In Vivo Phosphorylation and Protein Analysis of Hepatitis B Virus Core Antigen

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Received 25 July 1986/Accepted 5 December 1986

The C open reading frame of the hepatitis B virus contains two in-frame ATG codons that are separated by the precore region and encodes two major polypeptides that are antigenically distinct and that are probably synthesized from individual mRNAs. The precore region directs the secretion of the e antigen, whereas the core antigen can be expressed in the absence of these sequences. In this report a transient expression system was used to study the hepatitis B virus core antigen. By using a chimeric complex of adenovirus major late promoter-simian virus 40 enhancer sequences, we were able to achieve high levels of core antigen expression in transfected cells, permitting characterization of this protein and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The core polypeptide is a 20.9-kilodalton protein, and we show in this study that it is phosphorylated in vivo. Cell fractionation studies, the results of which are supported by indirect immunofluorescence, localized the phosphocore in the cytosol and the nucleus and indicated that it is associated with the membrane of transfected cells. Results of Triton X-114 solubilization studies indicated that the phosphocore is peripherally associated with cytoplasmic membranes. Expression of the membrane-associated phosphocore occurred in the absence of the precore sequences. The phosphocore also assembled into particles in the absence of other viral gene products or intact DNA.

The hepatitis B virus (HBV) is a small partially double-stranded DNA virus containing a viral core that encapsidates the genome and an envelope consisting of host-derived lipid and the viral surface antigen (HBsAg) (for a review, see reference 26). HBV is an important human liver pathogen that causes a wide range of clinical symptoms, and it has been epidemiologically linked to hepatocellular carcinoma. Information on the biology of the virus has been severely limited by its inability to multiply in a tissue culture system or a suitable laboratory animal.

Sequence analysis of cloned HBV DNA has identified four open reading frames (ORFs): S and preS, C, X, and P (26). Recent progress in understanding the molecular biology of HBV has been largely accomplished through the expression of cloned viral DNA in a variety of host cells. Polypeptides encoded by the S and C ORFs have been detected in expression systems by using both native and heterologous transcriptional control elements (4, 15, 18-20, 22, 27). The icosahedral capsid of HBV contains at least two virally encoded proteins, core and e (23), with reported M_r s of 19,000 to 21,000 and 14,000 to 16,000, respectively, which are both encoded by the C ORF (16). The C ORF contains two in-frame ATGs separated by an 87-base-pair region referred to as precore (preC). The e protein is probably generated by posttranslational cleavage of a precursor polypeptide that is translated from the entire C ORF. The carboxy terminus of e is located about 33 amino acids before the termination codon of the C ORF (24). Antibody to the e protein (anti-HBe) cross-reacts with the core protein, but human antibody to core does not cross-react with the native e antigen. Denaturation of the core protein can result in the loss of the core antigen (HBcAg) and conversion of the antigenicity to the e antigen (HBeAg).

The low expression levels of HBcAg and HBeAg directed by the native transcriptional control elements in heterologous cell lines have precluded a detailed biochemical analy-

sis of these proteins. The core protein, however, which has been purified from the livers of infected individuals, has been shown to bind DNA (17), which is consistent with its highly basic carboxy terminus, as predicted from the DNA sequence. Viral core particles have also been shown to contain a kinase activity which is capable of phosphorylating the core protein in vitro (1, 3, 17), although the origin of this activity is unknown.

Until recently the synthesis of detectable levels of HBcAg in transfected cells, under the direction of native transcriptional control elements, had not been achieved. We have shown previously that deletion of the precore sequences results in a dramatic increase in the synthesis of HBcAg (18). In contrast, constructs containing the preC region predominantly express and secrete HBeAg into the culture medium. The significance of the deleted sequences in the regulation of core synthesis is as yet unclear.

To better understand the origin of the HBcAg and HBeAg polypeptides, we continued to carefully examine the biosynthesis of C ORF-related polypeptides. Here we demonstrate the in vivo phosphorylation of the 20.9-kilodalton (kDa) core antigen polypeptide by using both homologous and heterologous systems. The e polypeptide does not appear to be phosphorylated. High-level expression with heterologous promoter-enhancer sequences and the in vivo phosphorylation of core enabled us to further characterize the core protein and to determine its subcellular location in transfected cells.

