain mammalian and avian species. Human hepatitis B virus (HBV) is the prototype of the *Hepadnaviridae*, while the woodchuck hepatitis virus (WHV) serves as an important model for infection (11). Hepadnaviruses contain within their envelope a small icosahedral nucleocapsid (or core) with a 30- or 34-nm diameter (8, 22). The core particles encapsidate a circular DNA genome of approximately 3,200-base length (31). This DNA is partially double stranded with a complete minus DNA strand, which is covalently bound at the 5′ end (12, 26) to the viral polymerase (4). In addition to the genomic DNA and viral polymerase, a protein kinase is present in the lumen of the core particles (2, 13), which has been identified as a protein kinase C (PKC) (19, 20).

Although all hepadnaviruses contain a DNA genome, they replicate via an RNA intermediate (34) that is synthesized by the cellular RNA polymerase II of the infected cell (23). Thus, the viral DNA has to be imported into the nucleus of the infected cell.

In an established infection with duck hepatitis B viruses (DHBV), approximately 20 to 50 copies of viral covalently closed circular DNA (cccDNA) can be found in the nucleus of an infected duck hepatocyte (35). The half-life of the cccDNA was determined to be 2 to 3 days (6, 7), implying an initial accumulation and permanent regeneration of the cccDNA pool. While primary hepatocytes lose their susceptibility to infection within 3 days after being put into culture (35), the main amplification of the cccDNA pool occurs 10 days after infection of primary hepatocytes. This observation implies that the cccDNA amplification is caused not by a reinfection of the cells but by reentry of the viral genomes from the mature core particles (36) which assemble in the cytosol (32).

* Corresponding author. Phone: 49-641-702 7386. Fax: 49-641-702 2870. E-mail: michael.kann@viro.med.uni-giessen.de.

In theory, there are at least three different possibilities for nuclear entry of the viral genome during de novo infection or intracellular amplification. (i) The viral DNA is released from the nucleocapsid or virion and deproteinized outside the nucleus, and then the genome is imported into the nucleus similarly to artificial transfection. (ii) The incoming virus loses the surface proteins during entry, and complete core particles are transported into the nucleus. (iii) A third possibility for the nuclear import of the viral genome would begin with disassembly of the core particles outside the nucleus. The subsequent transport of the genome could be mediated either by the covalently linked polymerase or by nonassembled core protein subunits attached to the polymerase-DNA complex.

Available hepatocyte lines are not susceptible to infection with hepadnaviruses, so there is no readily available system for studying nuclear entry. To study the process of nuclear import, we used the established system of permeabilized cell cultures which allow free access of macromolecules to nuclei and maintain active transport of nucleus-bound substances (1). As a source for viral components we used livers from WHV-infected woodchucks. The covalent polymerase-DNA complex and protein-free WHV DNA were prepared from WHV core particles and also subjected to the in vitro system for nuclear transport. The distribution of viral DNA between nuclei and surrounding medium was measured by PCR or by autoradiography of the viral DNA, which had been ^{32}P-labeled by the endogenous polymerase reaction. The release of the viral DNA from core particles made it accessible for DNase I. Our study suggests that the covalently bound polymerase mediates the nuclear transport of the hepadnavirus genome.

MATERIALS AND METHODS

Purification of the WHV core particles. Approximately 30 g of WHV-infected woodchuck liver, stored at −70°C, was thawed in ice-cold TKM buffer (50 mM Tris-HCl [pH 7.5], 10 mM KCl, 1 mM $MgCl_2$) and cut into small pieces. To remove anti-WHV antibodies, the liver pieces were washed several times in 200 ml of TKM buffer until the supernatant appeared clear. The sedimented liver

pieces were resuspended in 15 ml of TKMD (TKM buffer plus 1 mM dithiothreitol [DTT], 1% Nonidet P-40 [NP-40], 30 μg of aprotinin, 30 μg of pepstatin, 30 μg of leupeptin [Sigma, Deisenhofen, Germany]) and homogenized in a motor-driven homogenizer (Ultra-Turrax T25; IKA-Labortechnik, Staufen i. Br., Germany) (11 30-s cycles). The homogenate was precleared by centrifugation for 20 min at 1°C at 3,000 × g. The core particles in the supernatant were pelleted through an 8-ml 30% (wt/wt) sucrose cushion in phosphate-buffered saline (PBS) (171 mM NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.9 mM KH_2PO_4) for 24 h at 25,000 rpm at 4°C in an SW28 rotor (Beckman). The pellet was resuspended in TNE (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA) by 10 10-s cycles of sonification (Sonoplus HD70 with a UW70 head and a microtip [Bandelin, Berlin, Germany]) and subjected to ultracentrifugation through a sucrose gradient (10 to 50% [wt/wt] sucrose-PBS) for 14 h at 15,000 rpm at 4°C in an SW28 rotor. The gradient was harvested in 23 fractions of 1.6 ml. An endogenous polymerase reaction of a 10-μl aliquot of each fraction was carried out overnight at 37°C in the presence of 50 μCi of [α^{32}P]dCTP (3,000 Ci/mmol) (Amersham) 50 mM NH_4Cl, 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 7 mM $MgCl_2$, 0.1% NP-40, 10 μM dGTP, 10 μM dATP, and 10 μM dTTP in a volume of 30 μl. After the incubation, 10 μl of the reaction mixture was subjected to electrophoresis through a 1% agarose gel. The gel was fixed by two 10-min washes in 10% trichloroacetic acid (TCA) and two 5-min washes in 5% TCA, dried, and analyzed by autoradiography. Fractions 6 to 12 (33 to 24% sucrose), where a band appeared at approximately 3,000 bp, were pooled. To this pool of ^{32}P-labeled WHV core particles, TNE was added to a final volume of 4.4 ml, and the core particles were sedimented in an SW60 rotor for 24 h at 4°C at 45,000 rpm. To remove surface proteins, potentially adhering to the core particles, the pellet was resuspended in 150 μl of 1% NP-40–0.02% β-mercaptoethanol–1× PBS and incubated 60 min at 37°C. The nucleocapsids were sedimented for 60 min at 4°C at 30 lb/in^2 in an Airfuge (Beckman). The pellet was resuspended in 180 μl of ice-cold TNE and stored in aliquots at −20°C. The corresponding aliquots of nonlabeled core particles were treated in an analogous way without endogenous polymerase reaction.

