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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

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Assembly of replication-competent hepatitis B virus (HBV) nucleocapsids requires the interaction of the core protein, the P protein, and the RNA pregenome. The core protein contains an arginine-rich C-terminal domain which is dispensable for particle formation in heterologous expression systems. Using transient expression in HuH7 cells of a series of C-terminally truncated core proteins, I examined the functional role of this basic region in the context of a complete HBV genome. All variants containing at least the 144 N-terminal amino acids were assembly competent, but efficient pregenome encapsidation was observed only with variants consisting of 164 or more amino acids. These data indicate that one function of the arginine-rich region is to provide the interactions between core protein and RNA pregenome. However, in cores from the variant ending with amino acid 164, the production of complete positive-strand DNA was drastically reduced. Moreover, almost all positive-strand DNA originated from in situ priming, whereas in wild-type particles, this type of priming not supporting the formation of relaxed circular DNA (RC-DNA) accounted for about one half of the positive strands. Further C-terminal residues to position 173 restored RC-DNA formation, and the corresponding variant did not differ from the full-length core protein in all assays used. The observation that RNA encapsidation and formation of RC-DNA can be genetically separated suggests that the core protein, via its basic C-terminal region, also acts as an essential auxiliary component in HBV replication, possibly like a histone, or like a single-stranded-DNA-binding protein. In contrast to their importance for HBV replication, sequences beyond amino acid 164 were not required for the formation of enveloped virions. Since particles from variant 164 did not contain mature DNA genomes, a genome maturation signal is apparently not required for HBV nucleocapsid envelopment.

Hepatitis B virus (HBV) is the prototypic member of the hepadnaviridae, small enveloped DNA viruses which replicate through reverse transcription of an RNA intermediate (reviewed in reference 9). This reaction takes place inside the nucleocapsid, or core particle, which consists of a shell of a 180 subunits of the core protein enclosing the RNA pregenome and the product(s) of the viral P gene, which provide the enzymatic activities required for reverse transcription. The P gene products are also actively involved in pregenome encapsidation (1, 12). From the currently available data, it appears that assembly of replication-competent cores in vivo is controlled by the concentrations of core and P protein (1, 22). Specific packaging of the pregenome is mediated in *cis* by the encapsidation signal ε (14), which is recognized by the P or core protein (or a complex of P with core protein).

The core protein consists of two structurally and functionally distinct domains (24): the sequence from the N terminus to about amino acid (aa) position 144 is by itself sufficient for assembly competence of the protein, at least at the high protein concentrations present in heterologous expression systems, e.g., *Escherichia coli* (3, 8, 31). The following 39 aa contain 16 arginine residues, 14 of which occur in clusters of three or four. In the *E. coli* system, a core protein variant consisting of the first 164 aa formed particles with an RNA content similar to that observed for the full-length protein; by contrast, in particles from a variant ending with aa 149, the amount of RNA was reduced by 10- to 20-fold, suggesting that at least part of the basic C-terminal region is involved in nucleic acid binding (3).

In this study, I used a similar series of C-terminally truncated core proteins to examine the functional role of the arginine-rich region in the context of a complete, replication-competent HBV genome. Specifically, I examined whether the truncations would interfere with RNA encapsidation, packaging of the P protein, and the formation of enveloped virions. The data presented below confirm the importance of the poly-Arg region for pregenome encapsidation, but they also suggest that the core protein, via its C-terminal domain, takes an active part in HBV replication.

MATERIALS AND METHODS

Chemicals and enzymes. All enzymes for molecular cloning experiments were obtained from Boehringer (Mannheim, Germany) or New England Biolabs (Schwalbach, Germany) and were applied as recommended by the manufacturers. Oligonucleotides were prepared on an Applied Biosystems 380B synthesizer and purified by electrophoresis on urea-containing polyacrylamide gels. ^{32}P-labeled nucleoside triphosphates (NTPs) and ^{125}I-labeled protein A were purchased from Amersham (Braunschweig, Germany). Protein A-Sepharose and Sephadex G-100 were obtained from Pharmacia (Freiburg, Germany). Reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were from Serva (Heidelberg, Germany); agarose and low-melting-point agarose were from FMC Corp., Marine Colloids Div. (Rockland, Maine).

Bacterial strains, plasmids, and construction of mutants. For all cloning experiments, *E. coli* DH1 cells were used.

The bacteria were grown at 37°C in Standard I medium (Merck, Darmstadt, Germany). Plasmid pCHT-9/3091wt was assembled from the following fragments: a DNA fragment containing the human cytomegalovirus (HCMV) IE1 promoter (10) from nucleotides (nt) −592 to −14 was obtained by polymerase chain reaction with primers adding *Pst*I and *Sal*I restriction sites to the 5′ and 3′ ends, respectively. An HBV fragment carrying a *Sal*I site immediately upstream of nt 3091 was derived from plasmid pMH-9/3091 (13) by cutting with *Sal*I and *Eco*RI (nt 1280). These two fragments were combined with the large *Pst*I-*Eco*RI fragment of plasmid pHT-15/190 (22) such that the resulting plasmid, pCHT-9/3091wt, carried, under HCMV promoter control, a slightly overlength HBV genome from which a genomic RNA corresponding to the authentic pregenome is transcribed. The construction of the mutant C genes 1-138, 1-144, and 1-164 has been previously described (3). The corresponding pCHT-9/3091 derivatives were constructed by transferring the mutant C genes from the *E. coli* expression plasmids as *Drd*I (nt 247)-to-*Mam*I (nt 524) fragments into pCHT-9/3091wt. Plasmid pCHT-9/3091/173 was constructed by polymerase chain reaction, using a mismatched primer (23) which changes the Arg codon 174 (AGA) to a stop codon (TGA). None of the nucleotide exchanges alters the coding information of the overlapping P gene. The variant proteins are named according to the last amino acid that they contain; for example, variant 138 consists of the sequence 1 to 138.

To make M13 constructs, the large *Sal*I-to-*Fsp*I fragment from plasmid pHT-15/190 (22) was transferred into M13mp18 and M13mp19, which were cut with *Sal*I and *Bam*HI; the *Bam*HI overhangs had been blunt ended by using Klenow enzyme and dNTPs. Thereby, a unit-length HBV genome starting at nt 3084 (last nucleotide of the *Sal*I site) to 3084 (third nucleotide of the former *Fsp*I site) was present in these constructs in either the positive or the negative orientation.

For complementation experiments, plasmid pMTpol (28) was used to provide P protein, and plasmid pCS1C1 was used to provide wild-type (wt) core protein. This plasmid carries a synthetic C gene (20) under control of the HCMV IE promoter but no further HBV sequences. The polyadenylation signal is derived from simian virus 40.

