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Production of Infectious Duck Hepatitis B Virus in a Human Hepatoma Cell Line

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The differentiated human hepatoma cell line Hep-G2 was transfected with cloned duck hepatitis B virus (DHBV) DNA. Introduction of closed circular DNA into the human liver cells resulted in the production of viral proteins: core antigen was detected in the cytoplasm, and e antigen, a related product, was secreted into the medium. Moreover, viral particles were released into the tissue culture medium which were indistinguishable from authentic DHBV by density, antigenicity, DNA polymerase activity, and morphology. Intravenous injection of tissue culture-derived DHBV particles into Pekin ducks established DHBV infection. In conclusion, transfection of human hepatoma cells with cloned DHBV DNA results in the production of infectious virus, as occurs with cloned human hepatitis B virus DNA. Human liver cells are therefore competent to support production of the avian and mammalian hepadnaviruses, indicating that liver-specific viral gene expression is controlled by evolutionarily conserved mechanisms. This new DHBV transfection system offers the opportunity to rapidly produce mutated DHBV which then can be further investigated in Pekin ducks.

Hepadnaviruses are hepatotropic DNA viruses with a narrow host range which cause acute and chronic liver disease in humans and in a variety of animals. Chronic infection can result in primary liver cancer (2, 5). Most of the information on the biology of hepadnaviruses is derived from the animal-infecting members of this virus family. This is due to the fact that, until recently, tissue culture systems for propagation of the human hepatitis B virus (HBV), which is infectious only for humans and higher primates, were not available. Recently, it became possible to produce HBV in hepatoma cells in vitro by transfection (1, 4, 18, 23). However, these systems have the limitation that the hepatoma cell lines used for transfection experiments cannot be infected, and thus studies of the infectivity of mutated HBV still require chimpanzees, the only animal known to be susceptible to HBV infection. A way to overcome these limitations would be the use of a similar cell culture system for one of the animal-infecting members of the hepadnaviruses for which animals for in vivo experiments are more available. The only other tissue culture system described uses primary duck hepatocytes, which have been shown to support replication of duck hepatitis B virus (DHBV) in vitro upon infection with DHBV (10, 20). Unfortunately, transfection of these cells resulted only in production of viral components in quantities not sufficient for further studies (P. Galle, unpublished data).

The present study was conducted to combine the major advantage of in vitro transfection systems, i.e., the easy and rapid production of mutated viruses, with the availability of Pekin ducks for the subsequent in vivo investigation of these mutants. We used cloned DHBV DNA for transfection of human hepatoma cells. This resulted in the production of DHBV particles in vitro which were infectious in vivo. This new approach provides the unique opportunity to confirm and complement data derived from tissue culture experiments.

Moreover, this system can be used to study liver-specific gene expression. The human hepatoma cell line Hep-G2 supports not only replication of HBV (1, 18) but also, as demonstrated here, that of DHBV. Since the regulatory elements essential for tissue-specific gene expression are located within two very small viral genomes (about 3 kilobases) with low overall sequence homology (9), the HBV-DHBV expression system provides a valuable model for the study of liver-specific gene regulation.

MATERIALS AND METHODS

Cell culture and transfection. Hep-G2 and HeLa cells were grown at 37°C in Dulbecco minimal essential medium supplemented with 10% fetal calf serum, 5 mM glutamine, 100 μg of penicillin per ml, and 100 μg of streptomycin per ml and passaged two or three times per week at a 1:3 dilution. For transfections, closed circular DHBV DNA was prepared from plasmid pD16 (12), which carries a full-length DHBV type 16 (DHBV-16) genome in the *Eco*RI site of pUC13. Full-length DHBV DNA was excised with *Eco*RI, circularized (10 μg of total DNA per ml) with T4 DNA ligase (Boehringer GmbH, Mannheim, Federal Republic of Germany), and introduced into cells by calcium phosphate transfection (6). Subconfluent Hep-G2 or HeLa cells were passaged at a 1:5 dilution; 20 h later the medium was changed, and another 2 h later 1 ml of transfection cocktail (10 μg of DNA, 438 μl of Tris [pH 7.6], 62 μl of 2 M $CaCl_2$, 500 μl of HEPES-buffered saline [280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]) was added per 75-cm^2 dish. After 12 h, dimethyl sulfoxide was added to a final concentration of 10%, and the cells were incubated at 37°C for 20 min. The medium was changed afterwards. The cells and medium were harvested after 2 to 5 days.

