JOURNAL OF VIROLOGY, Mar. 1989, p. 1371–1376
0022-538X/89/031371-06$02.00/0

Vol. 63, No. 3

Characterization of the Major Duck Hepatitis B Virus Core Particle Protein

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Received 8 July 1988/Accepted 5 October 1988

The amino acid composition of the major duck hepatitis B virus (DHBV) core particle proteins was determined. The results of this analysis indicated that cores are composed of a single major protein that initiates translation from the second available AUG in the DHBV core gene. Proteins isolated from core particles purified from the cytoplasm of DHBV-infected duck hepatocytes exhibited heterogeneity in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, independent of the stage of viral DNA maturation. Incubation of native cores with alkaline phosphatase removed this heterogeneity, indicating that phosphorylation of external amino acids was responsible. Core protein isolated from mature DHBV purified from serum of infected animals did not display heterogeneity, suggesting a possible role for dephosphorylation in virus maturation.

Hepadnaviruses are composed of a nucleocapsid or core particle surrounded by a lipid membrane containing the viral envelope proteins. The core particle contains the viral DNA, a DNA polymerase, a protein kinase activity, and a protein covalently attached to the 5′ end of the DNA (6, 10). Core particles free of the viral envelope can be found in the cytoplasm of infected hepatocytes, and it is within these immature cores that the viral DNA is replicated, by a mechanism that involves reverse transcription of a viral RNA pregenome (20). A consequence of this replication process is that cores isolated from the cytoplasm of infected cells contain viral DNA at various stages of maturation. However, mature virus particles isolated from the serum of infected individuals contain only relatively complete viral DNA molecules. The mechanism by which core particles are packaged within the lipid envelope to produce complete virus is poorly understood. Summers and Mason (20) proposed in their description of the duck hepatitis B virus (DHBV) replication pathway that cores containing a mature viral genome might display some signal that targets them for packaging and export from the cell as mature virus. Such a signal may be expressed as a modification of the core particle protein. In this study, we attempted to characterize the major core particle protein of DHBV in both intracellular particles and mature virus, and we present evidence that there is a distinct difference in the nature of the core particle protein in these particles.

The nucleotide sequences of core antigen (cAg) genes from several cloned hepadnavirus genomes are now known (6, 9, 18). In each example, the cAg-coding sequence contains two in-phase initiation codons separated by a so-called precore region. This region encodes several hydrophobic amino acids and has recently been shown for hepatitis B virus (HBV) (13, 22) and DHBV (16) to function as a signal peptide in producing a secreted, soluble form of core protein referred to as e antigen (eAg). At the outset of the present study, it was thought that one role of a precore-core protein in hepadnavirus replication might be in targeting cores to the endoplasmic reticulum for packaging (13, 22). That DHBV cores might be composed of a mixture of precore-core and core proteins was suggested by the heterogeneity in migration of the major DHBcAg polypeptides observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Recently, studies with DHBV have shown that a frameshift mutation in the precore region destroys the ability to produce DHBeAg but does not destroy viability. These studies indicate that a precore-core gene product is not essential for virus replication and is thus not likely to constitute a major component of viral core particle protein. The present study lends biochemical support to the genetic analyses described above in that we demonstrated by amino acid analysis that the major polypeptides of DHBV core particles do not contain the precore domain.

MATERIALS AND METHODS

Purification of core particle protein. Core particles were partially purified from approximately 30 g of liver isolated from 3-week-old ducks congenitally infected with DHBV. The procedure has been described elsewhere (12), except that thiodiglycol (0.5%, vol/vol) was added to all buffers as an antioxidant. Core polypeptides were purified by reversed-phase high-pressure liquid chromatography (HPLC) on a column (10 by 250 mm) of Bakerbond WP-Octyl 5-μm resin (J. T. Baker Research Products, Phillipsburg, N.J.). Partially purified cores were solubilized by heating at 95°C for 5 min in SDS-PAGE sample buffer (1% [wt/vol] SDS, 20 mM Tris hydrochloride [pH 8.8], 10 mM EDTA, 5 mM dithiothreitol), injected in 44% acetonitrile (HPLC grade; Baker)–0.1% trifluoroacetic acid (Pierce Chemical Co., Rockford, Ill.), and eluted with a linear gradient to 56% acetonitrile–0.1% trifluoroacetic acid in 34 min at a flow rate of 3.3 ml/min. The main core polypeptide peak eluted at 48% acetonitrile. Appropriate fractions were collected and concentrated in a Speed Vac vacuum centrifuge (Savant Instruments, Inc., Farmingdale, N.Y.).

