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Hepatitis B Virus Nucleocapsid Envelopment Does Not Occur without Genomic DNA Synthesis

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Assembly of the enveloped hepatitis B virus (HBV) is initiated by packaging of the RNA pregenome and the viral reverse transcriptase-DNA polymerase into a nucleocapsid. The pregenome is then reverse transcribed into single-stranded minus-polarity DNA, which is subsequently replicated to double-stranded DNA. All replicative intermediates are observable in capsids within infected liver, but only relatively mature nucleocapsids containing partially double stranded DNA are found in secreted virions. This observation suggests that maturation of the genome within the capsid is required for envelopment and secretion. We show that the differential distribution of replicative intermediates between intracellular nucleocapsids and secreted virions is also observable in human hepatoma cells transfected with wild-type HBV genomes. However, nucleocapsids were not enveloped or secreted when they were produced by an HBV genome carrying a missense mutation in the DNA polymerase that eliminates all DNA synthesis. An HBV missense mutant defective in the RNase H activity of the polymerase which allowed minus-strand DNA synthesis but not formation of double-stranded DNA was able to form virion-like particles. These experiments demonstrate that immature nucleocapsids containing pregenomic RNA are incompetent for envelopment and that minus-strand DNA synthesis in the interior lumen of the capsid is coupled to the appearance of a signal on the exterior of the nucleocapsid that is essential for its envelopment.

Human hepatitis B virus (HBV) is the prototype of the recently defined virus family *Hepadnaviridae* (10). Other family members have been found in rodents (e.g., ground squirrel hepatitis virus and birds (e.g., duck hepatitis B virus [DHBV]). These viruses have a narrow host range and cause acute and chronic infections of the liver. The hepatitis B virion has a relatively simple structure: an icosahedral nucleocapsid with a diameter of 30 or 34 nm (5) is surrounded by a detergent-sensitive envelope consisting of envelope proteins and lipid. The shell of the nucleocapsid is formed by a single core protein and contains a circular partially double stranded DNA genome 3.2 kb in length and a virus-encoded DNA polymerase.

In 1982, Summers and Mason (20) unraveled the basic principles of the unique replication mechanism of the hepadnaviruses (see Fig. 7) by analyzing replicative intermediates in DHBV-infected duck livers (for reviews, see references 7 and 15). The covalently closed circular form of the genome in the nuclei of infected cells is transcribed by the host machinery to produce a terminally redundant RNA (the pregenome), which has a typical mRNA structure. The pregenome is encapsidated along with the viral DNA polymerase by core proteins into a nucleocapsid, where the viral DNA genome is synthesized by an asymmetric mechanism. First, the complete single-stranded minus strand of the viral DNA is generated by reverse transcription of the pregenome, which is concomitantly destroy by the RNase H activity of the viral polymerase. In a second step, the viral DNA plus strand is synthesized by the viral DNA polymerase and the genome is circularized. For unknown reasons, synthesis of the second DNA strand terminates prematurely, leaving a single-stranded region of variable length. The nucleocapsid can then either be enveloped at an internal cellular membrane and be actively secreted as a mature virion or be transported back into the nucleus, where the genome is repaired to yield covalently closed circular DNA, thereby amplifying the intracellular genome pool (21).

In HBV, and also in DHBV (13) and ground squirrel hepatitis virus (24), secreted virions do not contain a full complement of the intracellular nucleocapsids. In the infected liver, nucleocapsids containing DNA ranging from growing to complete single-stranded minus-strand DNA up to partially double stranded circular DNA can be detected at all levels of maturation. Virions, on the other hand, contain only relatively mature double-stranded DNA (dsDNA) (e.g., circular partially double stranded viral genomes), while the more immature nucleocapsids are excluded from the virions (1). This observation led Summers and Mason (20) to propose that completion of the minus-strand DNA or the initiation of plus-strand synthesis is associated with a structural change in the nucleocapsid that is required for it to be packaged into the viral envelope. Here we provide direct evidence for such a regulatory mechanism which links maturation of the viral genome to nucleocapsid envelopment.

MATERIALS AND METHODS

Plasmids. Plasmid pRVHBV1.5 contains 1.5 copies of the HBV genome, subtype adw2 (22), and has been described elsewhere (3). A nucleotide change from T to G has been introduced by site-directed in vitro mutagenesis into pRVHBV1.5 at position 196 (numbering of the plus strand of the HBV genome starts with the deoxycytidine of the unique *Eco*RI site), resulting in plasmid pRVenv$^-$, which carries a stop codon in the 15th triplet of the major surface protein S gene. This mutation is silent in the overlapping reading frame for the viral DNA polymerase (3). The RT$^-$ (reverse transcriptase-defective) mutation was introduced into pRVHBV1.5 by site-directed in vitro mutagenesis which changed the G residue at nucleotide (nt) 740 to C. An *Spe*I (nt 677)-to-*Hpa*I (nt 961) fragment carrying the RT$^-$ mutation was recombined into pRVenv$^-$, generating pRVRT$^-$env$^-$. The sequence between *Spe*I and *Hpa*I was verified by sequencing. The RH$^-$ (RNase H-defective) mutation (D750V) was created by

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site-directed mutagenesis of nt 1332 from A to T. The 305-nt region between the unique *Nsi*I (nt 1066) and *Nco*I (nt 1370) sites containing the mutation was verified by sequencing and was inserted into the overlength HBV vector pHBV1.5LE$^-$, resulting in plasmid pRH$^-$env$^-$L$^-$. Plasmid pHBV1.5LE$^-$ is a derivative of pRVenv$^-$ and contains an additional stop codon in the open reading frame for the surface proteins (in the 52nd codon of the pre-S1 region) which is silent in the overlapping reading frame for the polymerase (3). Plasmid pSV45H contains the whole pre-S1, pre-S2, and S open reading frames for all three HBV surface proteins and the HBV polyadenylation site under transcriptional control of a simian virus 40 early promoter and has been described elsewhere (16). The RNA probe for the RNase protection assay was transcribed from plasmid pHuprobeBglII (kind gift of Don Ganem), which carries a *Bgl*II fragment (nt 1982 to 2403) of the HBV genome inserted into the *Bam*HI site of the vector pGEM3Z.