Detection and characterization of DHBeAg and DHBcAg in culture medium and cell extracts by Western blotting (immunoblotting). Forty-eight hours after transfection, cytoplasmic extracts were prepared by lysing cells from 75-cm^2 dishes with 0.5 ml of 1% Nonidet P-40 (NP-40) in phosphate-buffered saline (PBS). Insoluble material was discarded. DHB core antigen (DHBcAg) from cell lysates and DHB e antigen (DHBeAg) from medium were concentrated by

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immunoprecipitation with a rabbit antiserum directed against bacterially synthesized DHBV core protein (J. Salfield, Ph.D. thesis, University of Heidelberg, Heidelberg, Federal Republic of Germany, 1986). Immunoprecipitation was carried out as described below, but the antigen-antibody reaction and subsequent washing steps were performed in 1% NP-40–PBS instead of PBS. The immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide (12.5%) gel electrophoresis and electrophoretically transferred to nitrocellulose filters by using the Bio-Rad electrotransfer system. The filters were then incubated with bovine serum albumin (1% in PBS) for 3 h followed by incubation with the anti-DHBcAg serum for 15 h. After being washed with 0.1% NP-40–PBS, the filters were incubated with 1 to 2 μCi ^{125}I-labeled protein A in 1% bovine serum albumin–PBS for 2 h, washed (two times for 30 min in 0.1% NP-40–PBS and two times for 30 min in PBS), dried, and autoradiographed.

Purification of DHBV particles from tissue culture medium. Particles from 100 ml of tissue culture medium were sedimented at 30,000 rpm for 17 h in a Beckman 45 Ti rotor at 4°C through a 10 to 30% sucrose gradient onto a 70% sucrose cushion. Samples of fractions were tested for DHBV DNA in a dot blot assay (see below). DHBV-DNA-positive fractions were pooled and dialyzed, and 50% of the material was subjected to isopycnic centrifugation in a preformed CsCl gradient (1.15 to 1.5 g/cm^3) at 30,000 rpm for 24 h in an SW40 rotor. Fractions (0.5 ml) were collected from the bottom of the tube. The density of each fraction was determined by refractometry. A sample of each fraction was assayed for DHBV DNA as described below.

Dot blot analysis for DHBV DNA and electron microscopy. The presence of DHBV-DNA-containing particles in gradient fractions was assessed by dot blot analysis essentially as described previously (16). Briefly, 5 μl from each fraction was spotted on nitrocellulose, denatured, and neutralized as described for Southern hybridization and baked for 0.5 h at 80°C. Hybridization was performed with nick-translated plasmid pD16. For dot blot analysis of sera from Pekin ducks, 1 μl of serum was used. Gradient-derived particles were examined by transmission electron microscopy after negative staining with 2% aqueous uranyl acetate on carbon-coated grids.

Immunoprecipitation of DHBV particles from tissue culture medium. Particles from tissue culture medium were subjected to immunoprecipitation with a rabbit antiserum directed against the pre-S portion of the viral envelope (11) 5 days posttransfection. Antibodies were adsorbed to preswollen protein A-Sepharose in RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate) for 1 h at room temperature. After a washing with PBS, immunoprecipitation was carried out for 16 h at 4°C with 25 μl of Sepharose per 10 ml of medium.

Endogenous DNA polymerase reaction. Particles immunoprecipitated with anti-pre-S serum and bound to protein A-Sepharose were washed twice with 1 mM Tris (pH 7.4)–10 mM EDTA. The endogenous polymerase reaction was performed essentially as described previously (8) but in the presence of 1% NP-40. After proteinase K digestion and phenol extraction, radiolabeled DNA was loaded on a 1.5% agarose gel, which was subsequently dried and autoradiographed.

Ducks and in vivo infection. One-day-old ducklings were purchased from a commercial supplier. Serum was obtained by bleeding the foot vein or by cardiac puncture and assayed for the presence of DHBV DNA by dot blot hybridization.

Four-day-old Pekin ducks found to be DHBV DNA negative in serum were injected intravenously with gradient-purified virus. For this purpose, fractions 11 to 15 (see Fig. 2) from the CsCl gradient were pooled and dialyzed against PBS; 20% of the material was injected per duck.

