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In Vitro Infection of Human Hepatoma (HepG2) Cells with Hepatitis B Virus

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An in vitro system for production of hepatitis B virus (HBV) was established by infection of human hepatoma (HepG2) cells. HBV particles obtained from the serum of a chronic hepatitis B surface antigen (subtype *ad*) carrier were used to inoculate HepG2 cells. HBV envelope and core proteins were synthesized de novo by the infected cells and secreted into the medium 3 to 6 days postinfection. Viral covalently closed circular DNA, the putative template for viral RNA transcription, accumulated in the cells with increasing time postinfection. The HBV-infected HepG2 cells were maintained for several months (HepG2-BV cell line) and continued producing viral antigens. Both HBV DNA replicative intermediates and major HBV transcripts were identified in HepG2-BV cells. Complete HBV particles, which contain HBV DNA and DNA polymerase activity and express the three antigenic specificities of the envelope (hepatitis B surface antigen, pre-S2, and pre-S1), were released into the culture supernatant. Thus, successful in vitro infection of transformed human hepatocytes raising stable HBV-producing cells was achieved for the first time. This strongly suggests that HepG2 cells have a receptor(s) for virus attachment and penetration. Such a system represents a significant advance for the study of HBV-target cell interactions as the early events of HBV infection.

The genome organization of hepatitis B virus (HBV) DNA has been extensively studied by recombinant DNA technology. However, our understanding of HBV replication and transcription at the molecular level has been hampered by the restricted host range of HBV, which infects only humans and chimpanzees, and by the lack of a cell culture system for productive viral infection. Infection by HBV causes acute and chronic hepatitis. Furthermore, HBV is known to be a major cause of human liver cancer. Human HBV is one of a family of small DNA-containing viruses that include woodchuck hepatitis virus (30), ground squirrel hepatitis virus (14), duck hepatitis B virus (17), and more recently, tree squirrel hepatitis B virus (5) and heron hepatitis B virus (28). The proposed replication cycle of these viruses was described by Summers and Mason (29) on the basis of observations of the in vivo replication of duck hepatitis B virus. Studies have been conducted on primary duck hepatocytes in vitro infected with duck hepatitis B virus (37) and on a mammalian model of woodchuck hepatocytes from animals naturally infected with woodchuck hepatitis virus (34). Very recently, in vitro HBV infection of adult human hepatocytes cultured in dimethyl sulfoxide (8) or of primary human fetal hepatocytes (19) was achieved. Propagation of HBV in cell culture has also been carried out by transfection of human hepatoma cell lines with closed circular HBV DNA (25, 32, 35).

In this report, we show that the human hepatoma cell line HepG2, having the biosynthetic capabilities of normal liver parenchymal cells (1, 2, 12) and known to support HBV replication following transfection by cloned HBV DNA (25, 32), can be infected with HBV virions. Evidence for persistent virus production is based on (i) immunological identification of HBV envelope and core proteins, (ii) detection of viral DNA and DNA polymerase activity in particles released into the medium, and (iii) identification of intracellular viral DNA forms and major HBV-specific transcripts. The advantage of this model over the one using transfection of HepG2 cells is that penetration and uncoating of the virus can be studied. Furthermore after infection, production of extracellular complete HBV virions (Dane-like particles) is maintained continuously for an extended time, which is not feasible with primary liver cells. Therefore, such an in vitro system for infection with HBV is suitable for studies of HBV-target cell interactions, as well as evaluation of neutralizing anti-HBV monoclonal antibodies or antiviral drugs.

MATERIALS AND METHODS

Cell cultures and inoculum preparations. HepG2 cells were grown in Dulbecco minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 4.5 g of glucose per liter, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cells were seeded at a concentration of 10^6 per dish (60-mm-diameter Falcon dishes from Becton Dickinson Labware) in 4 ml of complete culture medium. Cultures were observed daily with a phase-contrast microscope. The medium was changed every 1 or 2 days and stored at −20°C. HBV particles obtained from the serum of a chronic hepatitis B surface (HBs) antigen (HBsAg; subtype *ad*) carrier were used to inoculate HepG2 cells. In experiment 1, the inoculum used was HBV-positive fresh serum, undiluted and at a 1:100 final dilution. In the following experiments, the inoculum used was prepared from a freeze-stored sample of initially HBV-positive serum by clarification and ultracentrifugation of the resulting supernatant. The pellet was finally suspended in complete culture medium in one-fifteenth of the initial volume and used immediately for inoculation.