Cell culture, transfections, and hepatitis B surface antigen (HBsAg) assay. Huh7 and HepG2 cells were grown in 85-mm-diameter dishes in Dulbecco's modified essential medium–10% fetal calf serum at 37°C and 5% CO_2 and were split every third day. For transfections, cells were seeded at approximately 10^6 cells per 85-mm-diameter dish and were incubated overnight. Six micrograms of the plasmid carrying the HBV genome (mixed with 4 μg of plasmid pSV45H in case of cotransfections) was mixed with 0.5 ml of 250 mM $CaCl_2$. This solution was added drop by drop while stirring to 0.5 ml of 2× *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4 [pH 7.1]) and was incubated for 30 min at room temperature. The medium of the cells was changed, and 1 ml of the DNA precipitate was slowly added. The culture was incubated for 7 to 10 h, washed twice with phosphate-buffered saline (PBS), and incubated for 4 days with 10 ml of medium.

Transfection efficiencies and expression of the surface proteins in cotransfection experiments was assayed by measuring the HBsAg concentration in the culture medium at the end of incubation with an enzyme-linked immunosorbent assay (Sorin Biomedica, Saluggia, Italy). For quantification, a dilution series of an HBsAg standard serum was used.

Preparation of virus particles. The culture medium was collected, and cells were washed with PBS, placed on ice, and lysed with 5 ml of 150 mM NaCl–50 mM Tris-Cl (pH 7.5)–5 mM $MgCl_2$–0.2% (vol/vol) Nonidet P-40 (NP-40). The medium and cell lysate were cleared by low-speed centrifugation (15 min at 4,000 × *g*). Immunoprecipitations with a polyclonal rabbit antiserum against the HBV nucleocapsid antigen (anti-HBc; DAKO, Hamburg, Germany) or a polyclonal goat antiserum against the HBV surface antigen (anti-HBs; DAKO, Hamburg, Germany) were performed as described previously (2) except that for 5 ml of lysate or 10 ml of medium, 70 μl of a slurry suspension of protein A-Sepharose beads and 7 μl of antiserum were used. To some immunoprecipitations, NP-40 was added to a final concentration of 0.5% (vol/vol).

For isopycnic CsCl gradient centrifugation, 3.88 g of CsCl was dissolved in 10 ml of cleared medium or 5 ml of cleared lysate to which 5 ml of lysis buffer had been added. Ten milliliters of this solution was spun for 40 h at 40,000 rpm at 20°C in a Beckman 70Ti rotor. The tube was cut open at the top, and 10 1-ml fractions were taken from the meniscus by pipetting. Half of each fraction was diluted with 1 ml of PBS and 65 μl of 10% (vol/vol) NP-40, and 15 μl of a slurry suspension of protein A-Sepharose beads and 1.5 μl of rabbit anti-HBc antiserum were added for immunoprecipitation. After agitation overnight at 4°C, the beads were washed three times with PBS. The beads with bound viral particles were incubated for 30 min at 25°C in a mixture containing 0.2 ml of PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$ with 60 U micrococcal nuclease (Serva), and 40 U of RNase-free DNase I (Boehringer Mannheim). Then 10 μl of 0.1 M sodium ethylene glycol aminoethyl ether tetraacetate and 20 μg of tRNA were added. The encapsidated nucleic acid was isolated as described below.

For polyethylene glycol precipitation of nucleocapsids, the cells were rinsed with PBS and lysed with 1 ml of 10 mM Tris-Cl (pH 7.5)–1 mM EDTA–0.25% (vol/vol) NP-40–50 mM NaCl–8% (wt/vol) sucrose at 37°C for 10 min. The lysate was scraped from the plate and was clarified by centrifugation for 3 min at 12,000 × *g*. $MgCl_2$ was added to 7 mM, 50 μg of DNase I and 20 μg of RNase A were added to the supernatant, and the mixture was incubated at 37°C for 30 min. Nucleocapsids were precipitated with 333 μl of 26% (wt/vol) polyethylene glycol 8000–1.4 M NaCl–25 mM EDTA on ice for 30 min and were collected by centrifugation at 12,000 × *g* for 4 min. The pellet was suspended in 200 μl of 10 mM Tris-Cl (pH 7.5)–5.5 mM $MgCl_2$–1.5 μg of DNase I, and the mixture was incubated at 37°C for 15 min. DNase I digestion was terminated by addition of 2.5 μl of 0.5 M EDTA, and viral nucleic acids were isolated as described below.