Preparation of the polymerase-DNA complex and of deproteinized WHV DNA. Twenty microliters of the core preparation was incubated for 60 min at 37°C in 50 μl of 4 M urea–1% β-mercaptoethanol, 1× PBS. The released polymerase-DNA complex was sedimented through a 15% (wt/wt) sucrose-PBS cushion for 2 h at 135,000 × *g* in an Airfuge. The pellet was resuspended in 20 μl of 1× PBS and stored in aliquots at −20°C.

For preparation of the deproteinized WHV DNA, 20 μl of the core preparation was incubated in 100 mM Tris-HCl (pH 7.5)–12.5 mM EDTA–150 mM NaCl–1% sodium dodecyl sulfate (SDS)–100 μg of proteinase K (Sigma) per ml in a volume of 40 μl overnight at 56°C. The sample was phenol-chloroform extracted, and the supernatant was ethanol precipitated in the presence of 20 μg of glycogen (Boehringer Mannheim). The pellet was resuspended in 20 μl of 1× PBS and stored at −20°C.

In order to study whether core protein might reach the pellet during centrifugation and, thus, contaminate the polymerase-DNA complex preparation, 4.4 μg of *Escherichia coli*-derived core particles was phosphorylated by PKC in the presence of 10 μCi of [γ-^{32}P]ATP by the method of Kann and Gerlich (20). Half of the reaction mixture was subjected to the treatment described for the polymerase-DNA complex preparation. The rest of the reaction mixture was centrifuged without disruption of core particles. After sedimentation and resuspension of the pellets, protein-bound radioactivity was determined in the supernatants and in the resuspended pellets after TCA precipitation.

Detection of WHV DNA. Detection of WHV DNA by filter hybridization was carried out as described by Zyzik et al. (39) with the unlabeled components. As probe, the digoxigenin-labeled WHV DNA insert of plasmid pWHc2 (kind gift of S. Menne and M. Roggendorf), representing the core gene region, was used. Detection was performed by using a horseradish peroxidase (HRPO)-labeled antidigoxigenin antibody and 3′,3′-diaminobenzidine as substrate as recommended by the vendor (Boehringer Mannheim).

For PCR, samples were lysed in 0.1 M NaOH for 1 h at 37°C and neutralized by 1.5 volume of 0.2 M Tris-HCl, pH 7.5. Twenty microliters of the denatured, neutralized sample was subjected to the PCR, using 0.2 μM primer WHV11 (5′ GAGGAGGGCAGCATTGAT 3′ [positions 1901 to 1919]), WHV12 (5′ ATT CTTGAACTGTATGTT 3′ [positions 2372 to 2351]), 1× *Taq* buffer (Promega, Madison, Wis.), 1.5 mM $MgCl_2$, and 2.5 U of *Taq* polymerase (Promega) in 100 μl. The PCR was carried out in a thermocycler (Hybaid Omnigene) for 35 cycles (95°C for 60 s, 60°C for 60 s, and 72°C for 90 s) after initial denaturation for 3 min at 95°C. PCR was finished by a terminal incubation of 3 min at 72°C. The amplification products of 473 bp were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Immunoprecipitation. Five microliters of the core particles containing ^{32}P-labeled DNA was incubated with 2 μl (46 μg) of antibody to human hepatitis B core antigen (HBcAg) (anti-HBc), which cross-reacts with WHcAg (DAKO, Hamburg, Germany) or polyclonal rabbit antibody to woodchuck surface antigen (kind gift of T. Tolle and M. Roggendorf) for 2 h at 37°C. Immunocomplexes were bound to 30 μl of protein G-agarose (Sigma), which had been preincubated with 0.5 ml of 1% bovine serum albumin for 1 h at 4°C. Thereafter, the beads were sedimented and washed twice with 0.5% Tween 20–PBS, twice with 0.1% Tween 20–PBS, once with PBS, and once with H_2O (1 ml each time) before they were resuspended in proteinase K buffer. After incubation, as described for the deproteinization of the WHV DNA, the redissolved precipitate was separated on an agarose gel and the ^{32}P-labeled DNA was visualized by autoradiography.