Infection of cell cultures with HBV. At 2 to 6 days after plating, HepG2 cells were infected with 1 ml of HBV particles from patient serum. After 3 h of incubation at 37°C, the inoculum was removed. The cells were then extensively washed with phosphate-buffered saline, and complete cul-

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ture medium supplemented with 10^{-4} M dexamethasone and 10^{-5} M insulin was added to the cells. The cultures were incubated for 1 day, washed three times, and incubated again with fresh culture medium. The media were changed after 1 day, and the cells were maintained thereafter in complete medium supplemented with 10^{-6} M dexamethasone and 10^{-6} M insulin. The medium was then changed every 2 days, harvested, and tested for HBV antigens

Analysis of HBV-specific proteins by WIBA and radioimmunoprecipitation assay. For Western immunoblotting assays (WIBA), cells were washed in phosphate-buffered saline, and lysis buffer containing 50 mM Tris hydrochloride (pH 7.2), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 mM phenylmethylsulfonyl fluoride was added to the cells in plates before scraping. The cells were then harvested with a rubber policeman, shake for 30 min at 4°C, and centrifuged at 15,000 × *g* for 20 min. Cells extracts and virus pelleted by ultracentrifugation (280,000 × *g* for 5 h) from culture supernatant were incubated in SDS-polyacrylamide gel electrophoresis sample buffer and electrophoresed in 12.5% polyacrylamide gels, and the proteins were transferred onto nitrocellulose filters as previously described (21). The filters were incubated with rabbit anti-HBs immunoglobulins G (IgGs), and binding of HBV-specific antibodies was detected by incubation with the ^{125}I-labeled F(ab')$_2$ fragment of anti-rabbit immunoglobulins (Amersham France).

For radioimmunoprecipitation assays, HBV-exposed and control cells were incubated for 24 h with [^{35}S]methionine. Labeling of cells was performed 1 day postinfection, and preparation of cell extracts was done 2 to 6 days later. HBV-specific antigens from either cell extracts or virus pelleted from the culture supernatant were then immunoprecipitated with 10 μg of polyclonal IgGs adsorbed onto 100 μl of protein A-Sepharose. The immune complex was washed three times with buffer A (1 mM Tris hydrochloride [pH 7.4], 130 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 0.01% SDS, 1% deoxycholate, 1% Triton X-100) and then with buffer B (0.02 M Tris hydrochloride [pH 7.5], 0.05% SDS) and solubilized in SDS-polyacrylamide gel electrophoresis sample buffer for 15 min at 25°C, followed by heating at 100°C for 5 min. After centrifugation at 10,000 × *g* for 5 min, supernatants were analyzed by SDS-polyacrylamide gel electrophoresis.

Detection of HBsAg and pre-S antigens in culture medium by Mo-RIAs. Release of HBsAg and pre-S antigens (pre-S2 and pre-S1) into the medium was measured by using a monoclonal radioimmunoassay (Mo-RIA) system involving rabbit polyclonal anti-HBs IgGs on the solid phase and HBs-, pre-S2- or pre-S1-specific monoclonal antibodies in the revelation phase (21, 22), as described in detail elsewhere (M.-A. Petit, F. Zoulim, F. Capel, S. Dubanchet, and C. Trepo, Hepatology, in press). Binding of specific monoclonal antibodies was revealed by incubation with the ^{125}I-labeled F(ab')$_2$ fragment of anti-mouse immunoglobulins from Amersham.

DNA extraction and analysis. To obtain cellular DNA, cells were lysed in 0.5% SDS (10 mM Tris hydrochloride [pH 7.4], 10 mM NaCl, 10 mM EDTA) and incubated with 500 μg of proteinase K per ml for 2 h at 50°C (11). Extrachromosomal DNA was obtained by the method of Hirt (9). Cells were lysed with the above-described lysis solution and incubated for 12 h at 37°C. NaCl (1M) was added to the lysate, and the mixture was stored for 12 h at 4°C. Chromosomal DNA was pelleted at 27,000 × *g* for 40 mn, and the extrachromosomal DNA was extracted from the supernatant. Extraction for all samples was performed twice with phenol, followed by chloroform-isoamyl alcohol (24:1 [vol/vol]). The DNA solution was adjusted to a concentration of 0.3 M with sodium acetate (pH 6.5), precipitated with 2 volumes of cold ethanol, and then chilled to −20°C for 12 h. The precipitate was suspended in 10 mM Tris hydrochloride (pH 7.4) with 1 mM EDTA (TE buffer) and treated with 100 μg of RNase A (Boehringer Mannheim Biochemicals) per ml for 2 h at 37°C. The solution was extracted twice with phenol and twice with chloroform-isoamyl alcohol, and the DNA was precipitated as described above. The precipitate was suspended in TE buffer.

DNA isolation from virus in the cell culture medium and from gradient-purified virus was performed by the same procedure, except that the Hirt precipitation was omitted. Viruses were pelleted by ultracentrifugation at 280,000 × *g* for 5 h at 4°C. The supernatant was discarded, and the virus pellet was suspended in 10 mM Tris hydrochloride (pH 7.2) with 140 mM NaCl (TN buffer). Isopycnic centrifugation of the sedimented particles was then performed in a gradient of 20 to 60% (wt/wt) sucrose in TN buffer. The gradients were centrifuged at 230,000 × *g* for 17 h at 4°C. Fractions were collected and then analyzed for sucrose concentration, HBV antigens, DNA polymerase activity, and HBV DNA. High-molecular-weight DNA was digested with restriction endonuclease *Eco*RI (New England BioLabs, Inc.) by using 150 U of the enzyme for 15 μg of DNA in the appropriate reaction mixture for 17 h at 37°C. DNA was analyzed by electrophoresis on a 0.8 or 1.5% agarose gel and then transferred to a Hybond C filter (Amersham) by the method of Southern (27). The filters were hybridized with cloned ^{32}P-labeled HBV DNA at 65°C. The probe was labeled by nick translation to a specific activity of 0.4×10^9 to 1×10^9 cpm/μg. After hybridization using 10% dextran sulfate, the filters were washed, dried, and then exposed to Hyperfilm MP (Amersham) with an intensifying screen.