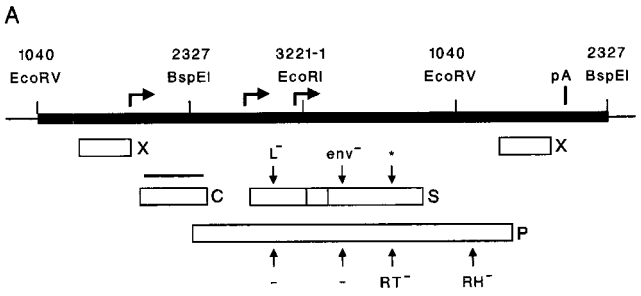
Southern blotting, RNase protection assay, and RNase H treatment. Nucleic acid was prepared by digestion of precipitated and nuclease-treated samples with proteinase K in sodium dodecyl sulfate (SDS) buffer, phenol-chloroform extraction, and ethanol precipitation. For the gel shown in Fig. 5, 1% agarose in 1× Tris-borate-EDTA buffer containing 40 μg of ethidium bromide per 100 ml was used; for the gel shown in Fig. 6, 1% agarose in 1× Tris-acetate-EDTA containing 50 μg of ethidium bromide per 100 ml was used. The radioactively labeled probe for the detection of both HBV DNA strands in Southern blotting was generated by random-primed DNA synthesis on isolated HBV dsDNA. The probe for detecting plus-strand DNA in Fig. 4 contained the complete HBV sequence found in pHBV1.5LE$^-$ and was generated by T7 RNA polymerase transcription of *Hin*dIII-linearized pHBV1.5LE$^-$ in the presence of [α-^{32}P]UTP. The radioactively labeled probe used for the RNase protection assays was transcribed from *Hin*dIII-linearized plasmid pHuprobeBglII with T7 RNA polymerase and was a minus-strand copy of part of the HBV core gene (nt 1982 to 2403). For the RNase protection assay, the nucleic acids isolated from virions or nucleocapsids from one 85-mm-diameter culture dish were dissolved in 29 μl of 40 mM HEPES (pH 7.9)–400 mM NaCl–1 mM EDTA, and 1 μl of RNA probe (500,000 cpm) was added. The mixture was incubated for 10 min at 80°C and then for 3 h at 65°C. Three hundred microliters of 300 mM NaCl–10 mM Tris-Cl (pH 7.2)–5 mM EDTA containing 20 μg of RNase A and 1,000 U of RNase T_1 (Boehringer Mannheim) was added. The mixture was further incubated at 30°C for 1 h and, after addition of 10 μl of 20% (wt/vol) SDS and 5 μl of proteinase K (10 mg/ml), was incubated for 15 min at 37°C. After addition of 2 μl of tRNA (10 mg/ml), the sample was extracted with phenol-chloroform and precipitated with ethanol by addition of 35 μl of 2.5 M sodium acetate and 750 μl of ethanol. The pellet was extensively washed with 70% ethanol, dissolved, heated, and loaded on a 5% polyacrylamide gel.

For RNase H treatment, isolated viral nucleic acids (20% of the yield from one plate) were incubated with 1.6 U of *Escherichia coli* RNase H for 60 min at 37°C in 40 mM Tris-Cl (pH 7.5)–4 mM $MgCl_2$–1 mM dithiothreitol–30 mM KCl–20 μg of bovine serum albumin per ml–5% (vol/vol) glycerol.

RESULTS

Virions secreted from cells transfected with the wild-type HBV genome contained predominantly mature genomes. A suitable animal or cell culture system which can be infected with HBV is not available. However, certain human liver cell lines secrete infectious virus after transfection of cloned viral DNA. To test whether selective envelopment of mature nucleocapsids can be observed in cell culture, we expressed HBV in HuH7 cells by transfection of an overlength copy of the wild-type HBV DNA (Fig. 1A). Nucleocapsids from detergent-lysed cells (Fig. 2, lane 6) and naked nucleocapsids from the medium (Fig. 2, lane 3) were immunoprecipitated with anti-HBc. Virions were immunoprecipitated from the medium with anti-HBs (Fig. 2, lane 5). In addition, naked nucleocapsids and nucleocapsids from virions were immunoprecipitated simultaneously from the medium with anti-HBc in the presence of the mild detergent NP-40 (Fig. 2, lane 4). The viral DNA from the particles was analyzed by Southern blotting. For reference, linearized HBV DNA 3.2 kb in length isolated from a plasmid was loaded on the gel in double-stranded (Fig. 2, lane 1) and heat-denatured single-stranded (lane 2) forms. The single-stranded DNA (ssDNA) appeared at a position corresponding to a 2.0 kb double-stranded linear DNA. The intracellular encapsidated viral DNA (Fig. 2, lane 6) formed three major bands corresponding, from bottom to top, to single-stranded linear, double-stranded linear, and double-stranded open circular HBV DNA. A smear to shorter molecular mass representing growing minus strands could also be observed. The ratio of dsDNA to ssDNA was approximately 1:4 (estimated from shorter exposures; data not shown). Virions in the culture medium contained predominantly dsDNA and much less ssDNA (Fig. 2, lane 5). Here, the ratio increased to approximately 10:1 (estimated from shorter exposures; data not shown). This result demonstrates that preferential envelopment of more mature nucleocapsids containing dsDNA versus immature nucleocapsids with ssDNA could also be observed in vitro. Naked nucleocapsids were also released into the medium by HuH7 cells (Fig. 2, lane 3) either by cell lysis or by another, unknown mechanism. In contrast, HepG2 cells release very low amounts of naked nucleocapsids into the medium (3, 4) (also below). The ratio of the different DNA forms in these naked nucleocapsids was identical to that in cytoplasmic nucleocapsids (seen on longer exposures; data not shown). The medium sample which was immunoprecipitated with anti-HBc in the presence of detergent (Fig. 2, lane 4) represented, as expected, the sum of naked and enveloped nucleocapsids (lane 3 plus lane 5).

Envelopment and secretion of nucleocapsids containing the RNA pregenome was inhibited. To test the hypothesis that genome maturation and nucleocapsid envelopment are linked,



C

mutation	nt change	aa change in P	aa change in S
RT⁻	G740C	D553H	W370C (•)
RH⁻	A1332T	D750V	—
env⁻	T196G	—	L189STOP
L⁻	G3006A	—	Y52STOP

FIG. 1. Map of HBV mutants used in this study. (A) Plasmids for HBV expression in transfected cells contained an overlength copy of the HBV genome, subtype adw2 (thick line). The positions of some restriction sites are indicated by thin vertical bars. The numbers refer to nucleotide positions of the 3221-bp-long HBV genome starting with the deoxycytidine residue of the unique *Eco*RI site. The horizontal arrows indicate promoters, from left to right, for the pregenomic RNA, the large surface protein mRNA, and the major surface protein mRNA. The thick vertical bar indicates the single polyadenylation site (pA) of the HBV genome. The open boxes show open reading frames for the genes of the core protein (C), the surface proteins (S), the DNA polymerase (P), and a protein of unknown function (X). The vertical lines in the S frame show the positions of the start codons for the middle and major surface proteins. The vertical arrows indicate point mutations introduced in this work (compare with Fig. 1C). The L^- and env^- mutations (stop codons in pre-S1 and S, respectively) are silent in the overlapping P frame (−). The RT^- mutation in P changes the YMDD motif in the active site of the polymerase to YMHD, which destroys the polymerase activity of the enzyme. This mutation changes the coding of the S gene (*). The RH^- mutation destroys the RNase H activity of the polymerase. The horizontal bar indicates the RNA probe used for the RNase protection assay. (B) The env^- mutation of the HBV genome was complemented in *trans* by cotransfection of the simian virus 40 early promoter (striped box) expression plasmid pSV45H, which directs the expression of all three HBV surface proteins from the S open reading frame. (C) Nucleotide and amino acid (aa; single-letter code) changes introduced by point mutations into the HBV genome.