Transport assay in permeabilized HuH-7 cells. The permeabilization and subsequent transport assay were performed by the method of Adam et al. (1). A 16-cm plate with 50 to 70% confluent HuH-7 cells was washed with 15 ml of serum-free Mixed Medium (1.972% Instamed Medium 199 Eagle [Seromed, Berlin, Germany], 8.32% Instamed RMPI 1640 [Seromed], 2% $NaHCO_3$, 20 mM L-glutamine [GIBCO BRL, Paisley, United Kingdom], 1× nonessential amino acids [GIBCO BRL], 10 mM sodium pyruvate). The cells were permeabilized for 5 min in 15 ml of serum-free Mixed Medium–40 μg of digitonin (Sigma) per ml at 37°C. After permeabilization, the cells were washed twice with 1× transport buffer (2 mM Mg acetate, 20 mM HEPES [pH 7.3], 110 mM K acetate, 5 mM Na acetate, 1 mM EGTA, 2 mM DTT) before they were harvested with a rubber policeman. The suspended cells were pelleted (for 1 min at 2,000 rpm at 4°C in an Eppendorf centrifuge) and resuspended in 1 ml of 1× transport buffer. A 50-μl aliquot of the suspension was used per assay. The cell aliquot was pelleted and resuspended in 34 μl of a transport mix, so that the assay after addition of the viral component contained final concentrations of 50% rabbit reticulocyte lysate (RRL) (Promega) (final concentration, 35 mg/ml), 50 mM creatine phosphate (Sigma), 10 U of creatine phosphokinase (CPK) (Sigma), and 1× transport buffer. In the assays using an ATP-depleting system, the resuspension mixture of 1× transport buffer and RRL without ATP, phosphocreatine, and CPK was preincubated for 15 min at 30°C in the presence of 100 mM glucose and 4 U hexokinase. Six microliters of the WHV component, containing approximately 2×10^7 WHV DNA copies was added immediately after the resuspension of the cells. After the samples were incubated for 20 min at 30°C the cells, were sedimented for 2 min at 2,000 rpm at 4°C. The supernatant was withdrawn and a 4-μl aliquot was diluted with 146 μl of 1× transport buffer. The pellet was washed gently three times with 40 μl of 1× ice-cold transport buffer before integrity of the nuclei was determined microscopically. Two aliquots of 4 μl were withdrawn and diluted in 146 μl of 1× transport buffer each. One additional 4-μl aliquot was used for disintegration of the nuclei, which was done by the method of Qiao et al. (30). To this end, the cells were diluted in 146 μl of H_2O and subjected to five freezing and thawing cycles. After microscopical control of the effect of disruption, the membranes were sedimented for 15 min at 15,000 rpm at 4°C and the supernatant, i.e., the nucleoplasm, was removed. The membrane pellets were washed twice in 200 μl of 1× transport buffer and finally resuspended in 150 μl of 1× transport buffer. From each sample, 7 μl was withdrawn and treated with 2 U of DNase I for 10 min at 37°C. To 7 μl of the original samples, 2 μl of PBS was added to adjust the volume, and these samples were also incubated for 10 min at 37°C. All samples were analyzed by PCR.

Purity of the nucleoplasma preparation. For controlling the separation between nucleoplasm and membranes, cells were permeabilized and subjected to a transport assay as described above. After the assay, the cells were washed and lysed by freezing and thawing cycles without dilution. The sedimented membranes were resuspended in the same volume of 1× transport buffer as the nucleoplasm. A dilution series of the resuspended membranes and an aliquot of the nucleoplasma were separated on an 10% SDS–polyacrylamide gel. Afterwards, the gel was blotted in a blotting device (TE Transphor electrophoresis series [Hoefer, San Francisco, Calif.]) overnight on a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, Mass.). The membrane was blocked by 5% milk powder–PBS for 1 h at room temperature before the anticalnexin antibody (antibody against the cytoplasmic tail of calnexin [kind gift of Ari Helenius]) was added in a final concentration of 1:200 for 2 h. The membrane was washed four times for 10 min each at room temperature in PBS–0.05% Tween 20–0.1% milk powder and incubated in the same buffer with a 1:2,500 dilution of alkaline phosphatase-labeled antirabbit antibody (Sigma) for 1 h at room temperature. After the membrane was washed as described above, detection of calnexin was performed with an ECL detection system, according to the instructions of the vendor (BM Chemiluminescence Blotting Substrate [Boehringer Mannheim]).

RESULTS

Characterization of WHV core particles. WHV core particles were purified from liver homogenate by several centrifugation steps. In order to ascertain the identity and purity of the viral components used for this study, the encapsidated viral DNA in the purified core particles was labeled with [^{32}P]dCTP by the endogenous polymerase. Separation of the labeled particles on an agarose gel showed a single band in autoradiography (Fig. 1A, lane 3). Phenol-chloroform extraction of the encapsidated DNA after proteinase K digestion showed that the core particles contained full-length genomes without a significant amount of heterogeneous replicative intermediates (Fig. 1A, lane 1). To confirm that the labeled DNA was encapsidated in the nucleocapsids, the core particles were immunoprecipitated with a polyclonal antibody against hepadnavirus