MATERIALS AND METHODS

Construction of recombinant plasmid vectors. All HBV fragments used in the construction of recombinant vectors were derived from a cloned HBV genome, subtype adw (21). Plasmid pQ3 (8) was obtained from R. Kaufman, Genetics Institute, Boston, Mass., and plasmid pML was from M. Botchan, University of California, Berkeley. All enzymes were obtained from either Boehringer Mannheim Biochemicals (Indianapolis, Ind.) or New England BioLabs,

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Inc. (Beverly, Mass.). The construction of pCEN and pSCΔPC has been described previously (18). For the construction of pAdΔPC, *EcoRI* linkers were added to a 1-kilobase-pair *RsaI-HhaI* fragment of HBV (nucleotides 1856 to 2812), and the purified fragment was cloned into the unique *EcoRI* site of pQ3 by standard methods (12). Plasmid pAdΔPC contained the adenovirus VA genes; the simian virus 40 (SV40) origin of replication, including the enhancer sequences; the adenovirus major late promoter and tripartite leader, with a 5' and 3' splice site; the C ORF, beginning from the second ATG (nucleotide 1907); a dihydrofolate reductase cDNA insert; the SV40 polyadenylation site; and the pBR322 origin of replication and tetracycline resistance gene. Plasmid pAdC contained the 1-kilobase-pair *MstI* to *HhaI* fragment of HBV (nucleotides 1806 to 2812), which was inserted at the *EcoRI* site of pMLAd with *EcoRI* linkers. Plasmid pMLAd contained an *EcoRI*, *SalI* fragment derived from pQ3, which does not include the dihydrofolate reductase and the SV40 polyadenylated sequences, but retains the entire adenovirus major late promoter-SV40 enhancer sequences, and the adenovirus VA I and II genes.

Cell culture and transfection. COS and Hep-G2 cells (10) were grown in Dulbecco modified Eagle medium supplemented with either 7% (COS) or 10% (Hep-G2) fetal bovine serum. COS cells were transfected at 40% confluency with 2 μg of plasmid DNA per dish (diameter, 100 mm) by the method described by Luthman and Magnusson (11). Hep-G2 cells were transfected at 60% confluency with 15 μg of plasmid DNA per dish (diameter, 100 mm) by the calcium phosphate precipitation method (5).

Protein analysis of HBcAg. Cells were radiolabeled with 200 μCi of ³²P_i (carrier-free; ICN Pharmaceuticals, Inc., Irvine, Calif.) or 250 μCi of [³⁵S]cysteine (>6.00 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 8 h, from 42 to 50 h posttransfection in 2.5 ml of appropriately depleted media. Culture supernatants were clarified at 12,000 × g for 10 min. Monolayers were washed twice with cold STE buffer (100 mM NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA), and cells were scraped off in 1.5 ml of STE and pelleted at low-speed centrifugation. Cells were suspended in 100 μl of hypotonic buffer (10 mM HEPES [N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; pH 7.9], 1.5 mM MgCl₂, 10 mM KCl), incubated on ice for 10 min, and lysed in a Kontes 1-ml Dounce homogenizer with a tight-fitting pestle until >90% of the cells were lysed (about 20 strokes). Nuclei were isolated by centrifugation at 4,500 rpm (Sorvall Tabletop centrifuge) for 5 min. Sonicated samples were suspended in 100 μl of TE buffer (10 mM Tris [pH 7.5], 0.1 mM EDTA) and sonicated for 15 to 30 s in a cell disruptor (185; Branson Sonic Power Co., Danbury, Conn.) at maximum output. All samples were immunoprecipitated by standard procedures (9) with either 10 μl of human polyclonal antibody that was positive for the core protein (anti-HBc) and e protein (anti-HBe) or 50 ng of ammonium sulfate-precipitated mouse ascites fluid containing monoclonal anti-HBc (anti-c/β) (24). Samples were analyzed by modified 18% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (25), followed by autoradiography.