we characterized an HBV mutant which was totally blocked in genomic DNA synthesis for its potential to form enveloped virions. Such a mutant has been generated by changing the YMDD motif in the active site of the viral reverse transcriptase to YMHD. This mutation does not interfere with pregenome packaging or nucleocapsid assembly; however, reverse transcription of the pregenome is completely blocked (17), and therefore the nucleocapsids remain in an immature state. We introduced the homologous point mutation into our HBV construct (RT^-) (Fig. 1A and C). The mutation affected also the overlapping gene for the surface proteins (9), changing amino acid 370 from tryptophan to cysteine in the large surface protein (Fig. 1A and C). To avoid an influence of this mutation on the phenotype of the HBV construct, we introduced into the genome a second mutation (env^- [Fig. 1A and C]) which prevented expression of the HBV surface proteins from this construct (3). The RT^-env^- HBV genome was cotransfected into HepG2 cells with a simian virus 40 expression plasmid (pSV45H) which provided the viral surface proteins in *trans* (Fig. 1B). Expression of surface proteins and their secretion as subviral particles could be verified by the detection of HBsAg in the medium. In the experiment shown in Fig. 3, 18 ng of HBsAg per ml of medium was measured. HBsAg concentrations in this range are typical for cotransfection experiments with plasmid pSV45H. Viral particles were immunoprecipitated from cells and medium, and the pregenome was detected by RNase protection assays. The pregenome was readily detectable in intracellular nucleocapsids (Fig. 3, lane 9), but the pregenome was undetectable in secreted enveloped nucleocapsids (Fig. 3, lane 6) in several repeated experiments. Release of naked nucleocapsids from the HepG2 cells could not be observed (Fig. 3, lanes 4 and 5). Identical results were obtained when viral particles were prepared by isopycnic CsCl gradient centrifugation (data not shown). These data demonstrate that the immature nucleocapsids were incompetent for envelopment, which implies that a signal for envelopment is generated on the surface of maturing nucleocapsids sometime during viral DNA synthesis (see Fig. 7).

Nucleocapsids containing minus-strand DNA were enveloped. Genomic maturation proceeds in two steps: reverse transcription yields minus-strand DNA, which is followed by plus-strand synthesis. We tried to genetically separate these two steps to test whether second-strand synthesis is necessary for nucleocapsid envelopment. This could be achieved by using the HBV point mutant D750V, which has a defect in the RNase H activity in the C-terminal portion of the viral DNA polymerase (17). This mutant allows first-strand synthesis but is defective in degradation of the RNA pregenome and in second-strand DNA synthesis (17). A homologous mutation (RH^-) was introduced into the HBV construct used in this work (Fig. 1A and C). This mutation did not change the coding of the surface protein gene. However, to allow direct comparability with the RT^-env^- construct described above, expression of the surface proteins was also blocked by introducing the env^- and L^- mutations into the RH^- strain. The $RH^-env^-L^-$ plasmid was

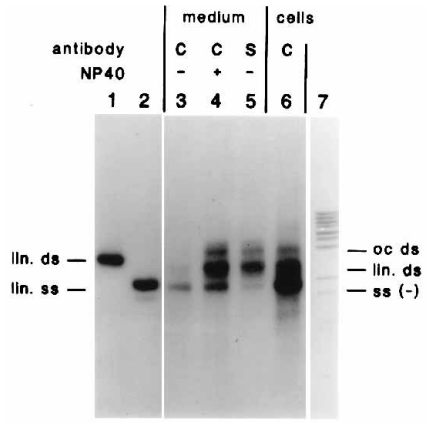


FIG. 2. Differential distribution of replicative intermediates between transfected cells and medium. HuH7 cells were transiently transfected with a replication-competent HBV wild-type construct (Fig. 1A). Nucleocapsids were immunoprecipitated from the cell lysate (lane 6) with anti-HBc (c). From the medium, virions and naked nucleocapsids were separately immunoprecipitated with anti-HBc and with anti-HBs (s), respectively. In addition, naked and enveloped capsids were immunoprecipitated simultaneously by anti-HBc in the presence of the mild detergent NP-40. Viral DNA was separated on an agarose gel and used for Southern blotting. Fifty picograms of linear double-stranded (ds) (lane 1) and heat-denatured single-stranded (ss) (lane 2) HBV DNA was applied for reference. In intracellular nucleocapsids, the single-stranded replicative intermediates of minus polarity were predominant relative to the linear (lin.) and open circular (oc) double-stranded forms (lane 6). The same distribution was found in naked nucleocapsids from the medium (lane 3). In virions (lane 5), the double-stranded forms prevailed over single-stranded forms. Lane 4 corresponds to the sum of the signals of lanes 3 and 5, as expected. Labeled *Bst*EII-digested phage lambda DNA served as a molecular mass standard (lane 7). The DNA in lanes 3 to 6 was extracted from one half of an 85-mm-diameter culture dish each, and the film was exposed for 2 days with an intensifying screen.