RNA analysis. Cells were lysed in a solution containing 4 M guanidium isothiocyanate, 0.5% sodium lauryl sarcosine, 25 mM sodium citrate (pH 7), and 100 mM 2-mercaptoethanol. The cell lysate was layered on a 3-ml cushion of 5.7 M CsCl in 50 mM EDTA (pH 7.8) in a Kontron TST 60 polyoallomer tube and centrifuged at 180,000 × *g* for 12 h at 20°C. The pellet was suspended in diethylpyrocarbonate solution adjusted to 0.3 M with 20× SSC (20× SSC is 3 M NaCl, plus 0.3 M sodium citrate) and precipitated with 2.5 volumes of cold ethanol. RNA preparations were kept in diethylpyrocarbonate solution at −20°C. RNA samples were electrophoresed through 1% agarose gels containing 1.1 M formaldehyde (13) and transferred to Hybond C filters for hybridization. Hybridization was performed as described above.

DNA polymerase assay. HBV endogenous DNA polymerase activity was determined directly on 30 μl of virus pelleted from culture supernatant by the method of Alberti et al. (3).

Electron microscopic examination. Culture supernatant was concentrated by ultracentrifugation, and then the resulting pelleted virus was suspended in TN buffer and observed directly after negative staining with a JEOL 100 CX electron microscope.

RESULTS

Determination of susceptibility of HepG2 cell cultures to infection by detection of HBV proteins. To determine when HepG2 cell monolayers were susceptible to HBV infection, cells were exposed to an inoculum containing HBV virions

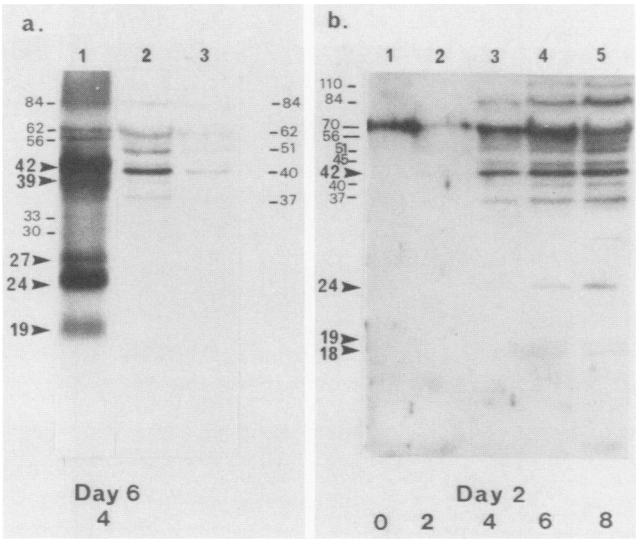

FIG. 1. WIBA of HBV surface proteins synthesized in HepG2 cells. (a) Cells were infected at day 6 after plating and extracted 4 days later. Lanes: 1, inoculation with undiluted virus from fresh, HBV-positive serum; 2, inoculation with a 1:100 dilution of virus; 3, incubation with normal human serum. (b) Cells were infected at day 2 after plating and extracted 0, 2, 4, 6, and 8 days later. Lanes 1 to 5, inoculation with undiluted virus. For panel a, lanes 1 to 3, and panel b, lanes 1 to 5, the filters were reacted with rabbit anti-HBs IgGs. Antibody binding was detected with the ^{125}I-labeled anti-rabbit immunoglobulin F(ab')$_2$ fragment. The numbers beside the panels indicate molecular sizes of polypeptides in kilodaltons.

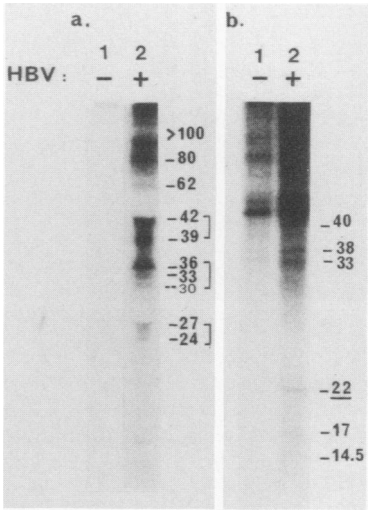

FIG. 2. Immunoprecipitation of newly synthesized HBV antigens. HBV-exposed (lanes 2) and control (lanes 1) cells were labeled for 24 h with [^{35}S]methionine. (a) Viral particles were pelleted from cell culture supernatant, and the pellet was suspended in one-fifteenth of the initial volume. After solubilization, viral proteins were immunoprecipitated with rabbit IgGs to HBsAg. (b) After solubilization of the cells, viral proteins were immunoprecipitated with human IgGs to hepatitis B e and core antigens. The numbers beside the panels indicate molecular sizes in kilodaltons.