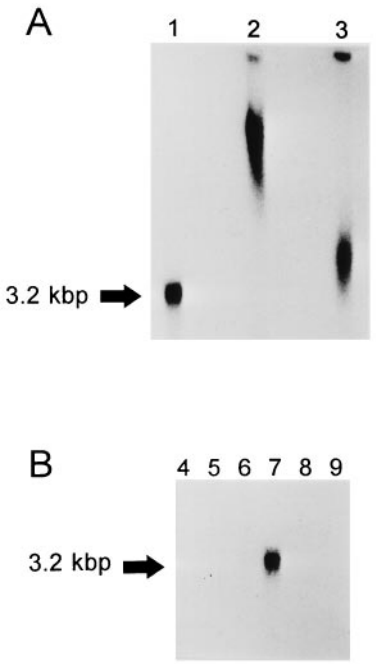

FIG. 1. Characterization of WHV DNA-containing samples. (A) Autoradiography of a 1% agarose gel of deproteinized WHV DNA (lane 1), WHV polymerase-DNA complex (lane 2), and WHV core particles (lane 3). DNAs of the three preparations were labeled with [α-^{32}P]dCTP by the endogenous polymerase reaction. All samples contained 0.1% SDS. (B) Autoradiography of a 1% agarose gel on which the DNA extracted from the immunoprecipitated ^{32}P-labeled preparations was separated. After immunoprecipitation, the samples were proteinase K digested, phenol-chloroform extracted, and ethanol precipitated. Lane 4, protein-free DNA as negative control, immunoprecipitated with anti-WHs; lane 5, protein-free DNA, immunoprecipitated with anticore antibodies; lane 6, WHV core particles, immunoprecipitated with anti-WHs; lane 7, WHV core particles, immunoprecipitated with anticore antibodies; lane 8, WHV polymerase-DNA complex, immunoprecipitated with anti-WHs; lane 9, WHV polymerase-DNA complex, immunoprecipitated with anticore antibodies.

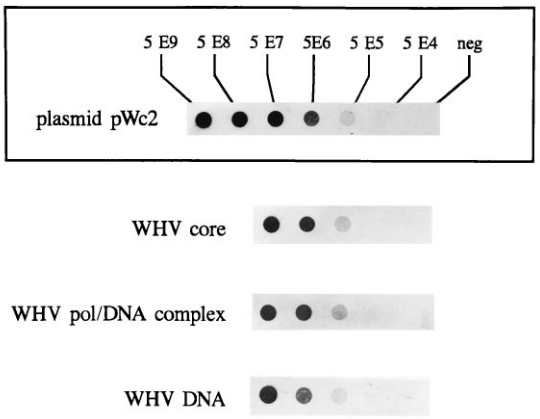

FIG. 2. Determination of the number of viral genomes by dot blot hybridization. Ten microliters of each component and their 10-fold dilutions were alkali lysed prior to blotting and hybridized with a digoxigenin-labeled WHV DNA fragment, covering the core region of a WHV isolate. The signal was visualized with an HRPO-labeled antidigoxigenin antibody. The upper row shows a dilution series of the plasmid standard. The numbering above this row gives the number of genomes per sample.

core antigen (Fig. 1B, lane 7). The absence of viral surface proteins (WHs) was verified by a negative result of the immunoprecipitation using a polyclonal anti-WHs antiserum (Fig. 1B, lane 6).

Isolation of the polymerase-DNA complex. Core particles containing ^{32}P-labeled DNA were treated with urea, in order to dissociate the core protein subunits. Since disulfide bonds between the core subunits might have been generated during core particle preparation, β-mercaptoethanol was added to prevent trapping of the polymerase-DNA complex within the cross-linked core protein subunits. Since the covalently linked polymerase-DNA complex sediments with 14S (12) faster than the core protein monomers or dimers, the reaction mixture was subjected to centrifugation through a sucrose cushion in a small ultracentrifugation rotor, leading to a complete sedimentation of the radioactive DNA after 2 h. To an aliquot of the resuspended pellet, agarose gel loading buffer was added, producing a final concentration of 0.1% SDS in the sample. The complex was separated on a 1% agarose gel. Autoradiography of the gel (Fig. 1A, lane 2) showed a slower migration of the protein-DNA complex than of the protein-free DNA. This confirmed the association of the released radioactive WHV DNA with a protein. To further study this association, the sample was extracted in the presence of SDS with phenol-chloroform and TCA precipitated in order to remove residual amounts of free ^{32}P-labeled nucleotides. TCA-precipitable radioactivity was extracted by phenol, confirming the presence of a covalent protein-DNA complex (12).

Since the migration of the polymerase-DNA complex in the native agarose gel was slower than the migration of intact core particles, the native structure of the nucleocapsids must have been disrupted and the DNA unfolded.

In order to exclude the presence of core particles in the polymerase-DNA preparation, an aliquot of the sample was immunoprecipitated with a polyclonal interspecies reactive antibody against HBcAg. Figure 1B (lane 9) shows that the complex was not precipitable, indicating the absence of core particles in the sample. To determine whether core protein might cosediment during pelleting of the polymerase-DNA complex, *E. coli*-derived core particles, radioactively labeled by protein kinase C were lysed and centrifuged as for preparation of the polymerase-DNA complex from liver-derived core particles. The TCA-precipitable radioactivity of ^{32}P-labeled core protein was found exclusively in the supernatant, whereas the sediment revealed a radioactivity slightly lower than the background (supernatant, 7,597 cpm; pellet, 118 cpm; background, 124 cpm). Thus, sedimentation of core protein subunits in the centrifugation appears unlikely, although a cosedimentation, caused by binding to the polymerase, could not be excluded by the use of the *E. coli*-derived core particles in this experiment. A further control experiment with phosphorylated nondenaturated core particles showed that 80% of the TCA-precipitable radioactivity was in the pellet.