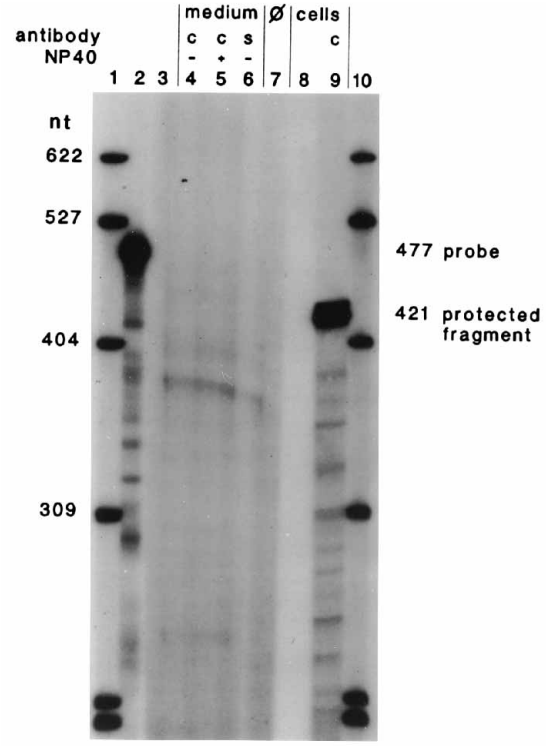


FIG. 3. Envelopment of immature nucleocapsids containing pregenomic RNA is blocked. HepG2 cells were transiently cotransfected with the RT⁻env⁻ HBV mutant and the simian virus 40 expression plasmid pSV45H, which complements the env⁻ defect (Fig. 1). Viral particles were immunoprecipitated from cells and medium as described for Fig. 2. The encapsidated pregenome was detected by an RNase protection assay by using a 477-nt RNA probe (lane 2) which hybridized to the 5′ end of the pregenome (Fig. 1A). The packaged pregenome could readily be detected in cytoplasmic cores, as is evident from the protected 421-nt fragment (lane 9). The nucleocapsids containing the pregenome were not enveloped and secreted into the medium (lanes 4 to 6). Lane 7 shows a minus-RNA control. Lanes 3 and 8 were not loaded. Lanes 1 and 10 contain a molecular mass standard (*Msp*I-cut pBR322). Sizes on the left are given in nucleotides (nt). Material isolated from one half of an 85-mm-diameter cell culture dish was loaded on each lane, and the autoradiogram was exposed for 6 days with an intensifying screen.

cotransfected with the expression vector pSV45H to provide the missing surface proteins in *trans.*

Southern blotting of DNA isolated from intracellular nucleocapsids with a full-length HBV DNA probe that detects both plus- and minus-polarity DNA revealed the production of open circular DNA, double-stranded linear DNA, and ssDNA for wild-type HBV (Fig. 4A, lane 5) but only the single- and double-stranded linear forms for the RH⁻env⁻L⁻ construct (lane 6). Probing an identical blot with a strand-specific probe that detects only plus-polarity DNA revealed the open circular and double-stranded linear forms for the wild-type construct (Fig. 4B, lane 5) but did not detect any DNA from the RH⁻env⁻L⁻ mutant (Fig. 4B, lane 6), even after much longer exposures (data not shown). To assess the extent of minus-strand synthesis, viral DNA from intracellular nucleocapsids was heat denatured or treated with RNase H prior to Southern blotting (Fig. 5). DNA from the wild type formed a strong band around 3.2 kb and a smear to lower molecular weight as a result of incomplete plus strands (Fig. 5, lane 5). This pattern did not change after RNase H treatment (Fig. 5, lane 7), as expected. Under the applied electrophoresis conditions, the viral ssDNA migrated slightly faster in the gel after heat denaturation (Fig. 5, lane 6). The RH⁻env⁻L⁻ mutant generated less viral DNA (Fig. 5, lane 8). After heat denaturation, the minus-strand ssDNA formed several bands (Fig. 5, lane 9) corresponding to full-length strands (compare lane 9 with lanes 4 and 6) and at least five bands of subgenomic size which seem to represent pausing sites of minus-strand synthesis. We estimated that approximately one-fifth of the minus strand was of full length. RNase H treatment (Fig. 5, lane 10) gave the same pattern as heat denaturation (Fig. 5, lane 9), confirming the RNase H-defective phenotype of the mutant. No signal was obtained in mock-transfected cells (Fig. 5, lanes 11 to 13).

The RH⁻env⁻L⁻ mutant was cotransfected with pSV45H into HepG2 cells. Southern blot analysis of viral DNA isolated from fractions of isopycnic CsCl gradients from a cell lysate showed nucleocapsids (Fig. 6, lane 8) at their characteristic density of 1.36 g/ml. Virion-like particles were found in the CsCl gradient from the culture medium at their typical density of 1.23 g/ml (Fig. 6, lane 6). Comparison of the patterns of heat-denatured viral DNA from virion-like particles (Fig. 6, lane 7) and from cellular nucleocapsids (lane 9) revealed no significant difference. The experiment suggests that second-strand synthesis or completion of first-strand synthesis was not required for envelopment of nucleocapsids.

DISCUSSION

Extracellular HBV virions contain relatively mature viral genomes, whereas the nascent viral cores within the hepatocytes contain DNA at all stages of maturation. This observation led Summers and Mason to propose that there is an orderly regulation of envelopment that selects only relatively mature, dsDNA-containing nucleocapsids for envelopment and excludes immature nucleocapsids (20). The results presented here provide experimental support for the existence of this regulatory mechanism in HBV virion formation and indicates that the mechanism functions coordinately with minus-strand DNA synthesis. The main evidence for this mechanism is the phenotype of the RT⁻env⁻ HBV genome cotransfected with the expression vector pSV45H for surface proteins (Fig. 1). As shown by two different techniques for the preparation of