Cells and transfections. HuH7 cells (19) were transfected with the appropriate plasmid constructs (20 μg of CsCl gradient-purified DNA per 10-cm dish) by the calcium phosphate method and lysed 3 days posttransfection as previously described (14, 22).

Immunological techniques. For Western immunoblotting, proteins were separated on 15% polyacrylamide gels containing 0.1% SDS and then transferred onto nitrocellulose membranes (BA85; Schleicher & Schuell, Dassel, Germany). Core proteins were detected by using a polyclonal rabbit antiserum elicited against denatured core protein expressed in *E. coli*, which recognizes all known forms of core protein, followed by incubation with ^{125}I-labeled protein A. For immunoprecipitations of core particles, the same antiserum, bound to protein A-Sepharose, was used (21). Envelope-associated cores were immunoprecipitated with a polyclonal rabbit antiserum raised against a phage MS2 replicase-pre-S1 (aa 55 to 121) fusion protein expressed in *E. coli*. The sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of HBV core antigen (HBcAg)- or HBV envelope antigen (HBeAg)-related antigens has been previously described (3).

RNase protection assays. Total RNA from the cytoplasm of transfected cells and encapsidated RNAs were isolated as previously described (14, 22). The probe RNA was derived from a plasmid carrying an HBV genome starting at nt 2922 and ending with the *Xba*I site at nt 241, immediately followed by a bacteriophage T7 promoter. For probe preparation, the plasmid was cut with *Dra*I (nt 3004 in HBV), and the runoff antisense transcript was labeled with [α-^{32}P]CTP (22). Fragments protected from RNase digestion were analyzed on 6% polyacrylamide gels containing 7 M urea.

Endogenous polymerase reaction. The conditions for the endogenous polymerase reaction have been previously described (28). In brief, immunoprecipitated cores were incubated in a total volume of 30 μl with a mixture of dCTP, dTTP, dGTP (12.5 μM, final concentration), and [α-^{32}P] dATP (1 μl of 3,000 Ci/mmol, 10 mCi/ml) in the usual buffer (without Nonidet P-40 and β-mercaptoethanol) for 2 h at 37°C. Subsequently, a chase was performed for 1 h by adding unlabeled dATP (12.5 μM, final concentration). Proteins were digested by proteinase K treatment and removed by phenol extraction. Unincorporated dNTPs were removed by gel filtration through Sephadex G-100. The material eluting in the void volume was concentrated by ethanol precipitation in the presence of 5 μg of herring sperm DNA as carrier. If desired, an aliquot of this material was further treated with avian myoblastosis virus reverse transcriptase (AMV-RT) as previously described (28). If virions were used for the endogenous polymerase reaction, Nonidet P-40 (1%) and β-mercaptoethanol (0.3%) were included in the incubation buffer to remove the envelope. The products were separated on SDS-containing 1% agarose gels and visualized by autoradiography.

Density gradient purification of cores and enveloped cores. Cell lysates were cleared by centrifugation at 12,000 × *g* for 5 min. Aliquots were removed for direct immunoprecipitation with anti-HBcAg/HBeAg antiserum; the remaining lysate was subjected to isopycnic centrifugation as described below for the medium-derived material. Cellular debris was removed from the cell culture supernatants by low-speed centrifugation. Particles were pelleted by centrifugation in an SW28 rotor for 4 h at 27,000 rpm and 4°C. The pellets were resuspended in 100 μl of phosphate-buffered saline (8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 140 mM NaCl) and then mixed with 3.8 ml of CsCl in TNE buffer (10 mM Tris-Cl, 100 mM NaCl, 0.1 mM EDTA, pH 7.5) with an initial density of 1.29 g/ml. The samples were spun at 4°C in an SW60 rotor for 6 h at 40,000 rpm and then for another 58 h at 28,000 rpm. Twelve fractions per tube were collected from the bottom and subjected to ELISA for HBV surface antigen (HBsAg), HBcAg, and HBeAg. Viral nucleic acid was detected by a dot blot procedure essentially as previously described (2). As probe, a mixture of in vitro-transcribed ^{32}P-labeled HBV RNAs which spanned nt 902 to 1280 and 283 to 1004 in positive orientation and nt 2447 to 84 in negative orientation was used.

Strand-specific hybridization of endogenous polymerase products to cloned HBV ssDNA. Equal aliquots of the endogenous polymerase products obtained from cores of the wt protein or of variant 164 were annealed as previously described (28) to excess M13mp18 or M13mp19 single-stranded DNA (ssDNA) harboring the complete HBV genome in either the positive or negative orientation. The products were separated on a 1% agarose gel and visualized by autoradiography.

Primer extensions. Core nucleic acid was isolated as previously described (22). RNA was hydrolyzed by alkali treatment at 95°C as described elsewhere (33). For the analysis of core DNA from variant 164, the nucleic acid from 5- to 10-fold more transfected cells was used than from the