Semiquantitative assay of WHV DNA. In order to determine the number of viral genomes in the various preparations, a dot blot hybridization of a dilution series of the different samples was performed. As a standard, purified plasmid DNA pWHc2, which contains the core gene region of WHV, was used. The number of genomes in the WHV core sample and in the preparations derived from them was adjusted to the final concentration of approximately 5×10^9 genomes per ml (Fig. 2).

Since the DNA distribution during the transport reaction was analyzed by single-round PCR, we examined the amplification efficiencies of the different components, by amplifying a dilution series of each sample. The result (Fig. 3), showed that the DNA of the polymerase-DNA complex and of the deproteinized WHV DNA was amplified slightly better than the

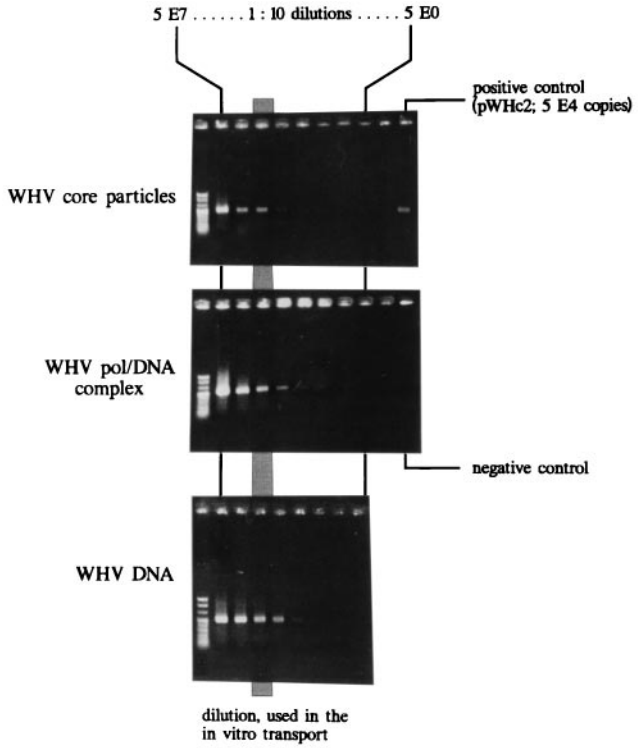

FIG. 3. Semiquantitative assays of WHV DNA in core particles (upper panel), the polymerase (pol)-DNA complex (middle panel), and free DNA (lower panel) using PCR. The amplification products were separated on a 2% agarose gel and stained by ethidium bromide. The left lanes in the panels show the DNA molecular weight marker (Boehringer VIII). The numbers at the top show the number of genomes per sample in a 10-fold dilution series.

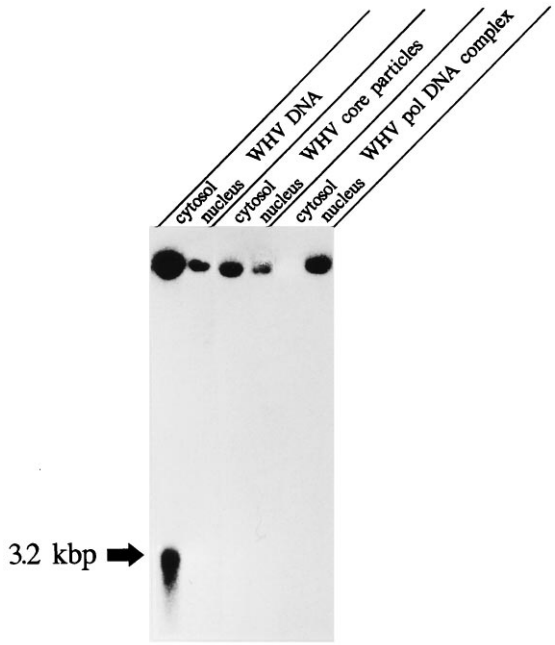

FIG. 4. Distribution of ^{32}P-labeled WHV DNA after transport assays with radioactively labeled preparations. The samples from the transport assay were separated into the nuclear fraction and the cytosolic fraction. The fractions were analyzed in the absence of detergent on a 1% agarose gel. The gel was TCA fixed and subjected to autoradiography. The radioactivity at the top of the figure indicates the amount of WHV DNA bound to structures which did not enter the gel, i.e., intact nuclei or cytosolic structures.

DNA within core particles. The detection limit ranged from 5×10^5 to 5×10^4 copies per sample. In all samples, the fluorescence intensity of the amplification products was proportional to the amount of the component within the range from 5×10^7 to 5×10^4 copies per sample.

In vitro nuclear transport of the components. The distribution of WHV DNA in the in vitro transport system was initially analyzed by using approximately 1,000 ^{32}P-labeled viral genomes per cell. After the transport reaction, the RRL as cytosol substitute was separated from the nuclei and both fractions were subjected to native agarose gel electrophoresis. With the protein-free WHV DNA, less than 10% of the ^{32}P was found in the nuclear fraction. In the cytosolic fraction, approximately 40% of the DNA migrated as free DNA and 60% remained in the pockets of the gel (Fig. 4), indicating formation of protein-DNA complexes. Addition of SDS to the sample resulted in a disruption of the complex and migration as in Fig. 1A (data not shown). In contrast to the distribution of the protein-free DNA, the polymerase-DNA complex was completely associated with the nuclear fraction (Fig. 4). The complex did not migrate and remained in the pockets of the gel (Fig. 4). When entire core particles were used in the assay approximately one-third of the radioactivity was found in the nuclear fraction (Fig. 4) and two-thirds in the cytosol. In both cases, the core particles were bound to large structures, preventing entry of labeled DNA into the gel.