either 2 or 6 days after plating (Fig. 1a and b). HBsAgs were then detected in infected cells by WIBA using HBs-specific rabbit polyclonal IgGs. Rabbit antisera were obtained by immunization with 22-nm HBsAg particles purified from hepatitis B e antigen-positive serum, and IgG specificity was described in detail previously (23). Briefly, these antibodies reacted with the three HBV envelope proteins (HBs, pre-S2, and pre-S1), as well as with human serum albumin, in WIBA, even after removal of antibodies to host proteins that contaminated the HBsAg preparation used for immunization. Anti-HBs IgGs gave a strong signal with albumin and reacted faintly with cellular components at 37, 40, 51, and 62 kilodaltons (kDa) in noninfected HepG2 cells (Fig. 1a, lane 3, and b, lane 1). However, when HepG2 cells were exposed to fresh, undiluted, HBV-positive serum 6 days after plating, virus-specific surface proteins at 19, 24, 27, 39, and 42 kDa were detected at 4 days postinfection (Fig. 1a, lane 1). When the HepG2 cells were exposed to virus 2 days after plating and extracted 0, 2, 4, 6, and 8 days later, synthesis of HBsAgs occurred by days 4 to 8 postinfection (Fig. 1b, lanes 3 to 5). Thus, HBV-specific envelope proteins in both cases were identified in infected HepG2 cell extracts 10 days after plating.

Active synthesis of HBV antigens was checked by labeling cells with [^{35}S]methionine 1 day postinfection, followed by immunoprecipitation of both cell extracts and concentrated culture supernatants 3 to 6 days postinfection. Figure 2a, lane 2, shows a complex pattern of specific labeled bands immunoprecipitated by rabbit anti-HBs IgGs in the pellet resulting from ultracentrifugation of media. These bands were not found in culture medium of uninfected HepG2 cells (Fig. 2a, lane 1). Surprisingly, the major HBs proteins (P24 and GP27) were less abundant than the middle (P30, GP33, and GP36) and large (P39 and GP42) pre-S proteins, as well as much larger polypeptides of 62, 80, and >100 kDa. These high-molecular-weight components probably correspond to antigenic dimers and polymers of HBsAg proteins resistant to dissociation in immune complex form (21, 23). Such a pattern is commonly found in HBV particles purified from serum which often remain associated with human serum IgM as HBV-IgM complexes after purification (21). Figure 2b, lane 2, shows labeled bands immunoprecipitated by human anti-hepatitis B e and core antigen IgGs in HBV-infected HepG2 cells. Bands upwards of 45 kDa were considered nonspecific for the virus (Fig. 2b, lane 1). The major HBV core protein of 22 kDa (P22c) was faintly detected, as were two other bands of 14.5 and 17 kDa, which could be hepatitis B X protein specific (data not shown). Three additional major labeled bands of 33, 38, and 40 kDa were observed. These high-molecular-weight components may correspond to dimeric forms of the hepatitis B core antigen or hepatitis B X antigen and also to X-C fusion proteins (M.-A.P., unpublished data).

Identification of HBV-specific particles released into the culture medium early postinfection. To further characterize the structures of extracellular HBV proteins and to determine whether the virus was released from in vitro-infected HepG2 cells, viral particles pelleted from culture medium 6 days postinfection were visualized by electron microscopic examination. The culture medium contained 22-nm-diameter spherical and 22-nm-long filamentous HBsAg particles and few 42-nm-diameter double-shelled HBV-like particles (Fig. 3). The relatively large number of filaments seems to confirm an excess of pre-S1 antigen production by HBV-infected HepG2 cells early postinfection. Detection of pre-S1-specific epitopes associated with HBsAg activity by our Mo-RIA in concentrated culture medium 4 to 8 days postinfection was therefore evidence of overexpression of pre-S1 proteins (data not shown).