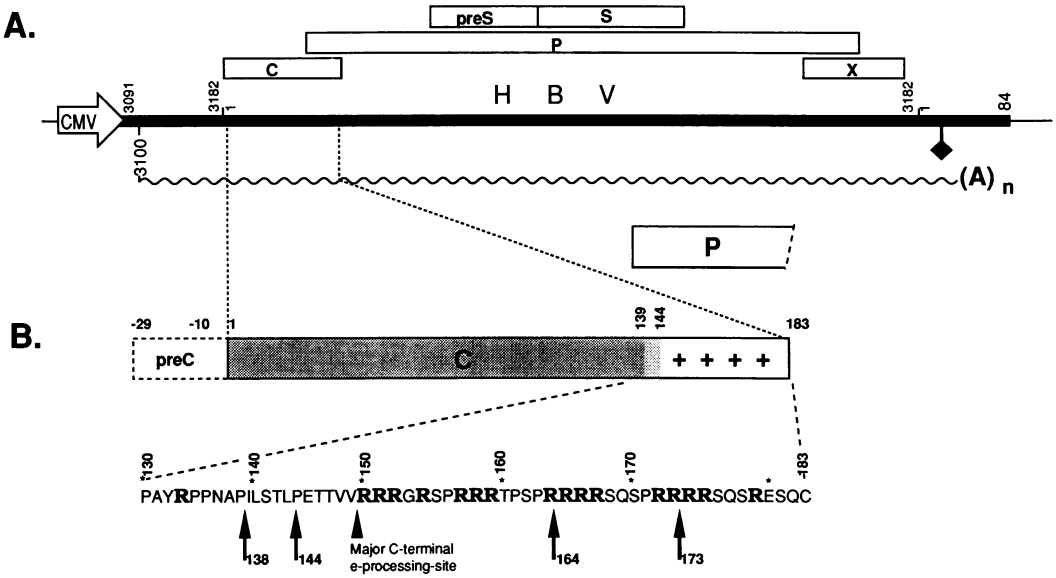

FIG. 1. Schematic view of the HBV genome and of the C-terminally truncated core protein variants used in this study. (A) General structure of the HBV expression plasmids. The boxes at the top represent the HBV genes. The thick black line represents the cloned HBV DNA consisting of a 1.1-unit-length genome. Transcription of a genomic RNA (wiggly line below) similar to or identical with the authentic pregenome is driven by the HCMV IE promoter (arrow marked CMV). The diamond depicts the HBV polyadenylation signal. The numbers are nucleotide positions; the numbering system is that of Pasek et al. (26), which starts with the initiation codon of the C gene. (B) Schematic representation of the HBV core protein. The numbers are amino acid positions. The shaded part of the closed box indicates the assembly domain; + symbolizes the clustered Arg residues. The box marked P shows the location of the N-terminal part of the P protein with respect to the core protein. The dashed box represents the pre-C region as present in the precore protein. The core amino acid sequence from position 130 to the C-terminal Cys-183 is shown below; arginines are highlighted in boldface. The arrows indicate the C-terminal borders of the truncated variants. The arrowhead indicates the major C-terminal processing site of HBeAg (36).

wt. Prior to primer annealing, the DNA was denatured by 0.2 M NaOH–1 mM EDTA at room temperature for 10 min and then precipitated by addition of 1/10 volume of 3 M sodium acetate, 1 μg of tRNA carrier, and 2.5 volumes of ethanol. The pellets were washed with 500 μl of absolute ethanol, briefly dried, and dissolved in 5 μl of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5). Annealing of the 5′-^{32}P-labeled primers and extension with AMV-RT were carried out as described elsewhere (33). The following primers were used: primer A, specific for HBV negative-strand DNA, covered nt 2978 to 2995; primers B and C, specific for positive-strand DNA, were complementary to nt 69 to 86 and 3070 to 3095, respectively. All primers were 5′ end labeled by using [γ-^{32}P]ATP and polynucleotide kinase.

RESULTS

Construction and expression of mutant HBV genomes encoding C-terminally truncated core proteins. On the basis of previous analyses using the *E. coli* expression system (3), I prepared plasmid constructs encoding core proteins ending with aa 138, 144, 164, and 173 (Fig. 1B). Variant 144, but not variant 138, had formed particles in the heterologous system; the longer variants appeared to package similar amounts of RNA as did the full-length protein. As the initiator codon of the P gene (nt 407) overlaps with codon 136 of the core protein, all of the introduced stop codons could potentially interfere with the coding information in the P gene. I therefore chose the nucleotide exchanges such that none of the amino acid residues specified by the P open reading frame was changed. The mutant C genes were introduced into plasmid pCHT-9/3091wt, which contains a slightly over-length HBV genome under control of the HCMV IE1 promoter (Fig. 1A).

A Western blot of the core-specific proteins present in lysates of HuH7 cells transfected with the appropriate constructs showed that all directed the synthesis of core proteins with the expected apparent molecular weights, except for protein 138, which was barely visible (Fig. 2A). All proteins detectable by Western blotting were also positive in an ELISA with an HBcAg-specific monoclonal antibody (3), suggesting that they formed core particles. Since the construct encoding protein 138 produced genomic RNA in similar amounts as did the other constructs (see below), this protein is presumably unable to assemble and is therefore subject to rapid degradation.

Analysis of the cell culture supernatants by ELISA and Western blotting (data not shown) revealed that they also contained core protein-related products, although the HCMV-driven constructs should not allow for the synthesis of the secretory C gene product, HBeAg (17, 25, 32). The proteins in the medium had the same apparent molecular weights as did those from the cytoplasmic lysates and therefore are likely to represent core proteins leaked out of lysed cells.

Amino acid sequences beyond position 144 of the core protein are required for efficient pregenome encapsidation. When aliquots of the total cytoplasmic RNA (15% of the lysates, containing both free and encapsidated RNAs) were analyzed by an RNase protection assay specific for the 5′ end of the pregenome, all constructs including the one encoding protein 138 gave rise to bands with a mobility expected for pregenomes with the correct start site at nt 3100

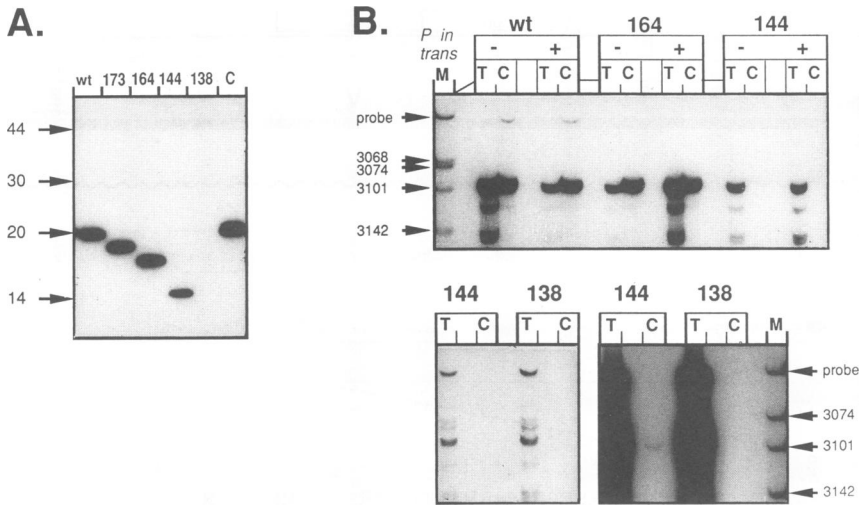

FIG. 2. Expression of truncated core protein variants and analysis of RNA pregenome encapsidation. (A) Western blot analysis of core protein variants expressed in Huh7 cells. Core proteins were immunoprecipitated from cell lysates with a polyclonal antiserum recognizing all known forms of C gene products and were detected on the blot by using the same antiserum. Individual variants are indicated above each lane. wt, core protein expressed from plasmid pCHT-9/3091wt; C, full-length core protein expressed from plasmid pCS-C1 which was used for complementation experiments. The arrows at the left show the position of marker proteins, with their relative molecular sizes given in kilodaltons. (B) RNase protection assay for the detection of encapsidated pregenomes. Individual core proteins are specified above the gels. P in trans indicates whether the transfections were performed in the absence (−) or presence (+) of plasmid pMTpol providing P protein in *trans*. Lanes T are derived from total cytoplasmic RNA; lanes C are derived from isolated core particles. M, RNA size markers obtained by hybridizing the probe RNA to in vitro-transcribed HBV sense RNAs, with the nucleotide positions of their 5′ ends indicated by the numbers at the left. probe, position of the undigested probe RNA. The lower two panels show a normal and a 10-times-overexposed autoradiograph for variants 144 and 138. Note the appearance of a signal at the position specific for the pregenome in lane 144 C but not in lane 138 C.