RESULTS

In vitro expression of DHBcAg and DHBeAg in human hepatoma cells. Cloned circular DHBV-16 DNA was transfected into the human hepatoma cell line Hep-G2 or the epithelial cell line HeLa. After 48 h, cell lysates and culture media were analyzed for the presence of viral proteins. We examined the production of DHBcAg and a related product, DHBeAg (12), since core gene expression in vivo is coupled to synthesis of the RNA pregenome required for viral replication (5). Large amounts of DHBcAg were detected in the cytoplasm of Hep-G2 cells (Fig. 1). Moreover, Hep-G2 cells secreted DHBeAg into the medium. In contrast, neither core-gene product was synthesized by HeLa cells. Therefore, the DHBV core gene is expressed in Hep-G2 cells but not in HeLa cells. This result is in agreement with the finding for HBV that core-gene expression is restricted to highly differentiated hepatoma cells (1, 4, 18, 23).

Production of DHBV particles in tissue culture. To determine whether in vitro production of DHBcAg and DHBeAg was accompanied by production and secretion of DHBV particles, tissue culture medium obtained 4 days posttransfection of Hep-G2 cells with DHBV DNA was subjected to CsCl equilibrium centrifugation (Fig. 2). DHBV-DNA-containing particles were detected by dot blot analysis at a buoyant density of about 1.15 g/cm^3 (fractions 12 to 15), which is the density of DHBV derived from infectious serum (11). Electron microscopy of the peak fraction (Fig. 2, fraction 13) revealed enveloped particles resembling authentic DHBV.

For further characterization of the viruslike particles, medium was subjected to immunoprecipitation with an antiserum directed against the viral pre-S protein, which is part of the viral envelope (11). The immunoprecipitated material was then used to carry out an endogenous polymerase reaction (8) in which radioactive nucleotides are incorporated into the partially single-stranded viral genome. The

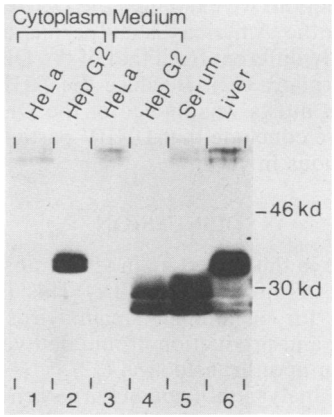

FIG. 1. Western blot analysis of tissue culture material (lanes 1 to 4) with an antiserum directed against DHBcAg/DHBeAg. Lanes: 1 and 2, cytoplasm of HeLa cells and Hep-G2 cells, respectively; 3 and 4, media of HeLa cells and Hep-G2 cells, respectively; 5 and 6, authentic DHBeAg and DHBcAg, respectively as detected in serum and liver from an infected duck. kd, Kilodaltons.