Phosphoamino acid analysis. The ³²P-labeled phosphocore was purified by sedimentation through a sucrose density gradient, as described above, and immunoprecipitated. A total of 5,000 cpm of the purified protein was subjected to acid hydrolysis, and phosphoamino acids were analyzed by two-dimensional thin-layer electrophoresis, as described previously (6). Unlabeled phosphoamino acids were used as markers and visualized with ninhydrin.

Detergent extraction of membrane proteins. Membranes were prepared from postnuclear supernatants by high-speed centrifugation in an airfuge (100,000 × g; Beckman Instruments, Inc., Fullerton, Calif.) for 11 min. The supernatant was removed and constituted the 0 detergent fraction. The pellet was sequentially suspended in TE buffer containing increasing concentrations of Nonidet P-40 (NP-40) and centrifuged in an airfuge. The supernatant from each centrifugation constituted the detergent fractions (0.05, 0.1, 0.25, and 0.5%). All fractions were immunoprecipitated and subsequently analyzed by SDS-PAGE as described above.

Triton X-114 phase separation. Phase separation was done by a modification of the method described by Bordier (2). Transfected cells were lysed by sonication in hypotonic buffer, and membrane fractions were prepared as described above. The supernatant contained soluble cytoplasmic components. The membrane pellet was suspended in 500 μl of ice-cold Triton X-114 buffer (50 mM Tris [pH 7.5], 300 mM NaCl, 5 mM EDTA, 1% recondensed Triton X-114) for 30 min with constant agitation at 4°C. Insoluble material was pelleted in the cold in a microfuge for 5 min. The supernatant was incubated for 1 min at 37°C, and the phases were separated by centrifugation in a microfuge for 1 min at room temperature. The detergent fraction was reextracted with 500 μl of Triton X-114 buffer, and the supernatants from both extractions were pooled; this is referred to as the aqueous fraction. All fractions were immunoprecipitated and analyzed by SDS-PAGE, as described above.

Sucrose density gradients. Total cell lysates (200 μl) were sonicated in the presence or absence of NP-40, layered over linear 15 to 40% sucrose gradients, and centrifuged in a rotor (SW55; Beckman) at 40,000 rpm for 2.5 h. Fractions of 400 μl were collected and immunoprecipitated with human anti-HBc and anti-HBe and analyzed by SDS-PAGE.

Immunofluorescence. COS cells were transfected as described above and plated in tissue culture chamber slides at 24 h posttransfection. At 48 to 60 h posttransfection, cells were fixed with acetone at -70°C for 10 min on ice. Incubation with the first antibody was at 37°C for 45 min by using the monoclonal antibody anti-HBc and anti-HBe (24) at a concentration of 10 μg/ml or a 1:100 dilution of the human antibody described above. Cells were washed three times with phosphate-buffered saline and incubated with 5 μg of RNase (Worthington Scientific Diagnostics; Freehold, N.J.) per ml for 15 min at room temperature to degrade the RNA. Ethidium bromide (concentration, 5 μg/ml) was included in the second antibody to counterstain the nuclei. Second antibody was either fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G [affinity-purified F(ab')₂ fragments; Cooper Biomedical] at a final concentration of 5 μg/ml or fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin diluted 1:100 (Calbiochem-Behring, La Jolla, Calif.). Cells were visualized with a microscope (16; Zeiss) with an IV fluorescent vertical illuminator and a Neofluor 40/0.75 objective. Exposures were for 1.5 to 2 min with using Kodak Ektachrome (400 ASA) film.

RESULTS

Expression vectors. In these studies we used two types of vectors for transient expression of the C gene in either human liver cells or in COS cells (Fig. 1). For expression in the human hepatoma cell line Hep-G2 (10), plasmid vectors pCEN and pSCΔPC were used, in which the C gene was under the control of the native promoter and the upstream

FIG. 1. Construction of recombinant plasmid vectors. (A) Schematic representation of the HBV genome subtype adw. C, preC, S, and preS refer to the ORFs of HBV. (B) HBV sequences in pCEN and pSCΔPC. Plasmid pSCΔPC contains a deletion of the sequences between the *Mst*I and *Rsa*I sites. (C) Linear representation of pAdC and pAdΔPC. The HBV sequences in pAdC are from the *Mst*I site to the *Hha*I site. The HBV sequences in pAdΔPC are from the *Rsa*I site to the *Hha*I site. Abbreviations: R, *Eco*RI; Hh, *Hha*I; Hp, *Hpa*I; M, *Mst*I; Rs, *Rsa*I; AdMLP, adenovirus major late promoter; Cp, C promoter; En, HBV enhancer element; SVori, SV40 origin of replication; SVpA, SV40 polyadenylation site; VA, adenovirus VA genes; ss, single stranded; DHFR, dihydrofolate reductase; t, triplicate leader sequence.