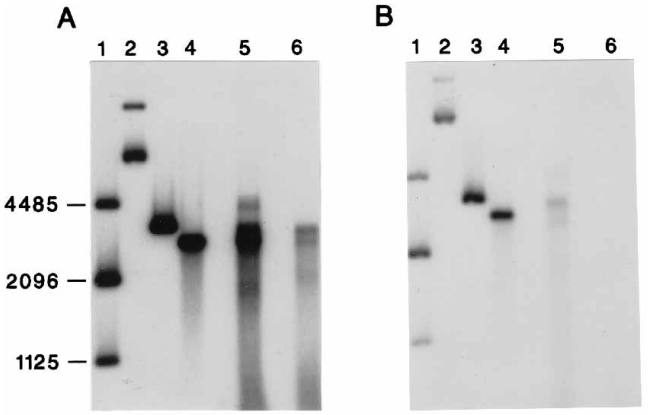


FIG. 4. The RH⁻env⁻ mutant generates no detectable HBV DNA plus strands. Plasmids pHBV1.5LE⁻ and pHBV1.5RH⁻env⁻L⁻ were transfected into HepG2 cells, and cytoplasmic cores were isolated. The viral DNAs were purified and analyzed by Southern hybridization. The order of the lanes is the same for both panels: dsDNA size marker (lane 1), uncut pHBV1.5LE⁻ (lane 2), double-stranded linear HBV monomer DNA (lane 3), heat-denatured single-stranded HBV linear monomer DNA (lane 4), and viral DNA from HBV1.5LE⁻ (lane 5) and from HBV1.5RH⁻env⁻L⁻ (lane 6). In panel A, the DNAs were probed with labeled HBV DNA generated by random-primed labeling from an HBV dsDNA template. The probe detects both minus- and plus-polarity HBV DNA. In panel B, the DNAs were probed with a single-stranded HBV RNA probe generated by T7 RNA polymerase transcription of pHBV1.5LE⁻. This probe detect plus-polarity HBV DNA only. For the mutant RH⁻env⁻L⁻ (panel B, lane 6), HBV plus-strand DNA could not be found. Sizes on the left are given in base pairs.

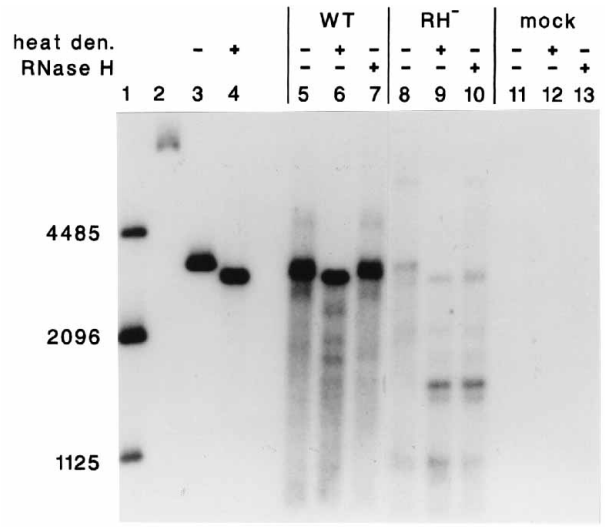


FIG. 5. RNase H-negative phenotype of the RH⁻env⁻L⁻ point mutant. HepG2 cells were transiently transfected with a wild-type (WT; lanes 5 and 7) or RH⁻env⁻L⁻ (RH⁻; lanes 8 to 10) HBV construct or mock transfected (lanes 11 to 13). Viral DNA was prepared from polyethylene glycol-precipitated nucleocapsids. One third of each sample remained untreated (first lane of each panel), one third was heat denatured (den.) (second lane of each panel), and one third was treated with RNase H (third lane of each panel). The blot was probed with a full-length double-stranded HBV probe that detects both plus- and minus-polarity DNA. For reference, a molecular mass standard (lane 1; the sizes of the fragments in base pairs are indicated at the left), the uncut wild-type construct pRVHBV1.5 (lane 2), and linearized monomeric double-stranded (lane 3) and single-stranded (lane 4) HBV DNA were used. RNase H treatment did not alter the migration of wild-type DNA, as expected (compare lane 7 with lane 5), but changed the migration of RH⁻env⁻L⁻ DNA to a pattern identical to that of heat-denatured ssDNA (compare lane 10 with lane 9). A substantial amount of RH⁻env⁻L⁻ minus-strand DNA was of full length.

viral particles, immunoprecipitation (Fig. 2) and isopycnic CsCl gradient centrifugation (data not shown), this mutant failed to produce secreted enveloped nucleocapsids. The trivial explanation that this negative result was due to inefficient complementation of the env⁻ mutation by the expression plasmid pSV45H is excluded because a second polymerase mutant (RH⁻env⁻L⁻) was efficiently complemented under identical conditions (Fig. 6) and expression of the surface proteins and their secretion as subviral particles of HBsAg were directly confirmed in the RT⁻env⁻ cotransfection. Virtually identical results were obtained by another laboratory for DHBV (23), indicating that this mechanism may well be conserved within the family *Hepadnaviridae.*

At which point during genome maturation is the signal for envelopment generated? The distribution of replicative intermediates in wild-type HBV-transfected HuH7 cultures (Fig. 2; compare lanes 5 and 6) suggests that nucleocapsids containing completed single-stranded minus strands can be enveloped. This observation correlates well with in vivo observations in which virions containing single-stranded minus strands could be found in rare cases (19). An HBV variant with a C-terminal 19-amino-acid truncation of the core protein which synthesized minus-strand DNA normally but produced very little plus-strand DNA formed enveloped virions (14). Similar observations with DHBV core mutants reveal a dependence of nucleocapsid envelopment on plus-strand synthesis (25); however, a direct effect of the core mutations on envelopment cannot be excluded in this case. The most detailed information concerning the timing of the envelopment signal comes from the RT⁻ and RH⁻ mutants (Fig. 3 and 6). The RT⁻ cores were not enveloped, indicating that envelopment occurs after initiation of reverse transcription. However, the envelopment of cores produced by the RH⁻ mutant argues that neither completion of the minus-strand DNA nor synthesis of the plus-strand DNA was required for envelopment (Fig. 7).