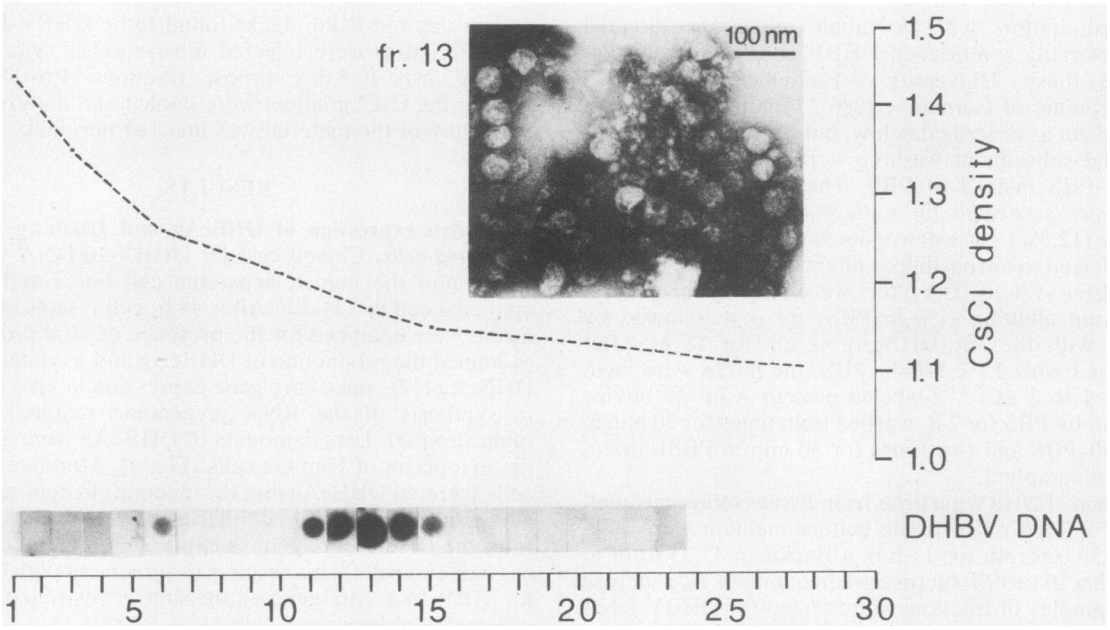

FIG. 2. CsCl equilibrium centrifugation of tissue culture supernatant. The densities of the fractions are indicated by the broken line. The fractions were analyzed for the presence of DHBV DNA by a dot blot assay (bottom inset). The top inset shows an electron micrograph of particles observed in fraction 13 (fr. 13).

same radiolabeled DNA species were obtained with the virus sample from tissue culture as with the authentic-DHBV control (Fig. 3). Restriction digests of the labeled DHBV DNA from medium-derived particles revealed bands as predicted for the cloned DHBV-16 DNA used for transfection. Thus, the viruslike particles released from Hep-G2 cells during transient expression of DHBV DNA can be immunoprecipitated with an antiserum specific for a viral surface protein and contain viral DNA and DNA polymerase.

DHBV particles produced in vitro are infectious in vivo. Finally, to investigate whether these particles were infectious, CsCl-gradient-purified particles were injected intravenously into five 4-day-old Pekin ducks. After 18 days, all five ducks had converted from DHBV DNA negative to DHBV DNA positive in serum and liver (Fig. 4). To prove that the infection was caused by the American DHBV-16 subtype used for transfection, we examined the DHBeAg phenotype. DHBeAg from the American subtype has a glycosylation pattern distinctly different from that of the DHBV-3 subtype endemic in Germany (12). Because the DHBeAg from the sera of infected ducks was indeed of the DHBV-16 phenotype (Fig. 4), we conclude that DHBV particles produced in vitro are infectious in vivo.

DISCUSSION

We introduce in this report an in vitro transfection system for production of infectious DHBV. This is the first cell culture system for an animal hepadnavirus which allows efficient testing and production of mutated variants by transient expression upon transfection (12).

A functional analysis of hepadnavirus genomes by transfection was first approached in vivo: intrahepatic injection of cloned hepadnavirus DNA into the host animal resulted in productive infection (13, 16, 21, 22), proving the infectivity of cloned DNAs. In these systems, however, a more detailed mutational analysis is possible only for mutants capable of replication. Replication-incompetent mutants can only be assessed as such, without further information as to the nature of their defects.

Testing of viral mutants in cultured cells competent to support hepadnavirus replication should allow a more thorough evaluation, including the examination of early genes. During the past 2 years, in vitro investigation of hepatitis B viruses has been approached successfully in two different ways. Primary duck hepatocytes were shown to support replication of DHBV (10, 20), and differentiated human hepatoma cell lines were proved capable of producing infectious HBV upon transfection with HBV DNA (1, 4, 18, 23). However, the first system cannot be used for transfection experiments and thus cannot be applied for production of mutated viruses. The latter system is limited in that subsequent in vivo investigations, e.g., to assess the infectivity of viral mutants produced in cell culture, depend on the chimpanzees, the only animals that can be infected with HBV. The DHBV expression system described here provides for the first time the possibility of easily testing a hepatitis B virus and its mutated forms in vitro and in vivo. Since DHBV is the animal hepadnavirus most distantly related to HBV, it seems very likely that Hep-G2 cells can be used to establish similar systems for ground squirrel hepatitis virus and woodchuck hepatitis virus.

Our data demonstrate that a differentiated human hepatoma cell line which has recently been shown to support replication of HBV upon transfection with HBV DNA (1, 18) is also competent for production of infectious DHBV. The finding that both a human and an avian hepadnavirus genome can replicate in Hep-G2 cells has further implications. First, it suggests that the host specificity of hepadnaviruses is not caused by intracellular factors but is due to selective interaction of viral envelope proteins, involving most likely the pre-S domain, with a yet undefined host cell receptor. Second, transcription of these distinctly different viral genomes must be controlled by regulatory elements which function equally well in human and duck liver cells. A