enhancer element, which is required for efficient activity of the C gene promoter (7). For high levels of expression the C ORF was placed downstream of the adenovirus major late promoter and the SV40 enhancer in plasmids pAdC and pAdΔPC, which were expressed in COS cells. Both pairs of vectors either included (pCEN and pAdC) or did not include (pSCΔPC and pAdΔPC) the preC sequences. We have shown previously that the predominant HBV-specific product from constructs that included the preC sequences is HBeAg, whereas when preC sequences are deleted the predominant product is HBcAg (18).

In vivo phosphorylation of the core protein. Results of previous studies have shown that the core protein can be phosphorylated in vitro (1, 3, 17). To determine if the core protein was phosphorylated in vivo, we labeled COS cells that were transiently transfected with pAdC and pAdΔPC with $^{32}\text{P}_i$. Cell lysates were immunoprecipitated with human sera containing anti-HBc and anti-HBe. Analysis by SDS-PAGE revealed a phospho-labeled band of 20.9 kDa in the samples from the pAdΔPC transfection (Fig. 2B, lane 1). The pAdC-transfected cells did not contain any immunoprecipitable phosphoprotein (Fig. 2B, lane 2), although HBeAg was being synthesized. This was detected by radioimmunoassay and SDS-PAGE analysis of a 16-kDa [^{35}S]methionine-labeled band (data not shown) and indicates that the e protein is not phosphorylated. Similar results were obtained in the human hepatoma cell line Hep-G2, which was

transfected with plasmids pCEN and pSCΔPC, in which expression of the C ORF was controlled by native promoter-enhancer sequences (Fig. 2A). We observed the 20.9-kDa phosphoprotein in pSCΔPC-transfected cells (Fig. 2A, lane 1) and at very low levels of pCEN-transfected cells (Fig. 2A, lane 2), but there was no evidence of a phosphorylated e protein. Because the pAdC construct does not direct the synthesis of detectable levels of the core, we examined the possible role of the core protein in the phosphorylation of e by cotransfection of COS cells with pAdC and pAdΔPC (Fig. 2D). We were still unable to detect any phospho-e, even though the phosphocore was synthesized during the cotransfection (Fig. 2D, lane 3).

We analyzed further the phosphocore using a monoclonal antibody against the core protein, which was raised against purified cores from human sera and which did not recognize the e protein (a generous gift of M. Mayumi, University of Tokyo, Japan) (24). Both human polyclonal (Fig. 2C, lane 3) and mouse monoclonal (Fig. 2C, lane 1) antibodies precipitated phosphocore, indicating that the phosphocore polypeptide retains the core epitope, which is recognized by the monoclonal antibody.

We analyzed the site of phosphorylation of the core protein by acid hydrolysis, followed by two-dimensional thin-layer electrophoresis (Fig. 3) as described above. We found only serine phosphorylation in the core protein, which is consistent with the in vitro phosphorylation site (3).

FIG. 2. In vivo phosphorylation of core polypeptide. Transfected cells were labeled with $^{32}\text{P}_i$, and cell lysates were immunoprecipitated with either human anti-HBc and anti-HBe (A, B, and C) or mouse monoclonal anti-HBc (D) and analyzed by SDS-PAGE. Autoradiographs were exposed for 3 days (A) or 12 h (B, C, and D). (A) Hep-G2 cells. Lane 1, pSCΔPC-transfected cells; lane 2, pCEN-transfected cells; lane 3, mock-transfected cells. MW indicates molecular mass markers (kd, kilodaltons). (B) COS cells. Lane 1, pAdΔPC-transfected cells; lane 2, pAdC-transfected cells; lane 3, mock-transfected cells. (C) Immunoprecipitation of COS cell lysates with monoclonal antibody. Lane 1, pSCΔPC-transfected cells, monoclonal anti-HBc; lane 2, mock-transfected cells, monoclonal anti-HBc; lane 3, pAdΔPC-transfected cells, human anti-HBc and anti-HBe; lane 4, mock-transfected cells, human anti-HBc and anti-HBe. (D) COS cell cotransfection. Lane 1, pAdΔPC-transfected cells; lane 2, pAdC-transfected cells; lane 3, cotransfected cells, pAdΔPC and pAdC; lane 4, mock-transfected cells.