(Fig. 2B, lanes T): the major band in each case comigrated with a band produced by hybridizing the probe to an in vitro-synthesized RNA starting at nt 3101. Thus, the HCMV promoter drives the transcription of genomic RNA which is essentially identical to the authentic pregenome.

A different pattern was visible when the RNA contained within isolated cores was analyzed (Fig. 2B, lanes C): both the wt protein and variant 164 gave signals which corresponded to the pregenome and did not differ markedly in intensity. By contrast, no pregenome specific signal could be detected for variants 144 and 138. However, on an overexposure of the autoradiogram, a faint band of the expected size became visible for variant 144 (lower right panel of Fig. 2B).

Efficient packaging of the pregenome requires the presence of P protein (1, 12). As the pregenome is also used as mRNA for core and P protein, the early translational stop in the construct encoding variant 144 could have interfered with the translation of the downstream P cistron (5). However, the same results were obtained when the HBV genomes carrying the mutant C genes were cotransfected with a construct that supplies P protein in *trans* (Fig. 2B, lanes P in trans +). This result indicates that the low amount of genomic RNA detectable in cores from variant 144 is not due to a lack of sufficient amounts of P protein.

Together, these data confirm the requirement for the arginine-rich region of the HBV core protein for RNA encapsidation, and they show that the 19 most C-terminal residues are dispensable for this purpose.

Efficient positive-strand DNA synthesis depends on the presence of C-terminal amino acids between positions 165 and 173. To test for the replication competence of the variant cores, I used the endogenous polymerase reaction (15). Adding the four dNTPs to isolated cores enables the P protein to reverse transcribe the encapsidated RNA or to fill the gap in the positive-strand DNA. The products obtained by this assay, after immunoprecipitation of core-related products from the cytoplasmic cell lysates, are shown in Fig. 3A. The wt HBV genome produced a mixture of linear DNA and relaxed circular DNA (RC-DNA) (lane wt, −); as

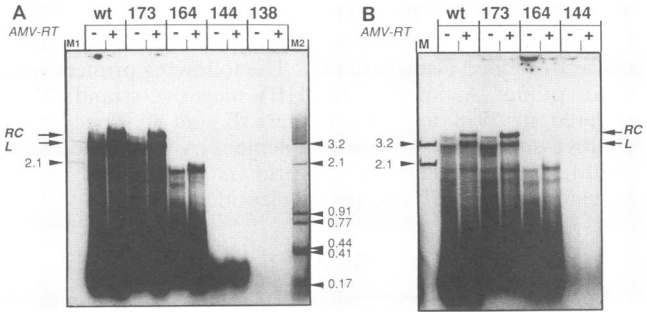

FIG. 3. Endogenous polymerase reaction products from cytoplasmic and enveloped cores. Cytoplasmic cores were immunoprecipitated from cell lysates by a core-specific antiserum; enveloped cores were isolated from the culture supernatants by immunoprecipitation with an antiserum directed against the pre-S1 region after removal of naked cores by immunoprecipitation with an anti-HBcAg antiserum. The core proteins encoded by the constructs used for transfection are specified above the gels. The endogenous polymerase reaction products were separated on 1% agarose gels and visualized by autoradiography of the dried gels. AMV-RT indicates whether the endogenous reaction was followed by a chase with exogenously added AMV-RT (+) or not (−). RC, RC-DNA; L, linear DNA. Lanes M, M1, and M2, ^{35}S-labeled marker DNAs, with their sizes given in kilobases. (A) Products from cytoplasmic cores; (B) products from medium-derived enveloped cores.

described earlier (28), AMV-RT increased the amount of RC form (lane wt, +). The assignment of the different DNA forms is based on a comparison with a linearized cloned HBV genome (lane M1). Basically the same pattern was observed for core variant 173 (lanes 173 − and +), indicating that all functions of the core protein required during replication were active in this variant; in particular, the presence of RC-DNA indicated that genome circularization had occurred which is therefore independent of the last 10 aa of the core protein.

Variants 144 and 138, expectedly, did not produce distinct bands (lanes 144 − and + and lanes 138 − and +). Variant 164, however, generated an unusual pattern: the major product was substantially higher in mobility than were the products from the wt protein. Since the mobility of this product, which did not change markedly after treatment with AMV-RT, was in the range expected for ssDNA, this result suggested that replication had been aborted before complete positive strands could be synthesized.

To test for the polarity of the endogenous polymerase products generated by variant 164, I hybridized an aliquot of the reaction products to single-stranded M13 phage into which unit-length HBV genomes had been cloned in either the positive or negative orientation. As a control, the endogenously filled-in products of cells transfected with plasmid pCHT-9/3091wt were analyzed in parallel. The radiolabeled DNA from variant 164 hybridized predominantly to the phage DNA carrying the HBV genome in the positive orientation; a signal of only about 10% the intensity (as measured by densitometric scanning of the autoradiograph) was obtained after hybridization to the phage carrying the HBV genome in the negative orientation (data not shown). For the wt construct, this ratio was about 2:1, indicating that the endogenous polymerase reaction products from cores of variant 164 contained predominantly negative-strand DNA and incorporated only 10 to 20% as much radioactivity in positive-strand DNA as did those from the wt protein. This result suggested that the major defect produced by the mutant core protein was on the level of second-strand DNA synthesis and, as seen from the RNase protection experiments described above, was not due to a defect in pregenome encapsidation.

Essentially identical results were obtained with enveloped cores isolated from the cell culture supernatants by immunoprecipitation with an antiserum directed against the pre-S1 part of the large surface antigen (Fig. 3B). Since the media contained naked cores, I first attempted to remove this material by immunoprecipitation with the polyclonal anti-HBcAg/HBeAg antiserum to ensure that nucleic acids labeled during the polymerase reaction originated indeed from enveloped particles. As a control, the medium after the anti-pre-S1 precipitation was again subjected to immunoprecipitation with the anti-HBcAg/HBeAg antiserum. When this material was analyzed by the endogenous polymerase reaction, no specific signals could be detected (data not shown).