Intracellular HBV DNA replication. To determine whether

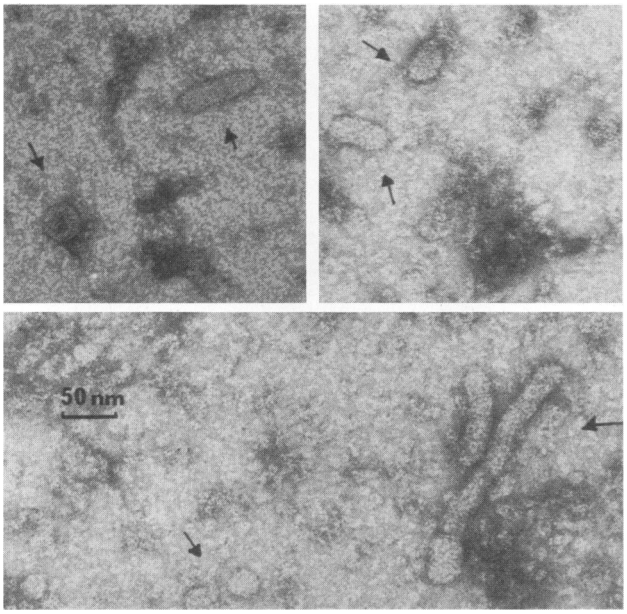

FIG. 3. Electron microscopic examination of viral particles released by HBV-infected HepG2 cells into the medium. Viral particles were sedimented from culture supernatant 6 days postinfection by ultracentrifugation at 280,000 × *g* for 5 h at 4°C. The resulting pellet was suspended in TN buffer, and then 0.05 ml was dropped on a carbon-coated grid and 1% uranyl acetate was added for negative staining. The preparation was examined with a JEOL 100 CX electron microscope. The arrows indicate HBV-specific particles.

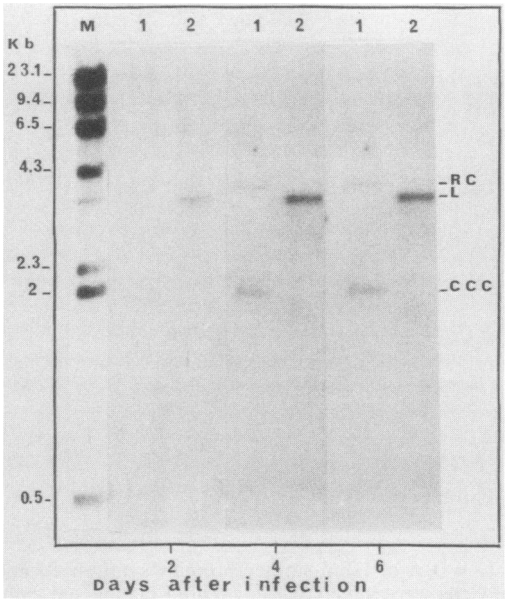

FIG. 4. Southern blot analysis of intracellular HBV DNA in infected HepG2 cells. Cells were harvested from 1 to 6 days postinfection. DNA was analyzed by Southern blotting with ^{32}P-labeled whole HBV DNA as the probe. Undigested DNA was loaded onto lanes 1, and DNA cleaved by *Eco*RI was loaded onto lanes 2. The positions of the RC, linear (L), and CCC species are indicated. The size markers (lane M) in kilobases (Kb) are *Hin*dIII-digested bacteriophage λ DNA.

HBV replicated in infected HepG2 cells, cells were harvested from 1 to 6 days postinfection. Nuclear DNA was extracted and analyzed by Southern blotting with ^{32}P-labeled whole HBV DNA as the probe. To detect the presence of the supercoiled covalently closed circular (CCC) form, the samples were undigested (Fig. 4, lanes 1) or digested with *Eco*RI (lanes 2). Undigested samples (lanes 1) contained two HBV-specific DNA components with apparent mobilities of 3.8 and 2 kilobases (kb). These bands correspond to the approximate mobilities expected for the relaxed circular (RC) DNA species and the CCC form of HBV DNA, respectively. Upon *Eco*RI digestion (lanes 2), both DNA forms shifted to the 3.2-kb position, which represents the linear double-stranded HBV genome. With increasing time postinfection, these nuclear virus-specific DNA replicative intermediates accumulated. The CCC form has been reported in actively infected human liver (18) and could serve as a template for DNA transcription (16). Therefore, our in vitro HBV infection system seemed to reproduce the full cycle of viral replication.

Maintenance of HepG2-BV cells and characterization of viral particles released into the culture medium. One culture of HBV-infected HepG2 cells, designated the HepG2-BV cell line, was maintained for 1 year after passage. By 4 to 6 days postinfection, cells were trypsinized, seeded in T-25 flasks (10^6 cells) in complete culture medium, and subcultured weekly thereafter. To characterize the viral particles released from HepG2-BV cells, supernatants (300 to 400 ml) from successive subcultures were pooled and subjected to isopycnic sucrose gradient centrifugation. Fractions were tested for sucrose concentration, analyzed for the combined presence of HBsAg, pre-S1 and pre-S2 antigens, DNA polymerase activity, and HBV DNA (Fig. 5).

By using Mo-RIAs, we showed that HBs, pre-S2, and pre-S1 antigenic activities were prominent in fractions 5 to 8 surrounding 40% sucrose, which corresponds to the banding position of the complete virions. In addition, DNA polymerase activity was detected in fractions 5 and 6. Thus, viral particles released from HepG2-BV cells contain endogenous DNA polymerase activity and express the three envelope specificities of HBV (Fig. 5a) in relative proportions similar to those found in complete virions isolated from infected human serum (20).