The molecular nature of the envelopment signal is unknown. The event which triggers the signal is linked to genomic replication in the interior of the capsid, and therefore the information must somehow be transmitted from the inside to the outside of the capsid in order for interaction with the envelope to occur. Several possible mechanisms are imaginable. For example, the different structural properties of the relatively rigid dsDNA compared with the flexible pregenome or single-stranded minus DNA strand may induce the envelopment signal, possibly through altering the capsid conformation. In this context, it is interesting that bacterially expressed HBV cores can assemble into two capsid structures with different sizes (5). Such a conformation-dependent mechanism has a precedent in poliovirus, in which the capsids express different antigens on their surface depending on their RNA content (18). This putative conformational change would offer a convenient explanation for the envelopment of the RH⁻ cores. In this scenario, envelopment would not be triggered by DNA synthesis per se but rather would be induced because the relatively rigid helical structure of the RNA-DNA hybrid in the RH⁻ mutant would mimic the structure of the dsDNA genome.

A second plausible mechanism for the envelopment signal depends on the ability of the arginine-rich C terminus of the core protein to bind to the pregenome during nucleocapsid assembly (6). It is possible that this binding dissociates during reverse transcription, resulting in a structural change in the core protein. This putative alteration may occur either directly or by unmasking a site for protein modification. One candidate for a putative modification is phosphorylation by a protein kinase activity associated with nucleocapsids that phosphory-

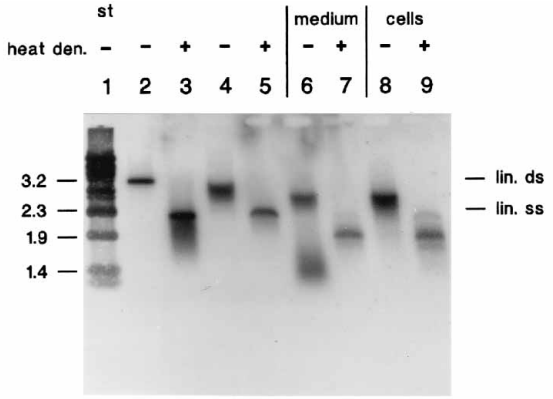


FIG. 6. Envelopment and release of the RH⁻env⁻L⁻ mutant nucleocapsids. HepG2 cells were transiently cotransfected with the HBV mutant RH⁻env⁻L⁻ and the simian virus 40 expression plasmid pSV45H for the surface proteins complementing the env⁻L⁻ mutations. The cell lysate and medium were used for isopycnic CsCl gradient centrifugation. Ten fractions were taken from the top. Viral DNA was isolated from the lysate gradient from combined fractions 8 and 9, having a density of 1.36 g/ml, which is typical for nucleocapsids (lane 8 and 9), and from the medium gradient from combined fractions 4 and 5, which had a density of 1.23 g/ml, indicative of virion-like particles (lane 6 and 7). One half of each sample was heat denatured (den.) prior the gel electrophoresis (lanes 7 and 9) and Southern blotting. For reference, radioactively labeled phage lambda DNA, *Bst*EII cut (lane 1), and 10 pg of linearized HBV DNA isolated from a plasmid without (lane 2) or with (lane 5) heat denaturation were loaded. Also, viral DNA isolated from 10 μl of serum from a highly viremic HBV carrier corresponding to approximately 3×10^6 genomes was loaded without (lane 4) or with (lane 3) heat denaturation. The extent of minus DNA strand synthesis in the virion-like particles was not significantly different from that in the intracellular nucleocapsids, indicating that completion of minus-strand or initiation of plus-strand DNA synthesis was not required for envelopment and secretion. lin. ds, linear double stranded; lin. ss, linear single stranded. Sizes of standards (st) are indicated in kilobase pairs.

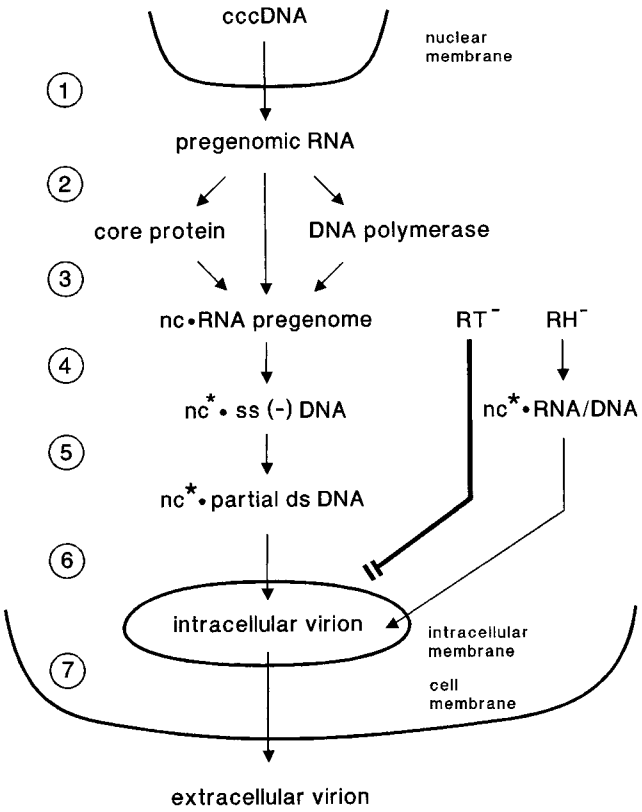


FIG. 7. Linkage of viral genome maturation and virion formation. The viral pregenomic RNA is transcribed in the nucleus from the covalently closed circular form (cccDNA) of the viral genome (step 1). The pregenomic RNA serves as a template for the translation of the DNA polymerase and core protein (step 2). Pregenome, DNA polymerase, and core proteins assemble to an immature nucleocapsid (nc; step 3). The pregenome is first reverse transcribed to ssDNA of minus polarity (step 4). Subsequently, the DNA plus strand is synthesized (step 5). Before completion of step 5, the mature nucleocapsid interacts with the viral surface proteins at an intracellular compartment to form a luminal virion (step 6), which is then actively secreted (step 7) (20). Here, we show that blocking the maturation of the viral genome at step 4 by a point mutation in the active site of the DNA polymerase (RT^- mutant) blocked the formation and secretion of virions as well (Fig. 3). We also show that a point mutation in the RNase H domain of the viral polymerase (RH^-) which led to the formation of minus-strand DNA–pregenome heteroduplexes (Fig. 4 and 5) was actively secreted (Fig. 6). The data suggest that minus-strand DNA synthesis is linked to a structural change in the nucleocapsid (indicated by asterisks) which is necessary for the interaction with viral surface proteins and envelopment.

lates serine residues in the C-terminal region of the core protein (8, 12). In DHBV, a different pattern of phosphorylation of the core protein in cytoplasmic capsids and secreted virions has been observed (26).