Amino acid analysis. Amino acid compositions were determined by using the PICOTAG method and instrumentation (Waters Chromatography, Div. Millipore Corp., Milford, Mass.) with minor modifications. Protein samples were dried

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from 1% acetic acid and hydrolyzed in batches together with amino acid and protein standards in the same reaction vial containing a small amount of constant boiling hydrochloric acid and 1 drop of phenol. After being flushed thoroughly with nitrogen, the vial was sealed under vacuum and incubated at 110°C for 17 to 18 h. For determination of cysteine as cysteic acid and methionine as the sulfone, the protein samples were oxidized with fresh performic acid (3% H_2O_2 in 80% formic acid) for 1 h at room temperature before hydrolysis. The amino acids were derivatized with phenylisothiocyanate and resolved by reversed-phase HPLC. The amount of each amino acid was calculated relative to the cohydrolyzed standards and is therefore corrected for hydrolytic losses of serine and threonine.

Alkaline phosphatase treatment of core particles. Partially purified core particles were stored at −70°C in HCB_2 buffer (50 mM NaCl, 20 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 0.1% [vol/vol] 2-mercaptoethanol, 0.1% [vol/vol] Triton X-100). Samples were incubated at 30°C with alkaline phosphatase (4 U/ml) (type III-N from *Escherichia coli*; Sigma Chemical Co., St. Louis, Mo.) in 50 mM Tris hydrochloride (pH 7.5)–5 mM dithiothreitol–10 mM $MgCl_2$–100 mM Na_2SO_4 (11). Aprotinin (20 μg/ml) and leupeptin (10 μg/ml) (Sigma) were added as protease inhibitors. Reactions were stopped by addition of an equal volume of SDS-PAGE sample buffer (8) and heating at 95°C for 5 min. Samples were analyzed on 10% (wt/vol) SDS-polyacrylamide gels. Gels were either stained directly (23) or proteins were transferred electrophoretically to a nitrocellulose membrane and incubated with rabbit antiserum to DHBcAg (provided by W. Mason) to detect core particle polypeptides by standard Western immunoblot procedures (17).

Fractionation of viral particles and analysis of viral protein and nucleic acid. DHBV particles were pelleted from sera isolated from congenitally infected ducklings and fractionated on 15 to 55% (wt/vol) sucrose gradients as previously described (14). Fractions containing DHBV were analyzed for viral cAg by SDS-PAGE and Western blot procedures (17). Nucleic acids were released from viral particles by incubation with lysis buffer (0.5 mg of pronase per ml, 20 mM Tris hydrochloride [pH 7.6], 10 mM EDTA, 0.15 M NaCl, 0.2% [wt/vol] SDS) for 1 h at 37°C. DHBV DNA was resolved by agarose gel electrophoresis and detected by Southern blot hybridization procedures as previously described (21).

Isopycnic centrifugation in cesium chloride was used to fractionate intracellular core particles. Solid CsCl was added to partially purified cores diluted in 2.5 ml of HCB_2 buffer to yield a solution with a density of 1.35 g/ml. The sample was overlaid with silicone oil and centrifuged to equilibrium in an SW56 Beckman rotor at 45,000 rpm for 24 h at 4°C. Fractions containing core particles were analyzed for DHBcAg protein by SDS-PAGE and silver staining of the gel (23). DNA contained within core particles was isolated and assayed as described above. Autoradiography was performed at −80°C with Kodak X-OMAT AR film and an intensifying screen.

RESULTS

Examination of the nucleotide sequence of the DHBcAg gene (9, 18) allowed us to search for differences between the predicted amino acid composition of proteins initiating translation from either the first or second AUG in the core open reading frame. This revealed that the precore region of the DHBcAg gene, with a coding potential for 42 amino acids, includes four of a total of five cysteines predicted for a protein containing all 305 amino acids encoded by the precore-core gene. Thus, to investigate whether intracellular core particles contain a significant amount of a protein that includes the precore region, we determined the amino acid composition of purified core particle protein.