Cell fractionation and detergent titration of the core. We examined the cellular location of the 20.9-kDa core protein by fractionating radiolabeled pAdΔPC-transfected COS cells. Cells were lysed by Dounce homogenization by hypotonic buffer, and nuclear fractions were isolated by low-speed centrifugation, as described above. Membrane fractions were prepared by high-speed centrifugation of postnuclear supernatants in an airfuge (Beckman). The supernatant of the high-speed centrifugation represented soluble cytoplasmic constituents, designated here as the 0 detergent fraction. We then suspended the membrane-enriched pellet in NP-40, centrifuged the sample in the airfuge, and repeated the procedure using increasing concentrations of NP-40. Supernatants were taken from each detergent-treated fraction after the centrifugation. All fractions, including the final pellet, the nuclear fraction, and the culture supernatant, were immunoprecipitated and analyzed by SDS-PAGE. We used cells labeled with either $^{32}\text{P}_i$ (Fig. 4A) or [^{35}S]cysteine (Fig. 4B) to determine if the phosphocore represented a subset of the core protein in the cell. In both instances we found core protein in the soluble cytoplasmic fraction (the 0

detergent fraction; Fig. 4A and B, lanes 1), the membrane fractions (Fig. 4A and B, lanes 2 through 5), and the nuclear fraction (Fig. 4A and B, lanes 8). We did not detect core polypeptide on subsequent washing of the first membrane pellet with TE buffer alone or with 0.01% NP-40 (data not shown). The majority of the 20.9-kDa protein was eluted from the membrane fraction with 0.05 and 0.1% NP-40 (Fig. 4A and B, lanes 2 and 3). These results indicate that the core protein might be membrane associated. When we further washed the nuclear fraction from Dounce-homogenized

FIG. 3. Phosphoamino acid analysis of core protein. Purified ^{32}P -labeled phosphocore was acid hydrolyzed and subjected to two-dimensional thin-layer electrophoresis as described in the text. Open circles represent migration of unlabeled markers. Abbreviations: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

FIG. 4. Analysis of membrane-associated core. Membrane preparations were suspended in increasing concentrations of NP-40, as described in the text. The samples were immunoprecipitated with human anti-HBc and anti-HBe and were analyzed by SDS-PAGE. (A) ^{32}P -labeled lysates. (B) [^{35}S]cysteine-labeled lysates. The gel was fluorographed in 0.5 M salicylate for 1 h. The lanes in A and B are identical. Lane 1, 0 detergent fraction (supernatant of high-speed centrifugation); lane 2, 0.05% NP-40; lane 3, 0.1% NP-40; lane 4, 0.25% NP-40; lane 5, 0.5% NP-40; lane 6, final pellet; lane 7, culture supernatant; lane 8, nuclear pellet. MW indicates molecular mass markers (kd, kilodaltons).

FIG. 5. Detergent solubilization of phosphocore. Details of the experiment are described in the text. After detergent treatments all fractions were immunoprecipitated with monoclonal anti-HBc and analyzed by SDS-PAGE. (A) Triton X-114 phase separation of phosphocore. Lane 1, soluble cytoplasmic fraction; lane 2, aqueous fraction of Triton X-114 phase separation; lane 3, detergent phase of Triton X-114 separation. MW indicates molecular mass markers (kd, kilodaltons). (B) NP-40 wash of nuclear fraction. Lane 1, cytoplasmic fraction of Dounce-homogenized cells; lane 2, NP-40 wash of nuclear fraction; lane 3, NP-40-insoluble nuclear fraction.