The linkage of genome maturation and envelopment of the nucleocapsid may provide a quality control checkpoint to guarantee that mainly replication-competent nucleocapsids are secreted and that the empty or replication-deficient nucleocapsids are retained within the cell. This may be an important advantage for HBV, whose transmission depends largely on the transfer of small volumes of blood containing only a few virions.

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REFERENCES

1. **Blum, H. E., A. T. Haase, J. D. Harris, D. Walker, and G. N. Vyas.** 1984. Asymmetric replication of hepatitis B virus DNA in human liver: demonstration of cytoplasmic minus-strand DNA by blot analyses and *in situ* hybridization. Virology **139:**87–96.
2. **Bruss, V., and D. Ganem.** 1991. Mutational analysis of hepatitis B surface antigen particle assembly and secretion. J. Virol. **65:**3813–3820.
3. **Bruss, V., and D. Ganem.** 1991. The role of envelope proteins in hepatitis B virus assembly. Proc. Natl. Acad. Sci. USA **88:**1059–1063.
4. **Bruss, V., and R. Thomssen.** 1994. Mapping a region of the large envelope protein required for hepatitis B virion maturation. J. Virol. **68:**1643–1650.
5. **Crowther, R. A., N. A. Kiselev, B. Böttcher, J. A. Berriman, G. P. Borisova, V. Ose, and P. Pumpens.** 1994. Three-dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy. Cell **77:**943–950.
6. **Galina, A., F. Bonelli, L. Zentilin, G. Rindi, M. Muttini, and G. Milanesi.** 1989. A recombinant hepatitis B core antigen polypeptide with the protamine-like domain deleted self-assembles into capsid particles but fails to bind nucleic acid. J. Virol. **63:**4645–4652.
7. **Ganem, D., and H. E. Varmus.** 1987. The molecular biology of the hepatitis B viruses. Annu. Rev. Biochem. **56:**651–693.
8. **Gerlich, W. H., U. Goldmann, R. Müller, W. Stibbe, and W. Wolff.** 1982. Specificity and localization of the hepatitis B virus-associated protein kinase. J. Virol. **42:**761–766.
9. **Heermann, K., U. Goldmann, W. Schwartz, T. Seyffarth, H. Baumgarten, and W. H. Gerlich.** 1984. Large surface proteins of hepatitis B virus containing the pre-S sequence. J. Virol. **52:**396–402.
10. **Howard, C. R.** 1995. Family Hepadnaviridae, p. 179–184. *In* F. A. Murphy et al. (ed.), Virus taxonomy. Springer-Verlag, Vienna.
11. **Huang, M. J., and J. Summers.** 1991. Infection initiated by the RNA pregenome of a DNA virus. J. Virol. **65:**5435–5439.
12. **Kann, M., and W. H. Gerlich.** 1994. Effect of core protein phosphorylation by protein kinase C on encapsidation of RNA within core particles of hepatitis B virus. J. Virol. **68:**7993–8000.
13. **Mason, W. S., C. Aldrich, J. Summers, and J. M. Taylor.** 1982. Asymmetric replication of duck hepatitis B virus DNA in liver cells: free minus strand DNA. Proc. Natl. Acad. Sci. USA **79:**3997–4001.
14. **Nassal, M.** 1992. The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. J. Virol. **66:**4107–4116.
15. **Nassal, M., and H. Schaller.** 1993. Hepatitis B virus replication. Trends Microbiol. **1:**221–228.
16. **Persing, D. H., H. E. Varmus, and D. Ganem.** 1986. Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. Science **234:**1388–1391.
17. **Radziwill, G., W. Tucker, and H. Schaller.** 1990. Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H activity. J. Virol. **64:**613–620.
18. **Roizman, B., M. M. Mayer, and P. R. Roane.** 1959. Immunochemical studies of poliovirus. IV. Alteration of the immunologic specificity of purified poliomyelitis virus by heat and ultraviolet light. J. Immunol. **82:**19–25.
19. **Scotto, J., M. Hadchouel, S. Wain-Hobson, P. Sonigo, A. Couroucé, R. Tiollais, and C. Bréchot.** 1985. Hepatitis B virus DNA in Dane particles: evidence for the presence of replicative intermediates. J. Infect. Dis. **151:** 610–617.
20. **Summers, J., and W. S. Mason.** 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell **29:**403–425.
21. **Tuttleman, J. S., C. Pourcel, and J. Summers.** 1986. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell **47:**451–460.
22. **Valenzuela, P., M. Quiroga, J. Zaldivar, R. Gray, and W. Rutter.** 1980. The nucleotide sequence of the hepatitis B viral genome and the identification of the major viral genes. UCLA Symp. Mol. Cell. Biol. **18:**57–70.
23. **Wei, Y., and D. Ganem.** Personal communication.
24. **Weiser, B., D. Ganem, C. Seeger, and H. E. Varmus.** 1983. Closed circular viral DNA and asymmetrical heterogeneous forms in liver from animals infected with ground squirrel hepatitis virus. J. Virol. **48:**1–9.
25. **Yu, M., and J. Summers.** 1991. A domain of the hepadnavirus capsid protein specifically required for DNA maturation and virus assembly. J. Virol. **65:** 2511–2517.
26. **Yu, M., and J. Summers.** 1994. Phosphorylation of the duck hepatitis B virus capsid protein associated with conformational changes in the C terminus. J. Virol. **68:**2965–2969.