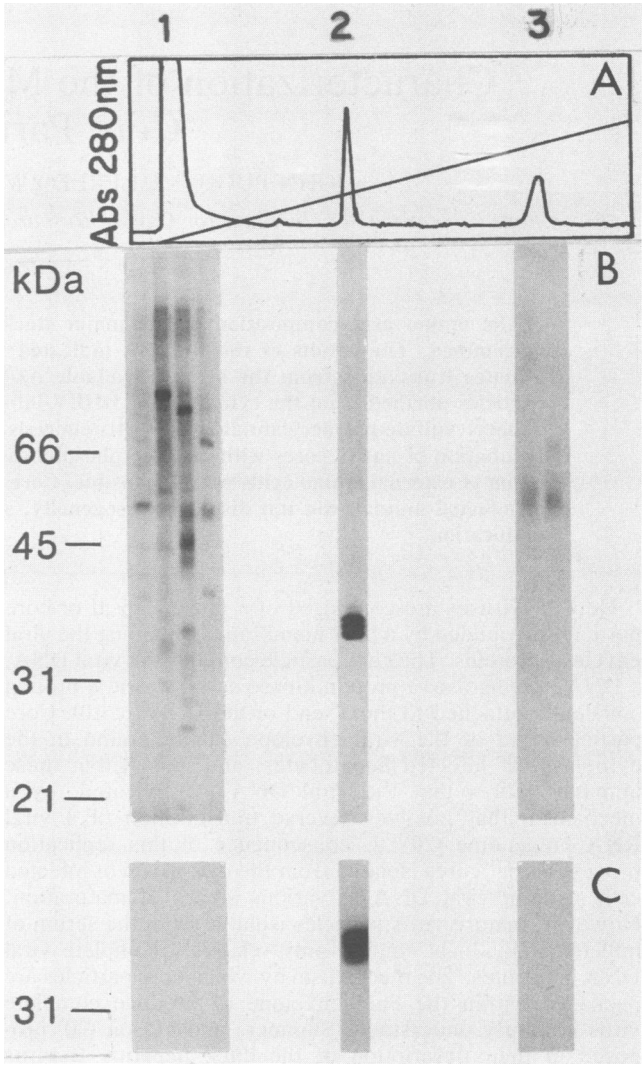

FIG. 1. Purification of DHBcAg protein by preparative HPLC. Partially purified core particle proteins were resolved by preparative HPLC (see Materials and Methods). The three peak protein fractions as assayed by A_{280} are shown in panel A. Proteins from each of the peak fractions were analyzed on 10% SDS-polyacrylamide gels and either silver stained (B) or transferred to nitrocellulose and incubated with a rabbit antiserum to DHBcAg proteins followed by ^{125}I-protein A (C). The position and size (kilodaltons [kDa]) of molecular size markers (Bio-Rad Laboratories, Richmond, Calif.) are shown on the left.

Purification of the major DHBcAg polypeptide. Core particles partially purified from the livers of ducklings congenitally infected with DHBV were heated at 95°C for 5 min in SDS-PAGE sample buffer (without bromophenol blue), and core particle protein was purified by preparative HPLC (see Materials and Methods). Peak protein fractions were detected by A_{280} (Fig. 1A), and proteins in each peak were analyzed on 10% (wt/vol) SDS-polyacrylamide gels. Proteins in the gel were either stained directly (23) (Fig. 1B) or transferred to nitrocellulose for immunoblotting to detect

TABLE 1. Amino acid composition of purified DHBV core particle polypeptides

Amino acid	mol%			
	1st AUG[a]	2nd AUG[a]	Exptl	±SD
Cys	1.6	0.4	0.4	0.05
Lys	4.9	5.7	5.7	0.82
Arg	9.2	10.3	12.4	1.18
His	3.3	3.8	4.2	0.27
Asx[b]	7.2	7.6	7.1	0.86
Glx[c]	8.6	9.9	10.0	1.69
Pro	7.9	8.4	8.0	0.73
Gly	4.3	4.2	4.6	1.13
Ser	6.9	6.5	6.6	0.51
Thr	8.5	8.0	7.6	0.30
Ala	7.2	7.6	7.7	0.60
Met	1.0	0.8	0.8	0.17
Leu	8.9	7.6	7.6	0.61
Ile	6.6	6.5	6.3	0.61
Val	5.3	4.6	4.8	0.67
Phe	2.6	2.3	2.3	0.28
Tyr	3.6	3.4	3.8	0.44
Trp[d]	2.3	2.3	ND	

[a] These values are those predicted from the nucleotide sequence of the DHBV core gene.
[b] Asx represents asparagine plus aspartic acid.
[c] Glx represents glutamine plus glutamic acid.
[d] Tryptophan residues were not determined (ND).