transfected cells with 0.5% NP-40 to remove any detergent-soluble components (Fig. 5B), we found the 20.9-kDa protein in the cytosol (lane 1), the nuclear fraction (lane 3), and the NP-40 wash fraction (lane 2). The nuclear fraction prepared by Dounce homogenization contained both detergent-soluble and insoluble forms of core protein. This pattern of phosphocore distribution shows that the protein is found free in the cytosol and the nucleus and is associated with detergent-soluble components (i.e., membranes) of the cells. Although we could not detect any core protein in the culture supernatants during this limited labeling time in this experiment, we have previously detected the core in culture supernatants as well as in cell lysates (18). Also, in our

recent studies accumulation of core particles is always seen (M. Roossinck, unpublished data). It is likely that the core accumulates in the culture supernatant more slowly than the labeling time used in these experiments.

Triton X-114 phase separation of membrane protein. To determine if the core protein was integrally or peripherally associated with the cellular membranes we used the low cloud point property of the detergent Triton X-114, as described previously (2). In this procedure, the detergent forms micelles at room temperature which can be separated from the aqueous phase of the detergent solution by centrifugation. Hydrophobic proteins partition into the detergent phase, and amphiphilic proteins partition into the aqueous phase. Cells transfected with pAdΔPC were radiolabeled with $^{32}\text{P}_i$, and the membrane fraction was isolated by high-speed centrifugation as described above. The membrane-associated core protein partitioned with the aqueous phase of the detergent-extracted membrane pellet (Fig. 5A, lane 2), indicating that it is peripherally associated with the membrane and is probably not an integral membrane protein.

Sucrose density gradient sedimentation of phosphocore. Phosphocore was analyzed by sedimentation through a sucrose density gradient to determine if it had assembled into particles. Cells were lysed by sonication, as described above, and lysates were fractionated by centrifugation on 15 to 40% sucrose density gradients. The presence or absence of NP-40 was used to distinguish between membrane-associated core, which would sediment with the microsomal fraction, and core particles. The 20.9-kDa phosphoprotein was found in the same fraction both with and without NP-40 (Fig. 6), indicating that the phosphocore is assembled into an ordered structure which is probably analogous to the virion core particle. We also observed the phosphocore in the pellet of the gradient, which could have resulted from further aggregation of the particles. Assembly of the core polypeptide into particles has been reported previously (13, 15, 16).

Immunofluorescence of core protein. To confirm the cellular location of the core protein that was found by cell fractionation, we examined pAdΔPC-transfected cells by indirect immunofluorescence. Cells were incubated with either mouse monoclonal antibody or human anti-HBc and anti-HBc, as described above. After a second incubation with appropriate fluorescein isothiocyanate-conjugated anti-

FIG. 6. Sucrose density sedimentation of phosphocore. Sucrose density gradient fractions were immunoprecipitated and subsequently analyzed by SDS-PAGE. Lanes 1 represent the fraction from the bottom of the gradient; lanes 12 represent the fraction from the top of the gradient. Gradients were designed such that fractions 8 to 9 corresponded to a sedimentation coefficient of about 90S. (A) Sedimentation in the absence of NP-40. MW indicates molecular mass markers (kd, kilodaltons). (B) Sedimentation in the presence of NP-40.

FIG. 7. Indirect immunofluorescence of core protein. Immunofluorescence was carried out on COS cells transfected with pAd Δ PC, as described in the text. Nuclei were counterstained with ethidium bromide. N is a nonimmunofluorescent, ethidium bromide-stained nucleus. (A) Monoclonal anti-HBc and anti-HBe as a first-stage antibody. (B) Human anti-HBc and anti-HBe as a first-stage antibody.

body, the cells were visualized by fluorescent microscopy (Fig. 7A and B). We observed immunofluorescence in both the cytoplasm and nuclei of the transfected cells, which is consistent with our cell fractionation data and results of studies of HBV-infected human liver (14).

DISCUSSION

We have demonstrated here that the HBV core protein is a nuclear-, cytoplasmic-, and membrane-associated phosphoprotein. We showed that *in vivo* phosphorylation of the core protein occurs in both heterologous (monkey) and homologous (human liver) host cells that were transfected with appropriate expression vectors. Results of the subcellular localization studies were consistent with those of studies of HBV-infected human liver, in which the core has been found in the nucleus and cytoplasm (14, 17). Because results of previous studies indicated that the HBV virion core contains a kinase activity that is capable of phosphorylating the core protein *in vitro* (1, 3, 17), we are currently investigating a possible role of the core protein in the phosphorylation.