Sucrose gradient fractions were further analyzed for the presence of HBV DNA by Southern blotting (Fig. 5b). A band of 3.8 kb, consistent with the mature viral DNA (RC form), was detected mainly in fractions 6 to 8, in which complete virus particles were identified. Two additional, smaller discrete bands (2.6 and 1.6 kb) were observed, possibly corresponding to supercoiled (CCC) and single-stranded HBV DNA forms.

Long-term presence of extrachromosomal forms of viral DNA in HepG2-BV cells. Six months after establishment of the HepG2-BV cell line, intracellular extrachromosomal HBV DNA was prepared from infected cells at 2, 4, 6, 8, and 10 days after seeding and analyzed by Southern blotting (Fig. 6). The DNA patterns showed a faint band at 3.8 kb, corresponding to the RC form of HBV DNA, and an intense band at the 3.2-kb position, with a smear downstream. These unintegrated forms (3.8 and 3.2 kb) and a short, intense smear without low-molecular-weight DNA forms were found in larger quantities at day 6 after seeding. In addition, a viral DNA component with an apparent size greater than 20 kb was also detected at the same intensity throughout the 10 days of passage, indicating that integration of the HBV genome in the cellular DNA probably occurred. This pattern differed somewhat from that found in liver with replicating

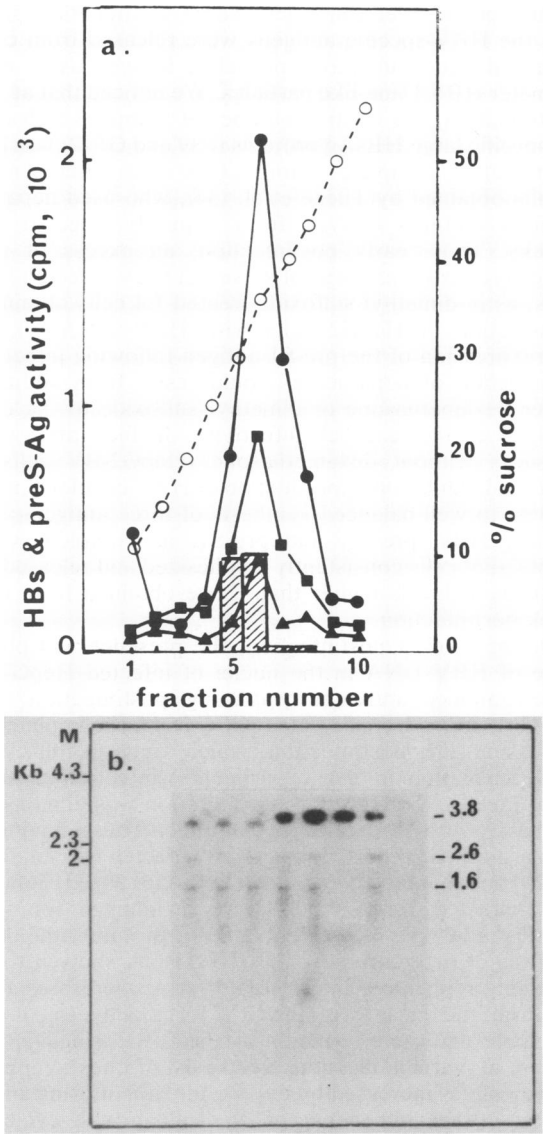

FIG. 5. (a) Immunochemical analysis of HBV proteins in a sucrose gradient of HepG2-BV particles obtained from a culture supernatant. The sedimented particles (concentrated 20-fold) were subjected to ultracentrifugation in a 10 to 60% (wt/wt) sucrose gradient for 22 h at 350,000 × *g*. Fractions were tested for HBs (●), pre-S2 (■), and pre-S1 (▲) antigen (Ag) activities by Mo-RIAs, for DNA polymerase (▨), and for sucrose density (○). (b) Southern blot analysis in a sucrose gradient. DNA was isolated from each fraction and separated on a 1.5% agarose gel, and HBV DNA was detected by Southern blotting. The size markers (M) are indicated as in Fig. 4.

HBV but was similar to that observed in transfected HepG2T4 cells (32). These results do, however, suggest maintenance of HBV replication in HepG2-BV cells.

Detection of HBV-specific transcripts in HepG2-BV cells. RNAs from HepG2-BV cells and noninfected HepG2 cells were isolated, Northern blotted, and hybridized to nick-translated full-length HBV DNA and to subgenomic fragments obtained after *Bam*HI digestion (Fig. 7). The selected *Bam*HI fragments corresponded to either nucleotides 1399 to 3105 (which represent the X and core regions) or nucleotides 3105 to 490 (which represent the pre-S1 and pre-S2 regions