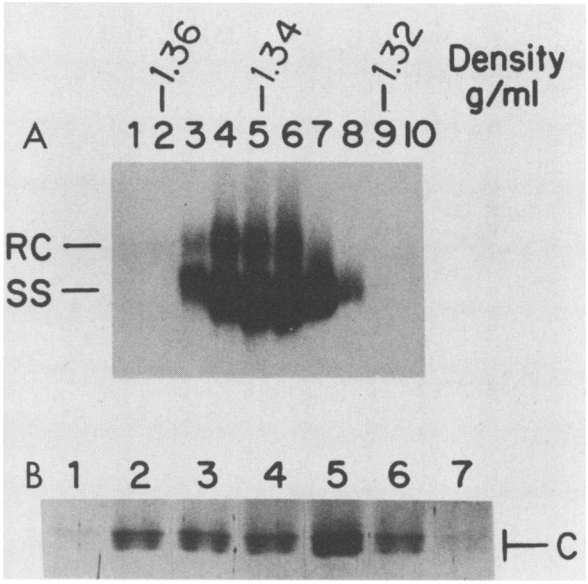

FIG. 2. Core particles were centrifuged to equilibrium in cesium chloride (see Materials and Methods). A sample (5 μl) from each 200-μl fraction was digested in SDS-pronase buffer to release viral DNA which was analyzed by Southern blot hybridization (A). Core particles were pelleted from each fraction, and DHBcAg proteins resolved on a 10% SDS-polyacrylamide gel were detected by silver staining (see Materials and Methods) (B). RC and SS, Relaxed circular and single-stranded DHBV DNA molecules, respectively. C, Position of DHBcAg polypeptide. The numbers above each lane refer to corresponding fractions from the gradient.

those fractions containing core protein (Fig. 1C). This analysis revealed that all the detectable DHBcAg protein eluted with 48% (vol/vol) acetonitrile (Fig. 1A, peak 2) and that this preparation was essentially free from contamination with other polypeptides. The core protein eluted in peak 2 clearly exhibited heterogeneity in SDS-PAGE, with at least three distinct bands detected by both staining and Western blot procedures (Fig. 1B and C, respectively). Proteins removed in the flowthrough fractions (Fig. 1A, peak 1) and in peak 3 (Fig. 1A) were not detected by the antiserum and most likely represent contaminating nonviral proteins. Core protein from peak 2 (Fig. 1A) was used for subsequent amino acid analysis.

Amino acid analysis of DHBV core particle protein. Amino acid analysis of protein from peak 2 (Fig. 1) was performed as described in Materials and Methods. The relative abundance of each residue, expressed as moles percent, is shown in Table 1. These are the mean values determined from seven independent analyses; the standard deviation is shown adjacent to each mean value. The predicted abundance of residues present in proteins encoded by the region extending from either the first or second AUG through to the end of the core coding region is also presented in Table 1. The relative abundance of cysteine residues in the core particle polypeptides suggests that the major protein initiates translation from the second AUG in the precore-core-coding region. If core particles were composed of a mixture of precore-core and core proteins, we would predict that the relative abundance of cysteine residues determined in our analysis would be intermediate between 0.4 and 1.6. For example, if cores contained a mixture of precore-core and core proteins in a 1:4 ratio, we would expect the relative abundance of cysteine residues to be 0.64%. The variation from the mean value for cysteine allows us to conclude that if a precore-core polypeptide constitutes more than about 5% of the total core particle protein we would be able to detect it by our assay procedure. Our results suggest that DHBV core particles are composed of a single major protein and that a precore-core protein is either absent or constitutes less than 5% of the total core particle polypeptides.