However, in some of several additional control experiments, a second anti-HBc/HBeAg immunoprecipitation directly after the first one yielded weak but specific signals in the endogenous polymerase reaction. To exclude the possibility that the signals obtained from the anti-pre-S immunoprecipitation in the above-described assay scheme were due to a cross-reactivity of this antiserum with naked cores remaining in the medium despite the prior anti-HBc/HBeAg precipitation, I used isopycnic density gradient analysis to independently test for the formation of enveloped cores from the wt HBV genome and the mutant specifying core protein 164. Particles from the cell culture supernatants were pelleted; one half of the resuspended pellets (marked Ma [medium a] in Fig. 4) was directly mixed with a CsCl solution in TNE buffer with a density of 1.29 g/ml, the other half was mixed after immunoprecipitation with anti-HBc/HBeAg antiserum (Mb [medium b] in Fig. 4), and the preparations were centrifuged in an SW60 rotor as described in Materials and Methods. Twelve fractions were collected from each tube and analyzed for HBcAg and HBsAg by ELISA (Fig. 4A) and for viral nucleic acid by dot blotting (Fig. 4B). In the untreated samples, wt cores banded in fractions 3 to 5, and those from variant 164 were slightly shifted to lower density (fractions 4 to 7). In the samples pretreated with anti-HBc/HBeAg antiserum, no distinct core-specific peaks were observed at these positions in the gradient, indicating that the immunoprecipitation had removed the majority of the naked cores. HBsAg was detected in the top fractions 10 to 12. The dot blot revealed the presence of two separate peaks of viral nucleic acid in the untreated samples: a major peak in the leading edge of the core peak (also slightly shifted to lower density in cores from variant 164) and a second peak in the HBsAg-containing top fractions. Only the lower-density nucleic acid peak was present in the samples pretreated with anti-HBc/HBeAg antiserum. Though the signals from variant 164 were rather weak, the blot showed a clear increase in signal strength toward the upper fractions, which was well separated from the core-associated nucleic acid by fractions 6 to 8 with their minimal nucleic acid content. Since virions have a density intermediate between those of cores and S particles, these data strongly suggest that cores from variant 164 are also enveloped.

The signals in the bottom fractions (particularly evident for the variant cores) were also removed by the anti-HBc/HBeAg antiserum; since their higher density indicates an increased nucleic acid-to-core protein ratio, they are likely to represent breakdown products of cores which could have arisen during centrifugation. The higher signal from the sample containing the variant cores might be a consequence of their lower stability under the centrifugation conditions.

Taken together, these result indicate that neither the deletion of half of the poly-Arg region by itself (variants 164 and 173) nor the inability of variant 164 to support formation of a mature DNA genome interferes with envelopment. Since the further-truncated variants were negative in the endogenous polymerase assay, I was unable to more completely delineate the potential sequence requirements for envelopment in the core protein.

The replication defects of core variants 144 and 164 can be complemented by full-length core protein. To positively prove that the replication defects seen for core variants 144 and 164 were indeed the result of the C-terminal truncations, I analyzed whether the wt phenotype could be rescued by complementation with a full-length core protein. The constructs harboring the mutant HBV genomes encoding proteins 144 and 164 were cotransfected with a construct carrying only the core gene. Cores were isolated from the cell lysates as described above and again subjected to the endogenous polymerase reaction (Fig. 5). Expectedly, neither variant 144 nor the core construct by itself produced specific signals; variant 164 gave the pattern observed before. However, in the cotransfection experiments, both variants now gave rise to 3.2-kb linear DNA and RC-DNA, demonstrating that the defects were directly correlated with the mutations in the core proteins. This result also shows that the mutant HBV constructs produce packaging-compe-

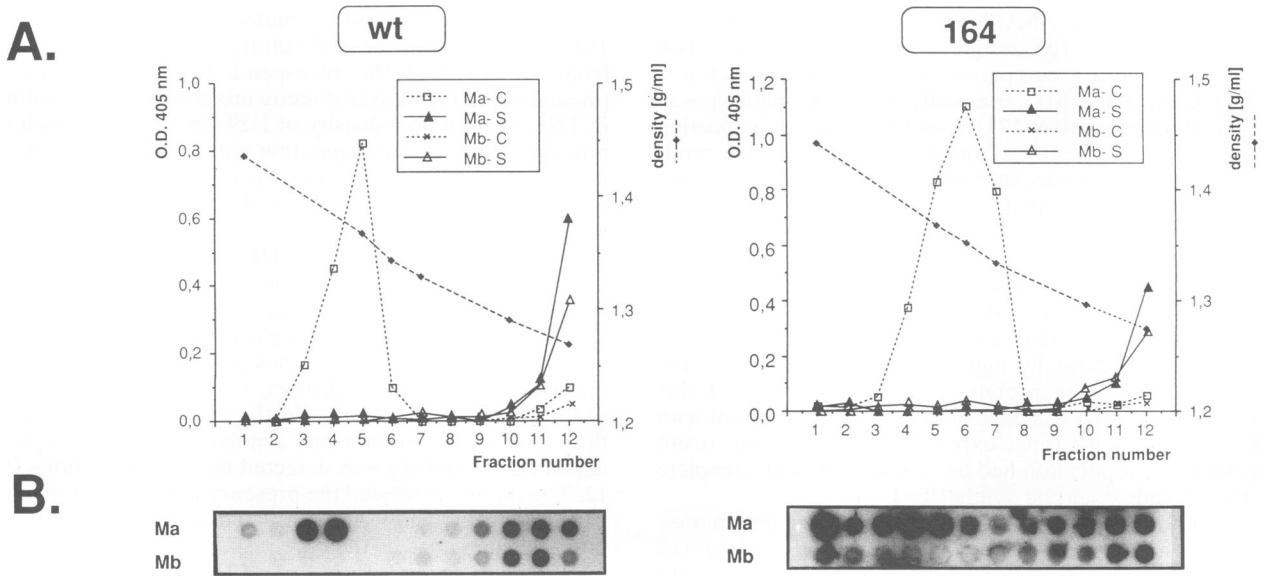

FIG. 4. Density gradient analysis of medium-derived HBV particles. Particles were pelleted from precleared supernatants of cells transfected with wt HBV DNA or the mutant encoding core protein 164; one half of the resuspended pellets was directly mixed with CsCl solution in TNE buffer (medium a [Ma]); the other half was mixed after immunoprecipitation with anti-HBc/HBeAg antiserum (medium b [Mb]). The samples were centrifuged in parallel in an SW60 rotor as described in Materials and Methods. Twelve fractions of about 350 μl were collected from each tube and analyzed for HBcAg and HBsAg by ELISA (A; the left abscissa shows the optical density (O.D.) readings in the ELISA for HBcAg [C] and HBsAg [S]). The density of individual fractions (right abscissa in panel A) was measured via the refractive index. One-fourth of each fraction was spotted onto nitrocellulose and probed with a mixture of three in vitro-transcribed ^{32}P-labeled RNAs (see Materials and Methods) (B).

tent pregenomes which can be replicated to full-length DNA genomes in the presence of intact core protein.

Negative-strand DNA synthesis in cores from variant 164 is initiated at the authentic sites, but positive-strand primer transfer to DR2 is impaired. By primer extension (Fig. 6), I analyzed whether negative- and positive-strand DNAs were initiated normally in cores from variant 164. Reverse transcription of the hepadnaviral RNA pregenome starts at the 3′ copy of the direct repeat sequence DR1 (labeled 1* in Fig. 6A), producing the first DNA strand of negative polarity. With primer A, located between DR2 and DR1*, both the DNAs from wt cores and those from variant 164 produced a series of extension products corresponding to negative-strand DNA 5′ ends around position 3109 (Fig. 6B). Hence, also in the mutant cores, negative-strand DNA synthesis starts at the expected positions (30, 37).