Expression of core protein leads to the formation of particles with a diameter of 27 nm in *Escherichia coli* (16) and in yeast (13). In our studies particles were also formed, and they represented the phosphorylated form of the core protein. Because of the ability of the core protein to spontaneously form a particle in the absence of other viral products, coupled with the finding of empty viral capsids in the cytoplasmic fraction of HBV-infected human livers (17), it can be suggested that during viral replication the genome may be packaged into a preassembled core particle.

The presence of the core protein in the soluble cytoplasmic as well as the membrane fraction of the transfected cell suggests that the protein is synthesized on free ribosomes and diffuses to the membrane. This is supported by the observation that the membrane-associated core protein separates with the amphiphilic fraction in Triton X-114 phase

separation, indicating that it is not an integral or transmembrane protein but that it is peripherally associated with the membrane. The precore sequences do not appear to be required for targeting the core protein to the membrane, as was reported previously (15). The e protein, which would likely be found in the endoplasmic reticulum because it is a secreted protein, could also be responsible for the previous observation, because the rabbit anti-HBc cross-reacts with the e protein (unpublished data). Because the two proteins cross-reacted antigenically, the distinction between core and e can be unambiguously made by SDS-PAGE.

We were not able to detect any 14- to 16-kDa protein that reacted with anti-HBe in cells transfected with pAd Δ PC, although we were able to detect such a protein in the culture supernatants of cells transfected with pAdC (Roossinck, unpublished data). From these data, it seems likely that the core protein synthesis uses only the second ATG codon, whereas the e protein uses the first ATG codon. Indeed, from S1 nuclease mapping of the C ORF-related mRNA species, two initiation sites beginning before and after the first initiation codon have been located (18). The absence of the 20.9-kDa core protein in cells transfected with pAdC probably reflects a regulation of core synthesis in HBV-infected human livers that we are unable to mimic in this system. Deletion of the precore sequences also removed the second copy of the viral direct repeat and the carboxy terminus of the X ORF, either of which could play a regulatory role in the biosynthesis of core and e.

Using the strong heterologous promoter, coupled with the *in vivo* phosphorylation of the core protein, we are currently investigating a number of other aspects of core biosynthesis and processing. Extracellular accumulation of HBcAg, as reported previously (18), is being investigated by pulse-chase experiments. It seems likely that the core protein does not leave the cell via a normal secretory pathway because (i) in a previous study (18) we did not observe accumulation of excess core in culture supernatants, as compared with cell lysates, and (ii) in this study the labeling time was not sufficient for detectable levels of core to accumulate in the culture supernatants. We are also examining the regulation

of expression of the core and e proteins by other HBV gene products and regulatory sequences.

ACKNOWLEDGMENTS

We thank E. J. Erikson, F. LaRosa, and R. Evans for invaluable assistance and advice with various procedures and J. Mapoles for helpful discussion and careful reading of the manuscript.

This study was supported by Public Health Service grants AI21182 and CA33135 from the National Institutes of Health to A.S.