DHBcAg protein heterogeneity is associated with all immature core particles. Hepadnavirus-infected hepatocytes contain immature viral core particles containing viral DNA at every stage of replication.The difference in the DNA content of these particles allows them to be separated owing to their slightly different densities in cesium chloride isopycnic gradients (J. Newbold, personal communication). Thus, DHBV cores containing exclusively single-stranded minus-strand DNA (Fig. 2A, lane 7) can be separated from those containing full-length relaxed circular DNA (Fig. 2A, lane 2). To determine whether heterogeneity of core particle protein was related to the degree of maturation of viral DNA, we analyzed protein isolated from cores fractionated by isopycnic centrifugation in cesium chloride (Fig. 2) (see Materials and Methods). Cores were pelleted from each fraction and suspended in SDS-PAGE sample buffer. After denaturation at 95°C for 5 min, proteins were resolved on a 10% (wt/vol) SDS-polyacrylamide gel and silver stained (Fig. 2). The results indicate that core particle protein displayed heterogeneity in SDS-PAGE independent of the stage of viral DNA maturation. Thus, heterogeneity is a characteristic feature of the major structural protein of all DNA-containing core particles isolated from the cytoplasm of DHBV-infected hepatocytes.

Heterogeneity of core particle protein is removed by phosphatase treatment. Since we were aware that HBV core

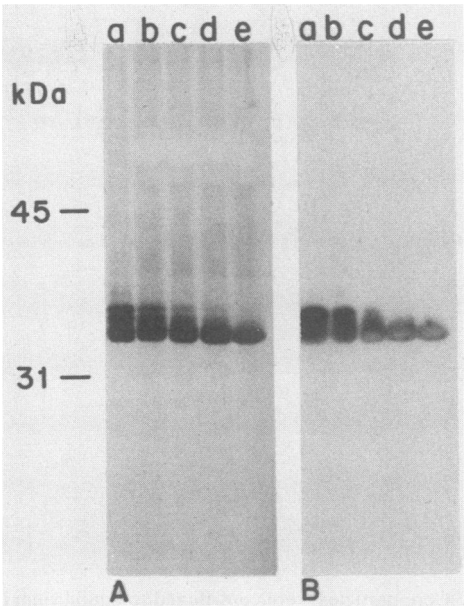

FIG. 3. Alkaline phosphatase treatment of native core particles. DHBcAg particles were incubated with bacterial alkaline phosphatase at 30°C; samples were removed after 0 (lane a), 0.5 (lane b), 1 (lane c), 3 (lane d), and 18 (lane e) h and added directly to SDS-PAGE sample buffer at 95°C for 3 min. Core proteins were resolved on a 10% (wt/vol) SDS-polyacrylamide gel and analyzed by silver staining (A) or Western blot (B) procedures. The positions of molecular size markers are shown on the left in kilodaltons (kDa).

particles exhibit an endogenous protein kinase activity (1) and can be labeled with $^{32}P_i$ in cultured cells (15), we investigated the possibility that phosphorylation was responsible for the observed heterogeneity. Native core particles purified from DHBV-infected liver were incubated with alkaline phosphatase for varying intervals, after which the reaction was terminated by boiling the sample in an equal volume of SDS-PAGE sample buffer (see Materials and Methods). Core particle protein was resolved on a 10% (wt/vol) SDS-polyacrylamide gel and detected either by silver staining (Fig. 3A) or by Western blot procedures (Fig. 3B) with a rabbit antiserum against DHBV core particle proteins. The results in Fig. 3 indicate that prolonged incubation of native cores with alkaline phosphatase resulted in complete removal of heterogeneity, with conversion of the multiple core polypeptide bands into a single species that comigrated with the fastest migrating of the undigested core protein bands. Cores incubated under identical conditions in the absence of alkaline phosphatase retained heterogeneity of the core polypeptides (data not shown). It appears that the phosphates responsible for the observed heterogeneity are located on the exterior of the particles, as the heterogeneity was removed following phosphatase treatment of native cores. Samples were negatively stained with uranyl acetate and examined by electron microscopy both before and after digestion (Fig. 4A and B, respectively). No evidence of damage to particle integrity was observed after a 10-fold, overdigestion of cores.