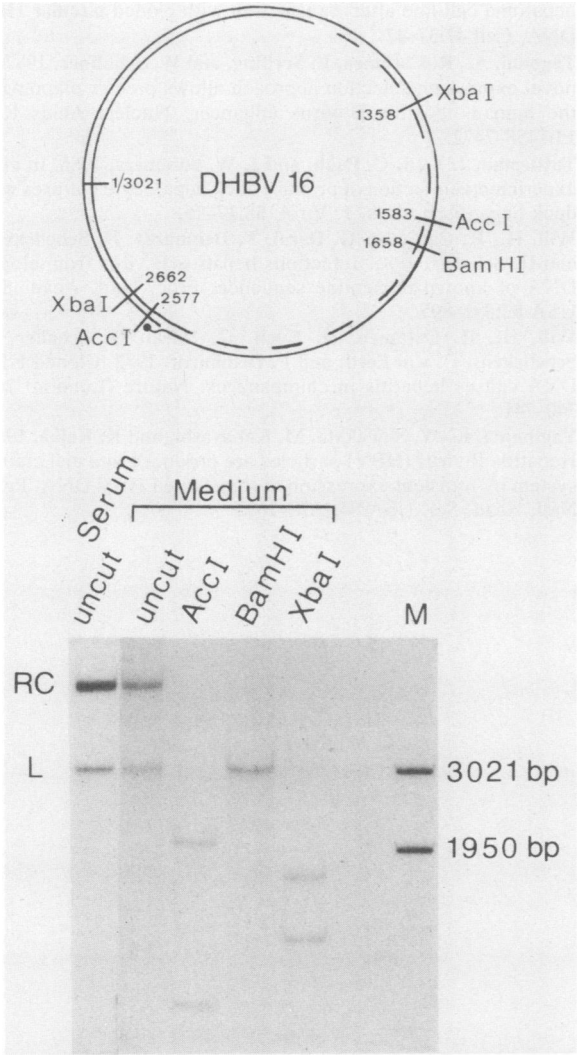

FIG. 3. Endogenously labeled DHBV DNA from serum-derived virus (Serum) and from particles immunoprecipitated from cell culture medium (Medium). The two left lanes show uncut DNAs. In the next three lanes DNAs from medium-derived particles are shown after cleavage with the restriction enzymes *Acc*I, *Bam*HI, and *Xba*I. The restriction sites of the enzymes in the viral genome are shown at the top. RC and L, Relaxed circular and linear forms, respectively, of the viral DNA. Lane M, Size markers. bp, Base pairs.

regulatory region upstream of the HBV core-gene promoter, which appears to enhance gene expression in liver cells (7, 15, 19), binds nuclear protein factors from differentiated hepatoma cells (14). In a gel mobility shift assay, nuclear proteins from duck liver bound specifically to two small sections of DHBV DNA upstream of the DHBV core-gene promoter (M. Fischer, diploma thesis, University of Heidelberg, Heidelberg, Federal Republic of Germany, 1987). Several of the protein-binding regions detected in the two viral genomes share the conserved motif TGATTGGA. Since a similar sequence is present in the proximal element in several mammalian albumin promoters (3), this motif might be a first candidate for a general liver-specific *cis*-acting signal predicted on the basis of our experiments. Since the viral genomes containing these elements are very small (about 3 kilobases) and have low overall sequence

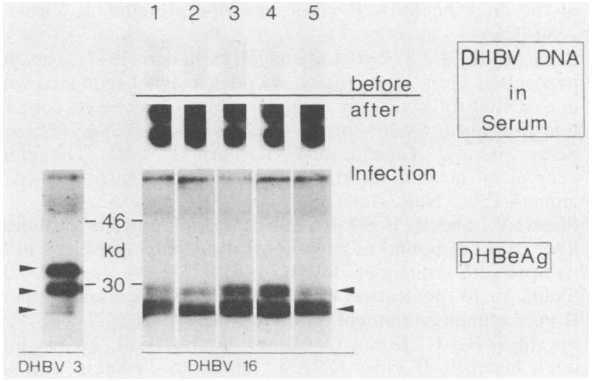

FIG. 4. Infectivity of DHBV-like particles in vivo. Particles from the CsCl gradient (Fig. 2, fractions 11 to 15) were injected intravenously into five Pekin ducks seronegative for DHBV DNA (top panel). Eighteen days later sera were assayed for DHBV DNA (middle panel) and DHBeAg (bottom right panel); the DHBeAg was of the DHBV-16 phenotype. The bottom left panel shows DHBeAg from the serum of a DHBV-3-infected duck. kd, Kilodaltons.

homology, the hepadnaviruses provide a particularly attractive system to study liver-specific gene expression.

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