To simulate the in vivo situation where only small amounts of core particles are present in the cytosol, further transport assays were performed, using approximately 10 nonlabeled genomes per cell. Due to this small amount of DNA, samples had to be analyzed by semiquantitative PCR. In an aliquot of the nucleus-containing fraction, the nuclei were disrupted by freezing and thawing and separated into nuclear membranes and the nucleoplasm. From each fraction an aliquot was diluted 1:37.5 before PCR to prevent inhibitory effects of substances in the transport assay. The volume of the aliquots for PCR was adjusted in order to allow detection of approximately 1/10 of the genomes added to the reaction mixture. Thus, a negative result in the PCR means that less than 10% of the genomes were present in the analyzed fraction.

To control the efficiency of the separation into nucleoplasm and into nuclear membranes, a Western blot was performed, using anticalnexin as marker for nuclear and endoplasmic reticulum (ER) membranes. Comparison of calnexin signal strength in a dilution series of the membrane fraction with the undiluted nucleoplasm revealed that the nucleoplasm contained at most 2% of the calnexin found in the nuclear membrane preparation (Fig. 5). Since 10% of the genome in an assay must be present for obtaining a positive result in the PCR, this result supports the conclusion that the WHV DNA detected in the nucleoplasmic fraction was indeed localized within the nucleoplasma.

The in vitro transport assay in the presence of an ATP-generating system confirmed the data of Fig. 4 that deproteinized WHV DNA almost completely remained in the cytoplasmic fraction (Fig. 6C). This suggests not only that protein-free WHV DNA is not transported or bound in a significant amount to the nucleus but also that the nuclear membrane remained essentially intact during the transport reaction. DNase I treatment prior to the PCR prevented the amplification of DNA (Fig. 6F), indicating that the WHV DNA does not form an undegradable complex with cellular proteins.

In the transport assay using the polymerase-DNA complex,

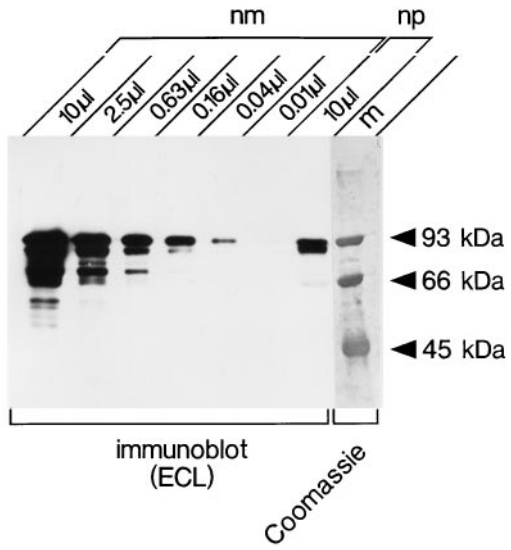

FIG. 5. Determination of contamination of the nucleoplasm with membranes. Ten microliters of the nucleoplasm and a dilution series containing the indicated amounts of the membrane fraction were separated on an SDS–10% polyacrylamide gel. After blotting on a PVDF membrane and reaction with anticalnexin and HRPO-conjugated anti-rabbit antibodies, detection of calnexin as a marker of nuclear and ER membranes was done by enhanced chemiluminescence (ECL). nm, nuclear membrane fraction; np, nucleoplasm; m, molecular weight marker.