Characterization of core protein in mature DHBV particles. The results presented in Fig. 2 indicate that the phosphates responsible for heterogeneity of the intracellular core protein in SDS-PAGE are not correlated with the maturity of the viral DNA in the cores. To determine whether core particle protein in mature extracellular virus also displays heterogeneity, we partially purified DHBV from the serum of congenitally infected animals by isopycnic centrifugation in sucrose gradients (see Materials and Methods), and viral core protein was detected in individual fractions by Western blotting (Fig. 5A). The distribution of viral DNA in the gradient is shown in Fig. 5B, indicating that the peak viral fractions correspond to a particle density of approximately 1.16 g of sucrose per ml. The results in Fig. 5A indicate that core particle protein in mature extracellular DHBV does not exhibit heterogeneity in SDS-PAGE (Fig. 5A, lanes 3 to 5) but comigrates with dephosphorylated intracellular core particle protein (Fig. 5A, lane 7). Protein from intracellular

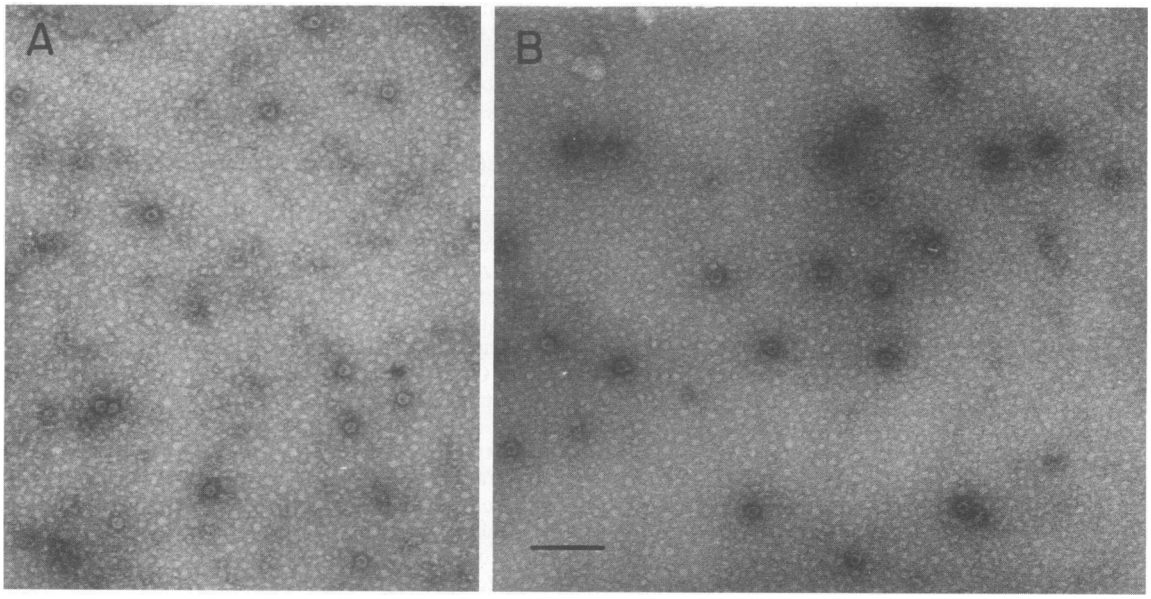

FIG. 4. Electron microscopy of core particles digested overnight in the absence (A) or presence (B) of alkaline phosphatase. Bar, 180 nm.