LITERATURE CITED

1. Albin, C., and W. S. Robinson. 1980. Protein kinase activity in hepatitis B virus. *J. Virol.* **34**:297-302.
2. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256**:1604-1607.
3. Gerlich, W. H., U. Goldmann, R. Muller, W. Stibbe, and W. Wolff. 1982. Specificity and localization of the hepatitis B virus-associated protein kinase. *J. Virol.* **42**:761-766.
4. Gough, N. M. 1983. Core and e antigen synthesis in rodent cells transformed with hepatitis B virus DNA is associated with greater than genome length viral messenger RNAs. *J. Mol. Biol.* **165**:683-699.
5. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456-457.
6. Hunter, T., and B. Sefton. 1980. The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA* **77**:1311-1315.
7. Jameel, S., and A. Siddiqui. 1986. The human hepatitis B virus enhancer requires *trans*-acting cellular factor(s) for activity. *Mol. Cell. Biol.* **6**:710-715.
8. Kaufman, R. J. 1985. Identification of the components necessary for adenovirus translational control and their utilization in cDNA expression vectors. *Proc. Natl. Acad. Sci. USA* **82**:689-693.
9. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115**:1617-1624.
10. Knowles, B., C. C. Howe, and D. P. Aden. 1980. Human hepatocellular carcinoma cell lines secrete the major plasma proteins the hepatitis B surface antigen. *Science* **209**:497-499.
11. Luthman, H., and G. Magnusson. 1983. High efficiency polyoma transfection of chloroquine treated cells. *Nucleic Acids Res.* **11**:1295-1308.
12. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. Miyanohara, A., T. Imamura, M. Araki, K. Sugawara, N. Ohtomo, and K. Matsubara. 1986. Expression of hepatitis B virus core antigen gene in *Saccharomyces cerevisiae*: synthesis of two polypeptides translated from different initiation codons. *J. Virol.* **59**:176-180.
14. Mondelli, M., R. S. Tedder, B. Ferns, P. Pontisso, G. Readli, and A. Alberti. 1986. Differential distribution of hepatitis B core and e antigens in hepatocytes: analysis by monoclonal antibodies. *Hepatology* **6**:199-204.
15. Ou, J. H., O. Laub, and W. J. Rutter. 1986. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. *Proc. Natl. Acad. Sci. USA* **83**:1578-1582.
16. Pasek, M., T. Goto, W. Gilbert, B. Zink, H. Schaller, P. MacKay, G. Leadbetter, and K. Murray. 1979. Hepatitis B virus genes and their expression in *Escherichia coli*. *Nature (London)* **282**:575-579.
17. Petit, M.-A., and J. Pillot. 1985. HBc and HBe antigenicity and DNA-binding activity of major core protein P22 in hepatitis B virus core particles isolated from the cytoplasm of human liver cells. *J. Virol.* **53**:543-551.
18. Roossinck, M. J., S. Jameel, S. H. Loukin, and A. Siddiqui. 1986. Expression of hepatitis B viral core region in mammalian cells. *Mol. Cell. Biol.* **6**:1393-1400.
19. Siddiqui, A. 1983. Expression of hepatitis B virus surface antigen gene in cultured cells using recombinant plasmid vectors. *Mol. Cell. Biol.* **3**:143-146.
20. Siddiqui, A., S. Jameel, and J. Mapoles. 1986. Transcriptional control elements of hepatitis B surface antigen gene. *Proc. Natl. Acad. Sci. USA* **83**:566-570.
21. Sninsky, J., A. Siddiqui, W. S. Robinson, and S. Cohen. 1979. Cloning and endonuclease mapping of the hepatitis B viral genome. *Nature (London)* **279**:346-348.
22. Standring, D. W., W. J. Rutter, H. E. Varmus, and D. Ganem. 1984. Transcription of the hepatitis B surface gene in cultured murine cells initiates within the presurface region. *J. Virol.* **50**:563-571.
23. Takahashi, K., Y. Akahane, T. Gotanda, T. Mishiro, M. Imai, Y. Miyakawa, and M. Mayumi. 1979. Demonstration of hepatitis B e antigen in the core of Dane particles. *J. Immunol.* **122**:275-279.
24. Takahashi, K., A. Machida, G. Funatsu, M. Nomura, S. Usuda, S. Aiyagu, K. Tachibana, H. Miyamoto, M. Imai, T. Nakamura, Y. Miyakawa, and M. Mayumi. 1983. Immunochemical structure of hepatitis B e antigen in the serum. *J. Immunol.* **130**:2903-2907.
25. Thomas, J. O., and R. D. Kornberg. 1975. An octamer of histones in chromatin and free in solution. *Proc. Natl. Acad. Sci. USA* **72**:2626-2630.
26. Tiollais, P., C. Pourcel, and A. Dejean. 1985. The hepatitis B virus. *Nature (London)* **317**:489-495.
27. Will, H., R. Cattaneo, E. Pfaff, C. Kuhn, M. Roggendorf, and H. Schaller. 1984. Expression of hepatitis B antigens with a simian virus 40 vector. *J. Virol.* **50**:335-342.