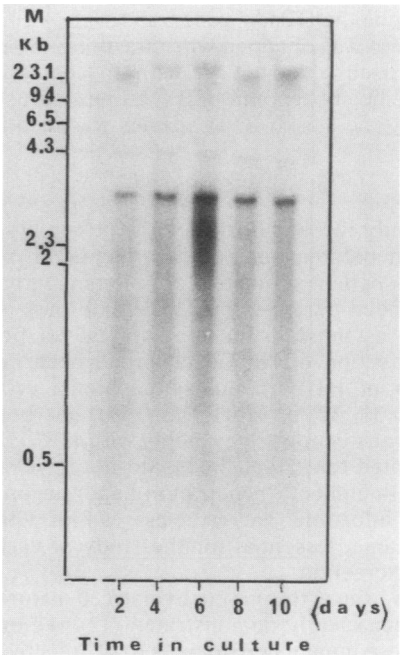

FIG. 6. Kinetic analysis of intracellular extrachromosomal HBV DNA. Extrachromosomal DNA from HepG2-BV cells was isolated as described by Hirt (9), on days 2, 4, 6, 8, and 10 after plating. A 10-μg sample of undigested DNA was loaded in each lane and analyzed by Southern blotting by using ^{32}P-labeled whole HBV DNA as the probe. Size markers (M) are indicated as in Fig. 4.

and a portion of the S region) on the physical map of the HBV genome. With whole HBV DNA as the probe (Fig. 7a, lane 2), two major transcripts of 3.5 and 2.1 kb were detected, as well as three additional minor bands with apparent lengths of 2.7, 2.3, and 1.9 kb. The 3.5-kb species may represent the reported pregenomic RNA, and the 2.1-kb

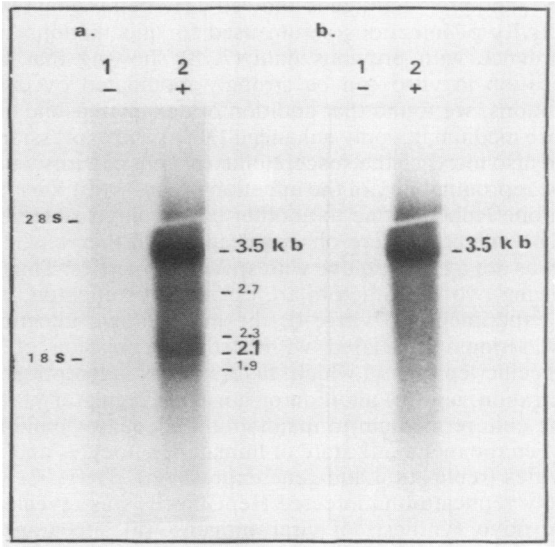

FIG. 7. Northern (RNA) blot analysis of total RNA from HepG2-BV cells (lanes 2) and control cells (lanes 1) with different regions of HBV DNA as probes. (a) Hybridization with the entire cloned HBV DNA. (b) Hybridization with a probe corresponding to the X-core region (as described in the text). Symbols: +, HBV infected; –, control.

species may be the HBsAg gene transcript. A similar hybridization pattern was obtained when the pre-S–S-specific HBV probe was used (data not shown). In contrast, when the core-X-specific subgenomic HBV probe was used (Fig. 7b, lane 2), only the 3.5-kb RNA species was identified.

DISCUSSION

In this study, we demonstrated the possibility of infecting the human hepatoblastoma cell line HepG2 (1, 2) with HBV virions from patient serum and of obtaining virus replication for an extended period (HepG2-BV cell line). All previous attempts to establish an in vitro system for production of HBV by infection of HepG2 cells had been unsuccessful. Propagation of HBV in human hepatoma cell lines, like HepG2 (25, 31, 32) or HuH-7 (35, 39), has been achieved only after transfection experiments with HBV DNA. It was thus postulated that HepG2 cells do not have a receptor(s) for virus attachment, penetration, and fusion leading to productive infection. Nevertheless, an in vitro infection system remained essential for the study of early events of virus-cell interaction.

Therefore, since the parenchymal cell nature of HepG2 cells has been clearly demonstrated (12), we examined the ability of these human hepatocytes to be infected with HBV, and we defined the conditions required for successful HBV infection. (i) The antigenic and morphologic characteristics of HBV virions used for inoculation were analyzed in detail and shown to be critical (20). We demonstrated previously that the serum expression of pre-S1-specific epitopes involved in recognition of HBV by hepatocyte receptors (22) correlated well with the level of HBV replication and thus with the infectivity of serum (Petit et al., in press). In addition, we showed that the pre-S-coded part of the HBV envelope proteins, which is sensitive to protease in vitro, could be easily released from virus particles in vivo, inducing destabilization of the native HBV structure (21) and affecting viral infectivity (20). The Mo-RIAs developed by us (Petit et al., in press) for simultaneous detection of HBsAg, pre-S2, and pre-S1 antigens allowed us to better quantify the infectivity of infectious serum used for inoculation. (ii) In accordance with previous data (7, 8) showing that HBV replication in vitro can be strongly modulated by culture conditions, we found that addition of dexamethasone to the culture medium not only enhanced HBV gene expression but could also increase the susceptibility of cells to virus, as well as the reproducibility of the infection process. (iii) Variations from one HepG2 clone to another concerning susceptibility to HBV infection were observed, although the HepG2 cell line was not a unique clone with special properties. Thus, the following two main factors are essential for effective infection experiments in vitro: (i) the morphologic integrity of HBV virions associated with strong expression of pre-S1-specific epitopes, which favors virus adsorption and penetration, and (ii) addition of hormone regulatory factors to the culture medium to maintain an adequate equilibrium between the metabolic state of human hepatocytes and viral activities (replication and gene expression).