For positive-strand DNA synthesis, a short RNA primer derived from the 5′ end of the RNA pregenome, which carries the 5′ copy of DR1, is transferred to DR2; elongation of the primer and circularization lead to the characteristic RC form of the DNA genome (Fig. 6A). As was recently shown for the duck hepatitis B virus (DHBV) (33), a substantial fraction of the RNA primers fails to be translocated to DR2, resulting in in situ priming.

To map the 5′ ends of the positive-strand DNA and to distinguish between normal and in situ priming, I used two different primers (Fig. 6A). Primer B, located downstream of DR1, on in situ-primed positive-strand DNA will yield an extension product of about 150 nt, terminating at DR1; positive-strand DNA initiated at DR2, and elongated after circularization, will give rise to an extension product of about 390 nt, terminating at DR2. Primer C, positioned about 215 nt downstream of DR2, should detect all DR2-primed positive strands, independent of circularization.

The results of these experiments are shown in Fig. 6B. DNA from wt cores with primer B gave two distinct signals: a band with a length of about 390 nt expected for products from DR2-initiated circular positive-strand DNA molecules, and a multiplet of bands of similar intensity, with the major extension products terminating at positions 3114 and 3116, i.e., within DR1. Products extending to about position 3100 were obtained when the RNA portion of the positive-strand DNA was not hydrolyzed by prior alkali treatment at 95°C (data not shown). The result with primer C confirmed this assignment, as the major extension products terminated

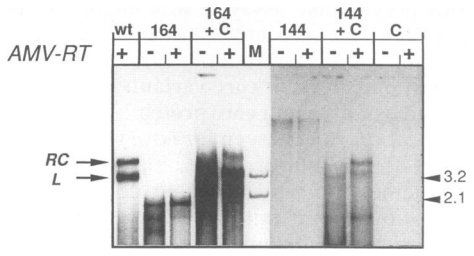

FIG. 5. Complementation of replication defects in variants 144 and 164 by wt core protein provided in *trans*. Plasmids harboring HBV genomes encoding core variants 144 and 164 were transfected either alone (lanes 164 and 144) or together with plasmid pCS-C1 harboring the C gene only (lanes 164 + C and 144 + C). As controls, pCS-C1 (lane C) alone and a corresponding plasmid carrying the wt HBV genome (lane wt) were used. Cores isolated from cell lysates were analyzed by using the endogenous polymerase reaction and analyzed directly (lanes AMV-RT −) or after a chase with exogenously added AMV-RT (lanes AMV-RT +). The arrowheads on the left show the sizes, in kilobases, of the marker DNA (lane M). RC, relaxed circular DNA; L, linear DNA.

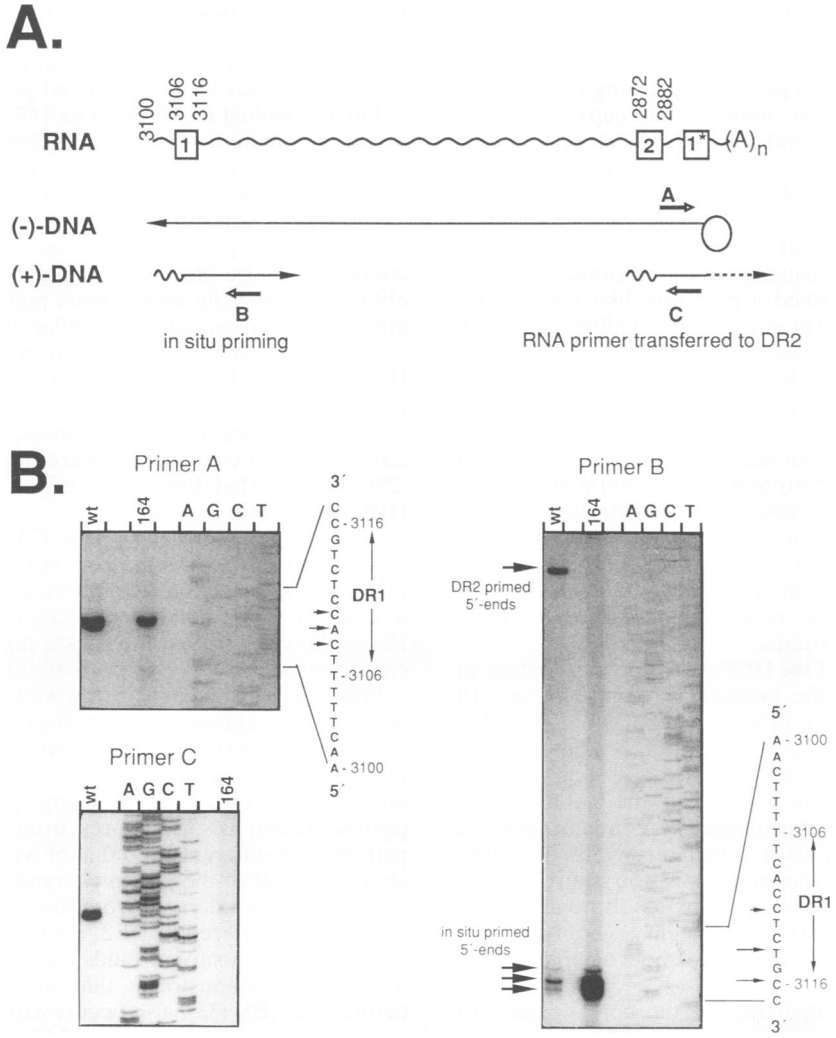

FIG. 6. Mapping of the 5′ ends of negative- and positive-strand DNA present in cores from variant 164 by primer extension. (A) Schematic view of HBV replication. The terminally redundant RNA pregenome carries a 5′ and a 3′ copy of DR1 (boxes marked 1 and 1*) and DR2 (box marked 2). Negative-strand DNA [(−)-DNA] initiates, presumably by protein priming via the terminal protein (open sphere; see reference 2), at the 3′ copy of DR1. Positive-strand DNA [(+)-DNA] synthesis is primed by short RNAs (wiggly lines at 5′ ends of positive-strand DNA) derived from the 5′ end of the pregenome. Transfer of the RNA primer to DR2 yields, after circularization, RC-DNA. In situ priming results from extension of RNA primers not transferred to DR2. The arrows marked A, B, and C symbolize the primers used for 5′-end mapping. Primer A spanned nt 2978 to 2995, primer B was complementary to positions 69 to 86, and primer C was complementary to positions 3070 to 3095. Primer B will, after circularization, also detect positive-strand DNA initiated at DR2. (B) Primer extension products obtained with DNA from cores from wt core protein and variant 164. Primer extensions were performed with 5′-end labeled primers A, B, and C as indicated, and the products were run in parallel with sequencing ladders generated using the same primers on a plasmid containing a cloned HBV genome (lanes A, G, C, and T). For primers A and B, the sequence around DR1 is shown at the right of each gel. For simplicity, the complementary sequence is given for primer B. Because of the limited resolution of the gels, the major start sites indicated by the arrows may vary by 1 nt. The major start site detected by primer C was around nt 2882, at the 3′ end of DR2. Five- to tenfold more core DNA was used from variant 164 than from the wt cores.

around the 3′ end of DR2. Thus, HBV wt cores contain a mixture of positive-strand DNA initiated correctly at DR2, together with similar amounts of DNA arising from in situ priming at DR1.