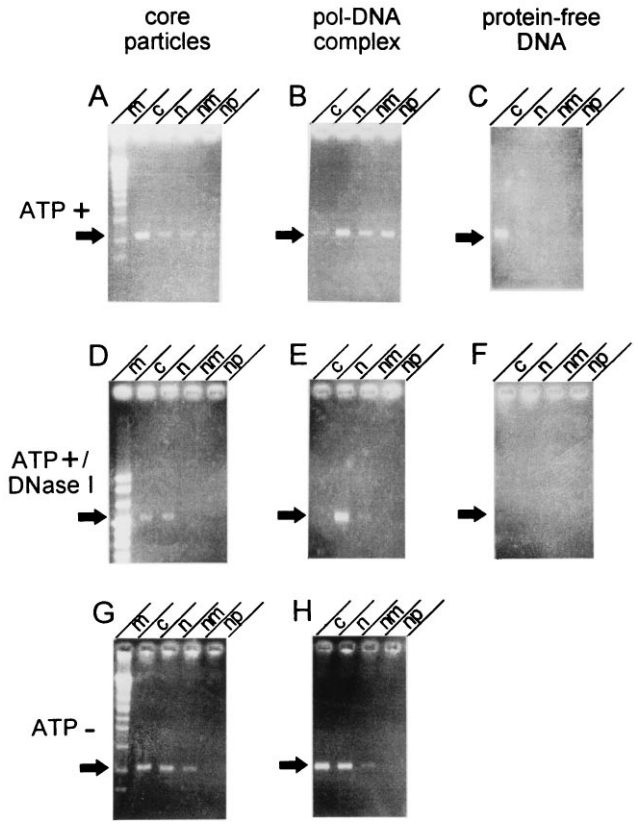

FIG. 6. PCR analysis of WHV DNA distribution in transport assays. PCR amplification products were separated on a 2% agarose gel and stained by ethidium bromide. (A to C) Transport assay in the presence of an ATP-generating system; (D to F) transport assay with DNase I digestion prior to the amplification; (G and H) transport assay in the presence of an ATP-depleting system. (A, D, and G) Distribution of DNA added as component of core particles; (B, E, and H) distribution of the polymerase (pol)-DNA complex; (C and H) distribution of the protein-free DNA. Lane m in panel D, DNA molecular weight marker VIII (Boehringer); lanes m in panel A and G, DNA molecular weight marker VII; lanes c, PCR of the RRL representing the cytosol; lanes n, intact nuclei; lanes nm, nuclear membranes; lanes np, nucleoplasm.

the viral genomes were found to be associated mainly with the nuclei (Fig. 6B, lane n). PCR after separation of nuclear membrane and nucleoplasm showed that the major part of the viral DNA was in the nucleoplasm (Fig. 6B, lane np). Since the nucleoplasm represents the supernatant after several steps of pelleting, an accidental copurification with nuclei due to the stickiness of the polymerase-DNA complex could be excluded. Another part was bound to the membrane (Fig. 6B, lane nm). These findings showed that (i) the in vitro transport system is applicable to high-molecular-weight complexes containing DNA, (ii) the permeabilization led to holes in the cellular membrane, large enough to allow the diffusion of the polymerase-DNA complex into the cytosol, and (iii) the polymerase-DNA complex was efficiently transported into the nucleus. The resistance of the viral DNA in the nuclei against DNase I digestion of the samples showed that the genomes were protected within the nuclei (Fig. 6E, lane n). The viral DNA was, however, digestible after disruption of the nuclei (Fig. 6E, lanes nm and np). These data show that viral DNA within the polymerase-DNA complex is accessible to DNase I and confirm the intranuclear localization of the viral DNA after the transport reaction. Furthermore, the observations show that during the short incubation period no significant diffusion of the DNase I through the nuclear pores into the nucleoplasm occurred.

A different cellular distribution of the viral genomes was found when the entire core particles were used in the transport assay. Although the majority of the genomes were found in the cytoplasm, a significant proportion of WHV DNA appeared in the nuclear fraction. Separation of this fraction into nuclear membranes and nucleoplasm revealed that WHV genomes were present in both samples (Fig. 6A). The DNase I digest of the same samples showed that the genomes, associated with the nucleus, were protected against the DNase I treatment when the intact nuclei were used (Fig. 6D, lane n) but were digestible after disruption of nuclear structure (Fig. 6D, lanes np and nm). Since the DNA inside the core particles is DNase I protected (Fig. 6D, lane c), these findings indicate that a disintegration of the core particles had taken place prior to or during nuclear transport. In contrast to the association of the core particles with the nuclear membrane, the amplification products derived from the nucleoplasm did not appear in all of the performed experiments (data not shown). Thus, a nuclear binding of the core particles has been demonstrated, but efficient release and transport of DNA to the nucleoplasm is not yet proven.

ATP dependence of nuclear import. To verify whether the observed nuclear import of the polymerase-DNA complex was an active process, the assay was performed with RRL, which was ATP depleted. In these assays, neither the genome of the polymerase-DNA complex nor the DNA in the core particles was transported into the nucleoplasm (Fig. 6G and H), indicating that ATP is a prerequisite for the transport. Thus, passive diffusion of the DNA into the nucleus could be excluded. Nevertheless, besides a significant fraction in the cytosol, an association of the WHV genomes with the nucleus was found in both assays, with core particles and the polymerase-DNA complex. Further fractionation showed that the DNA was exclusively bound to the nuclear membrane, indicating ATP-

independent binding of both components to membranes (Fig. 6G and H, lanes n and nm).

DISCUSSION

Previous studies have shown that core protein (9, 29, 37), as well as hepadnavirus polymerase (10, 31a), contains one or more nuclear localization signals (NLS). Since the NLS of the core protein overlaps and interferes with the nucleic acid binding domain (5, 17, 28), the NLS may not be exposed to the surface of the core particles. However, phosphorylation of the core protein in vitro leads to disappearance of the RNA encapsidation capacity (20), which is in accordance with the observation that in vivo-phosphorylated core protein does not bind nucleic acids (17, 25). Thus, phosphorylation may allow translocation of the NLS to the surface of the core particles. In any event, the nuclear membrane is an impermeable barrier for the intact core particles in transgenic mice (16). Thus, transport of the viral genome within the complete core particle into the nucleus is highly unlikely during natural infection. It is possible that core protein dimers (38) mediate the transport of the viral genome by binding the viral DNA with one core subunit and exposing the NLS with the second subunit. This possibility is consistent with the observation that in vitro only every second core molecule can be phosphorylated by PKC (20). If core protein mediates viral genome transport, a partial or complete disintegration of the core particle structure in the cytosol, in subcellular organelles, or at the nuclear membrane is implied.