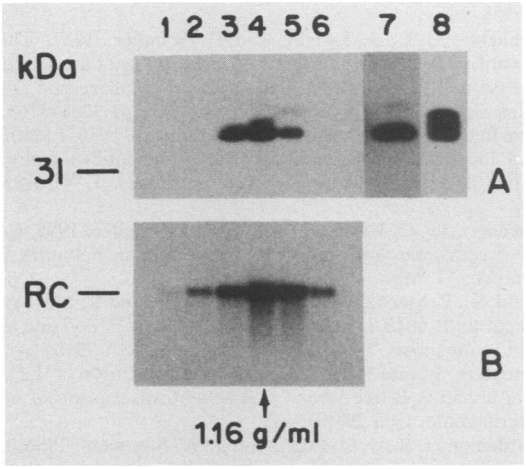

FIG. 5. DHBcAg protein in serum-derived DHBV particles. DHBV particles were partially purified by isopycnic centrifugation in sucrose. Lanes 1 to 6 represent adjacent sucrose gradient fractions of increasing density. Lanes 7 and 8, respectively, represent core particle protein from intracellular cores incubated with or without alkaline phosphatase. Samples from each fraction were analyzed for DHBcAg protein by SDS-PAGE and immunoblot procedures (A) and for DHBV DNA by Southern blot hybridization of viral DNA released by SDS-pronase digestion (B) (see Materials and Methods). The position of relaxed circular (RC) DHBV DNA is shown on the left, and the peak viral DNA fraction is shown corresponding to a density of 1.16 g of sucrose per ml (panel B, lane 4). kDa, Kilodaltons.

core particles that were not incubated with alkaline phosphatase was run on the same gel as a control (Fig. 5, lane 8). Thus, we conclude that the external phosphates responsible for the heterogeneity displayed by intracellular core particle protein are not present on core particles that are packaged within the viral envelope and secreted from the cell.

DISCUSSION

Recent studies have demonstrated that the eAg produced during hepadnavirus infection is derived from a precore-core protein that is processed through a secretory pathway independent from that which generates the structural viral core protein (2, 13, 16, 22). The two products are likely translated from two distinct mRNAs with different 5′ ends, but a naturally occurring transcript with the potential for encoding a precore-core product has been identified only in ground squirrel HBV-infected liver (5).

Expression of the HBcAg gene alone in *E. coli* results in the formation of core particles structurally and serologically indistinguishable from particles isolated from infected liver (4, 19). Thus, although expression of the precore region is not required for core particle assembly, it was not clear at the outset of this study whether the viral cores produced in a natural infection were composed primarily of the polypeptide translated from the first or second AUG. From the amino acid analysis presented in this study, it appears that the major structural protein of the core particle is that translated from the second AUG. Recent genetic studies with DHBV suggest that the precore region is not essential for core particle formation, viral DNA replication, or the formation of infectious virus (3, 16). The only clearly defined role for the precore region at present is that it is responsible for secretion of eAg. The function of eAg in hepadnavirus infection and pathogenesis is not known.

A protein kinase activity associated with HBV core particles was described several years ago (1). This activity is capable of labeling core particle protein in vitro and appears to be endogenous since ^{32}P label incorporated during the in vitro reaction is resistant to removal by alkaline phosphatase treatment of core particles (7). A similar kinase activity has been described associated with core particles purified from DHBV-infected duck liver, and the incorporated ^{32}P label also appears to be resistant to treatment of cores with exogenous phosphatase (J. C. Pugh and J. W. Summers, unpublished data). In vivo phosphorylation of HBcAg protein transiently expressed in tissue culture cell lines under the control of heterologous and native HBV promoter elements has also been reported (15). In this study, we described DHBcAg phosphorylation that appears to be distinct from that associated with the endogenous kinase activity. DHBcAg polypeptide exhibits heterogeneity in SDS-PAGE that can be completely removed following treatment of native core particles with alkaline phosphatase (Fig. 3), suggesting that the phosphate moieties responsible reside on the exterior of core particles.

Interestingly, core protein from mature virus particles does not exhibit the heterogeneity displayed by intracellular core particle protein. Provided that the mature viral cores are derived by maturation of the pool of intracellular cores, dephosphorylation at these sites must occur, whether before or after secretion of mature virus. The significance of this dephosphorylation is not known. Dephosphorylation may occur as a prelude to or a part of the process of packaging of cores within the lipid envelope. In this case, it may act as a signal to allow packaging to occur or to provide a driving force for packaging. Alternatively, dephosphorylation may occur after budding or secretion, as an extracellular step of virus maturation. In such a case, dephosphorylation may be required for initiation of a subsequent round of infection by the mature virus.

ACKNOWLEDGMENTS

We thank Anthony Pomenti for HPLC analyses, Sally Shephardson for electron microscopy, and Laura Coates, Carol Aldrich, and Carrie House for help with the ducks. We also thank Marianne Piatek for secretarial services and the Fox Chase special services department.

The work was supported by Public Health Service grants CA-06927 and RR-05539 from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania. J.C.P. was supported by Public Health Service grant CA-42542 from the National Institutes of Health.

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