In marked contrast, nucleic acid from the variant cores gave basically only a signal for in situ-primed positive strands, with a pattern very similar to that for DNA from wt cores. Only very faint signals were visible in the region corresponding to positive-strand DNA starting at DR2. The same result was obtained with primer C. Hence, in cores from variant 164, positive-strand DNA arises almost exclusively from in situ priming, suggesting that a major component of the replication defect in this variant is the failure to initiate positive-strand synthesis at DR2 and, as a consequence, to produce RC-DNA. However, even in situ-primed positive strands should be extended during the endogenous polymerase reaction, yielding linear 3.2-kb DNA molecules, in contrast to my observation. This suggests that a second major aspect of the defect is that the in situ-primed positive strands are not properly elongated.

DISCUSSION

Viral capsid proteins are primarily designed to provide a protective shell for the virus genome, escorting it out of one host cell into another; this implies that capsid proteins exhibit affinity for nucleic acid. For the HBV core protein, data previously obtained by expressing variants of the protein in *E. coli* (3, 8, 31) had shown that the C-terminal 39 aa are not required to make the protein assembly competent. This C-terminal sequence, which forms a structurally independent entity (24), is unusually rich in arginine residues; therefore, it has been termed a protaminelike region (26), implying that it is involved in nucleic acid binding. This view was confirmed by the expression studies in *E. coli*, in which the nucleic acid content of the particles, and concomitantly their stability, correlated with the presence of at least part of the arginine-rich region (3, 8). However, only limited conclusions can be derived from such heterologous systems in the absence of the viral P protein and the authentic pregenome, which are both essential components for the assembly of replication-competent cores (1, 12, 14). In this study, therefore, I transiently expressed in animal cells complete HBV genomes encoding truncated core proteins to examine the role of the protaminelike region on virus replication and formation of enveloped virions.

The C-terminal region of the HBV core protein including aa 164 is sufficient for specific pregenome encapsidation. All C-terminally truncated core proteins except for variant 138 were able to assemble into particles, in agreement with the data obtained from the *E. coli* expression system. Variant 138 did not detectably accumulate in the animal cells, although similar amounts of genomic RNA and core mRNA were produced as in the cells transfected with the other constructs. Presumably, the protein is too short to fold properly and is rapidly degraded. This result confirms our previous mapping of the HBV core protein assembly domain to the sequence from the N terminus to about aa position 144 (3).

An analysis of the competence of the core variants to encapsidate the RNA pregenome by an RNase protection assay showed that particles from variant 164 encapsidated similar amounts of the pregenome as did those formed by the full-length protein. However, RNA from cores of variant 144 gave no specific band on normally exposed autoradiograms, suggesting that these capsids harbor no or drastically less genomic RNA than do cores from variants containing at least half of the Arg-rich C terminus. These results correlate well with our previous data on RNA packaging obtained from expressing the variant proteins in *E. coli*. Thus, the presence, in the eukaryotic system, of P protein and the genomic RNA carrying the encapsidation signal did not enhance the amount of RNA detectable in the variant cores.

On overexposed films, however, a faint band corresponding in mobility to that expected for the pregenome could be visualized for variant 144, which might imply that the particles contain small amounts of genomic RNA, similar to the low amounts of nucleic acid present in particles from this variant produced in *E. coli* (3). I cannot rule out, however, the possibility that the faint signal originates from contaminating, i.e., nonencapsidated, genomic RNA. Obviously, in view of these results and previously reported, controversial in vitro data (8, 18, 27), the question of whether variant 144 retains a basal nucleic acid binding activity, i.e., a nucleic acid binding site independent of the poly-Arg region, needs further investigation.

In summary, these results confirm that the arginine-rich region in the core protein is essential for proper interaction with nucleic acid, but only half of the C-terminal region is sufficient for specific encapsidation of the pregenome with an efficiency similar to that of the wt protein.

The C-terminal region from aa 165 to 173 of the HBV core protein is required for proper replication. All core proteins that efficiently encapsidated the genomic RNA also contained P protein, as detected by its DNA polymerase activity, in accord with previously published data which demonstrated that P protein and pregenome encapsidation are coupled (1, 12). However, the patterns of labeled DNA obtained during the endogenous polymerase reaction were different: from variant 173, similar amounts of linear DNA and RC-DNA were produced as from the full-length protein. Hence, the last 10 aa of the core protein are not required for proper viral DNA synthesis. The observation that DHBV virions containing replication-competent cores from a similarly truncated core protein were apparently not infectious (29) suggests that this region might be important also for HBV infectivity.

In marked contrast to variant 173, the endogenous polymerase products obtained from cores of variant 164 exhibited a drastic reduction in the ratio of labeled positive strands to negative strands, suggesting that the absence of aa 165 to 173 in the core protein imposes a replication defect preferentially on the level of second-strand DNA synthesis.

Primer extension analyses showed that this reduction was not due to improper, i.e., nonspecific, priming of DNA synthesis. First-strand DNA synthesis in the variant cores, as in the wt, occurred at the 3′ copy of DR1, in accord with previous data (30, 37). The mapping of the 5′ ends of the positive-strand DNA of cores from variant 164 showed a pattern very different from that of wt particles. In the latter, about one half of the positive strands initiated at DR2; i.e., the RNA primer derived from the 5′ end of the pregenome had been transferred to DR2, eventually yielding RC-DNA. The remaining positive strands started at the 5′ copy of DR1. This result demonstrates that in situ priming, observed before in DHBV (33), also occurs with HBV. However, with core nucleic acid from variant 164, almost exclusively signals from in situ-primed positive strands were detected. Thus, in these cores the RNA primer remains at DR1. Therefore, primer transfer to DR2, a prerequisite for the formation of RC-DNA and hence replication-competent cores, is drastically impaired in cores lacking the sequence between aa 164 and 173, suggesting that these residues are involved in preparing the negative-strand template for the primer transfer reaction.