A second possibility is that the nucleocapsids are completely disintegrated prior to the transport, leading to a release of the polymerase-DNA complex. In this second model, the viral polymerase would be the mediator for the nuclear import of the viral genome. Studies showing the presence of an NLS in the polymerase (10, 31a) support this theory, especially since the polymerase is, in contrast to the core protein, covalently bound to the minus DNA strand of the virion-encapsidated form of the genome. To investigate whether the viral polymerase is sufficient for mediating the nuclear import of the WHV genome or whether the presence of the entire nucleocapsid is a prerequisite for nuclear transport, we performed an in vitro transport assay using digitonin-permeabilized cells with both structures and, for control, with protein-free WHV DNA. The results obtained with the polymerase-DNA complex indicate that the transport system was highly efficient, even with a nucleoprotein of 2.2 MDa, whereas until now, this transport system was established only for the transport of smaller proteins. We could show that core particles were absent in this preparation and, thus, a transport involving particle structure could be excluded. Due to the limited sensitivity of the detection system, a contamination of the polymerase-DNA complex with a few core protein subunits per complex could not be excluded. Since core protein from liver does not bind nucleic acids (25), copurification of core proteins caused by binding to the WHV DNA is unlikely, but binding of a core protein monomer or dimer to the polymerase cannot be excluded. While the results suggest that the polymerase is essential for mediating the transport of the genome in vitro, it may in fact be mediated by the polymerase-bound core protein subunits. Additionally, a redundancy of karyophilic targeting of the genome, as suggested for HIV MA protein and Vpr (18), by both polymerase and core protein may exist.

An active nuclear import of the polymerase-DNA complex was confirmed by its ATP dependence. Inside core particles, the viral polymerase seems to be uncleaved (3). Thus, the observation of Foster et al. (10), who showed nuclear import of the amino-terminal portion of the polymerase, suggests that proteolytic cleavage of the polymerase after nucleocapsid disassembly prior to nuclear transport may occur. The transport activity of the polymerase in our in vitro transport system could, furthermore, be induced by the treatment with urea which was used for dissociation of the core proteins from the complex.

The observed binding of the polymerase-DNA complex to the nucleus in the absence of ATP most likely indicates that the complex binds in an ATP-independent manner via its NLS to the nuclear pore complex, without being imported. This assumption is in accordance with the observations of Moore and Blobel (27) and Görlich et al. (14), who showed similar reactivity patterns for the nuclear transport of fluorescein isothiocyanate-labeled peptide conjugates and nucleoplasmin with and without ATP. Nevertheless, binding to other copurified membrane-bound structures cannot be excluded by our experiments.

Both PCR and autoradiography after the transport assays using complete core particles revealed that only one-third of the genomes were bound to the nuclear fraction. The majority of the genomes remained in the cytosol. Since core particle-encapsidated viral DNA in the reticulocyte lysate was DNase I resistant, a disassembly of the majority of the core particles in the lysate is unlikely. In contrast, the viral DNA present in or at the nuclei was DNase I resistant only as long as the nuclei were intact. Thus, nuclear import occurs most probably only by disassembly of the core particles which were bound to the nucleus. This hypothesis is in accordance with recent findings (18a) where phosphorylation-dependent binding of core particles to the nuclear pore complex could be directly shown. The low efficiency of genome transport from core particles into the nucleoplasm in our system, in contrast to the efficient transport of the polymerase-DNA complex, suggests that the core particles are only slowly degraded. This assumption is in accordance with the report of Qiao et al. (30), who found intracellular accumulation of core particles after abortive infection of an established cell line (HepG2) but no nuclear import. Furthermore, these findings are consistent with the observation that in transfected HepG2 cells only a small pool of approximately 10 copies of viral cccDNA exists (33). The relatively inefficient release of hepadnavirus genomes from core particles might be one of several reasons for the nonsusceptibility of the established hepatocyte lines for hepadnaviruses. On the other hand, the poor nuclear import of the hepadnavirus genome in this assay may be caused by absence of the natural entry pathway of the core particles through another cellular compartment or by the lack of hepatic factors. For adenoviruses it has been shown that reductive activity in the endosomes led to a destabilization of nucleocapsids (15) and may lead to an activation of a sulfhydryl-dependent protease, which seems to be a prerequisite for nuclear import of the adenovirus genome.

Two main strategies of genome transport to the nucleus for viral capsids have been suggested. Our results are consistent with the expectation that opening of the core particles occurs prior to nuclear transport of the genome. The genome of influenza virus is released in the cytoplasm of the infected cell (21, 24). The finding that the released hepadnavirus polymerase-DNA complex could be transported into the nucleus resembles the transport of the influenza virus ribonucleoprotein into the nucleus (21, 24). On the other hand, the observed association of the hepadnavirus nucleocapsids with the nuclear membrane (30) shows more similarity to the disassembly of adenoviruses, where the nucleocapsid is destabilized in the cytoplasm and then bound to the nuclear pore complex and the genome is then released into the nucleoplasm. Since there is

no evidence for passage of the hepadnavirus core particles through an acidic compartment, at least during the intracellular amplification cycle, the destabilization of the nucleocapsids is probably mediated by another mechanism. A candidate for such a mechanism is the phosphorylation of the core particles during genome maturation, since phosphorylation leads to inhibition of the nucleic acid binding (20, 25) of the core particles. Thus, the stabilization of particle structure by its nucleic acid binding (5) would be terminated. This mechanism would provide a signal that nucleocapsids with the mature DNA genome could release their genome for nuclear transport. Further mechanisms like proteolysis of the polymerase or structural changes may be involved in the nuclear transport of the viral genome and must be subjected to further studies.

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