HBV replication in infected HepG2 cells was revealed by (i) de novo synthesis of viral antigens, (ii) appearance of intracellular specific HBV DNA replicative forms, (iii) identification of viral transcripts, and (iv) release of complete HBV particles (Dane-like particles). As previously described for duck HBV (37), synthesis of HBV envelope antigens (HBs, pre-S1, and pre-S2) and HBV core antigens (P22c and perhaps hepatitis B x proteins) was detected only 4 to 8 days postinfection. Electron microscopic examination showed that the HBV-specific antigens were released from cells in spherical and filamentous particles, as well as in 42-nm-diameter HBV Dane-like particles. We noticed that at 7 days postinfection, the relative number of filaments rich in pre-S1-specific large HBsAg proteins (P39 and GP42) was higher than that of complete virions. This is in agreement with results obtained by Thézé et al. (34), who used hepatocyte primary cultures derived from naturally infected woodchucks. Thus, early postinfection, an excess of pre-S1 antigens over HBsAg was observed in HBV-infected HepG2 cells, as in dimethyl sulfoxide-treated E4 cells obtained by transfection of HepG2 cells with HBV DNA (7). Such overexpression of the pre-S1 antigen following infection or transfection of hepatocytes could result from addition of either dexamethasone or dimethyl sulfoxide to the culture medium to increase cell infectivity. In fact, after several passages without dexamethasone (HepG2-BV cells), we observed regulation of production of HBV envelope proteins leading to well-balanced synthesis of three antigenic specificities, HBs, pre-S2, and pre-S1, as complete virions. HepG2-BV cells consistently synthesized and released HBV particles at levels higher than those obtained from cells 1 week postinfection.

During week 1 postinfection, the presence and physical state of HBV DNA in the nuclei of infected HepG2 cells were examined, and as early as 2 days postinfection, the RC and CCC forms were detected. CCC DNA accumulated with increasing time postinfection, which favors a full cycle of viral replication in our experimental infection system of HepG2 cells. No HBV DNA integration was demonstrated in the DNA of the cells at that time. These findings are similar to those reported in actively infected human liver (4, 18, 24) and in infected duck liver cells (16, 29, 33, 36). When HBV-infected HepG2 cells were maintained for several months after passage (HepG2-BV cell line), the kinetic analysis of extrachromosomal HBV DNA showed that the following replicative intermediate forms were present: the RC form, the linear form, the CCC form, and a smear due to the molecular heterogeneity of the HBV single-stranded region of variable length. Synthesis of these replicative intermediates increased by day 6 after subculturing and thus appears to be dependent on the cell growth cycle. The respective amount of the 3.2-kb double-stranded linear DNA was high. Moreover, a high-molecular-weight DNA component was detectable at a constant level, as in transfected HepG2T14 cells (32), suggesting the presence of integrated HBV DNA sequences. Analysis of the RNA of HepG2-BV cells revealed the presence of two major species (3.5 and 2.1 kb), corresponding to the most abundant mRNAs which are products of HBV transcription in infected liver (10). The 3.5-kb mRNA is used as pregenomic RNA for replication of HBV DNA and as a template for translation into viral core proteins. The 2.1-kb mRNA codes for synthesis of the major HBsAg protein. These results indicate active replication in HepG2-BV cells according to the model proposed by Summers and Mason (15, 29) and others (4, 6, 38).

The HBV particles persistently released by HepG2-BV cells into the medium have the same antigenic (HBs, pre-S2, and pre-S1 activities), morphological, and biological (DNA polymerase activity) properties as virions found in infectious human sera. They contain mainly mature RC forms of viral DNA. Minor components identified as CCC and single-stranded DNAs could result from release of either immature viral particles or intracellular viral DNA forms as a result of cell death. Thus, we demonstrated that the HepG2-BV cell

line can support the assembly and secretion of intact virus particles and are competent for HBV production as a transfected HepG2 cell line (25, 32).

Successful in vitro infection of HepG2 cells strongly suggests that such human hepatoma cells display a receptor(s) that is necessary for attachment and penetration of HBV and leads to infection. Binding experiments carried out by us with serum-derived HBV particles and HepG2 cells (20) support this hypothesis. This is the first report of long-term culture establishment of HBV-infected HepG2 cells, hepatoblastoma cells of human origin which support complete-cycle infection. This long-sought-after in vitro infection system of human hepatocyte cultures with HBV seems to be functionally stable and capable of maintaining virus activity. Therefore, this model allows the study of the early events of HBV infection and represents a powerful tool for gaining a fuller understanding of (i) HBV-target cell interaction, (ii) neutralizing properties of human polyclonal or mouse monoclonal HBV-specific antibodies, and (iii) the mechanism of action of antiviral agents used for treatment of chronic hepatitis B.

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