On the other hand, the trimming of the genomic RNA to yield the positive-strand primer appears to work properly in cores of variant 164, since the initiation sites are basically the same as those observed for in situ-primed positive strands from wt cores. The markedly reduced incorporation of radioactivity into positive-strand DNA during the endogenous polymerase reaction is therefore likely to be the result of an impaired chain elongation rather than incorrect initiation, suggesting that the core protein's C terminus is required for efficient positive-strand extension. An alternative explanation could have been that primer transfer to DR2 occurred efficiently, but circularization would depend on core sequences beyond aa 164. Then, DR2-initiated positive strands could be extended only to the 5′ end of the negative strand, generating a short strong-stop positive-strand DNA. Such molecules, however, if present in substantial amounts, would have produced a much stronger signal in the primer extension with primer C than was observed.

An issue which is currently unclear is why the in vitro reaction with AMV-RT does not, at least not under the standard conditions used, produce full-length linear double-stranded DNA (dsDNA) from the endogenous polymerase products of variant 164. Either most of the positive-strand primer is lost during the workup procedure or, more likely, the enzyme is unable to read through secondary structures on the negative strand. In wt but not in the variant cores, these regions would already have been copied by the HBV P protein during the endogenous polymerase reaction. A variation in the reaction conditions or the use of a different polymerase enzyme might help to clarify this point. Since all other results are consistent with the presence of the 5′ and 3′ sequences essential for replication of the RNA pregenome, the data presented above suggest, in summary, that the first half of the core protein's C terminus (to aa 164) is required and sufficient for RNA encapsidation, that the amino acids from positions 163 to 173 act as an auxiliary component in replication, in particular during second DNA strand synthesis, and that the extreme C terminus might be important only during or following infection.

In my view, two mechanisms could conceivably account for the apparent involvement of the core protein in hepadnavirus replication. First, the core protein may initially help to package the genomic RNA and subsequently, during replication, condense the largely double-stranded mature DNA genome into a more compact form compatible with the restricted volume inside the viral capsid. In this process, the C-terminal arginine-rich domain might act like histones in chromatin. The SPRRR motif present in three copies in the poly-Arg region does indeed occur in histones (7, 11, 35). As the RNA pregenome is presumably degraded concomitantly with negative-strand DNA synthesis, no full-length duplex nucleic acid is initially produced during reverse transcription, and first-strand DNA synthesis can proceed normally even in the absence of the condensing function of the core protein. By contrast, positive-strand synthesis produces dsDNA which, if not condensed, would use up too much space inside the particle and restrict the ability of the P protein to move along its template. Hence, in cores from variant 164, positive-strand synthesis would cease after a certain average length of dsDNA had been produced. A speculative extension of this concept would lead to a model for uncoating: if the dsDNA genome fits only into the core since it is condensed via the interaction with the poly-Arg region, then an inhibition of this interaction, e.g., by serine phosphorylation, might contribute to efficient uncoating. It has been experimentally shown that phosphorylation of the serine residues in the S-P-X-K/R motifs present in certain histones does indeed modulate their interaction with DNA (11), and recently, a decreased affinity for DNA of phosphorylated core protein has been demonstrated by in vitro binding experiments (16). Alternatively, and not mutually exclusively, the arginine-rich region could be more actively involved in replication in a way similar to that for ssDNA-binding proteins, e.g., phage T4 gene 32 protein, *E. coli* SSB protein, or adenovirus DBP (6). These proteins are essential for replication, in that they prevent the formation of DNA structures inhibitory to the progressing DNA polymerase, or they directly stimulate their cognate polymerase by protein-protein interactions. In the case of the HBV capsid, the cooperativity usually required for the stimulatory activity of these binding proteins would be naturally given by the fact that HBV DNA synthesis takes place inside complete capsids, providing 180 repeats of the C-terminal poly-Arg regions. An in vitro replication system consisting of genomic RNA and isolated P and core protein would be useful to distinguish between these possibilities.

HBV nucleocapsid envelopment is independent of the core protein sequence beyond aa 164 and independent of the presence of a mature DNA genome. All core protein variants that scored positive for DNA synthesis in the endogenous polymerase assay when the intracellular cores were analyzed were also positive with the medium-derived material, suggesting that all of these variant cores were enveloped. While several control experiments showed that the immunoprecipitation scheme used to isolate virions did indeed remove the vast majority of naked cores from the media, a slight contamination with some cores remaining in the media after the first anti-HBc/HBeAg immunoprecipitation could not be completely excluded. Given a cross-reactivity with core particles of the anti-pre-S antiserum used to precipitate envelope-associated cores, the rather weak signals in the endogenous polymerase assays from the media (usually, the ratio between radioactivity incorporated into high-molecular-weight DNA in anti-pre-S-precipitated material from the media to that in cytoplasmic cores was in the range of 1%, both for the wt and for the variants) could have arisen from contaminating naked cores. However, density gradient analysis provided independent evidence that cores from variant 164 were indeed enveloped.

Hence, the extreme C terminus of the HBV core protein is itself not essential for virion formation. Furthermore, variant 164 was obviously unable to produce mature genomes, and yet it was enveloped.

Yu and Summers (38) recently reported a similar analysis of the effects of C-terminal deletions in the DHBV core protein on the replication and envelopment of this hepadnavirus, which is relatively distantly related to HBV. In particular, the core proteins of both viruses differ markedly, not only in length (183 aa for the HBV core protein and 261 aa for the DHBV core protein) but also in their C-terminal sequences. The C terminus of the DHBV protein is also highly basic, but the corresponding amino acid residues are not arranged in similar clusters. Despite these differences, and in accord with an earlier report by Schlicht et al. (29), the replication phenotypes observed for mutant DHBV core proteins were similar to my results. Deletion of only a few amino acids resulted in normal negative- and positive-strand DNA synthesis, whereas further truncations yielded complete negative strands, but positive-strand elongation was progressively inhibited. Hence, the C-terminal regions of the HBV and DHBV core proteins seem to achieve the same function by using a substantially different primary sequence.

However, for DHBV a rather strict correlation between genome maturation and envelopment has been observed (38), and it has been hypothesized that a packaging or genome maturation signal transduced to the capsid surface would be required for efficient envelopment (34). The results reported above do not support this model for HBV, and independent evidence that a mature DNA genome is not required for HBV capsid envelopment was recently obtained by the direct detection of enveloped core protein in transfection experiments with constructs lacking a functional P gene or genomic RNA (4), which therefore cannot produce mature DNA. Whether this difference indicates that such a signal does exist in DHBV, but not in HBV, or whether the different results reflect the fact that the determinants for the interaction between capsid and envelope reside in different parts of the primary sequences of the HBV and DHBV core protein remains to be established.

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