Duck Hepatitis B Virus (DHBV) Particles Produced by Transient Expression of DHBV DNA in a Human Hepatoma Cell Line Are Infectious In Vitro

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Transfection of the human hepatocellular carcinoma cell line HuH7 with a plasmid containing a tandem copy of the duck hepatitis B virus DNA sequence resulted in transient replication of the virus. Viral particles secreted by transfected HuH7 cells exhibited physical properties similar to those of serum-derived duck hepatitis B virus and were infectious in primary duck hepatocyte cultures.

The hepadnaviruses are a family of small enveloped DNA viruses which can produce both acute and chronic liver diseases in their respective animal hosts. The lack of a suitable tissue culture system with which to study hepadnavirus infections in vitro is a problem only recently circumvented by the discovery that certain human hepatocellular carcinoma cell lines can support hepatitis B virus (HBV) replication after transfection with cloned HBV DNA (2, 12, 14). These cell lines may be useful for testing the effects of mutations in viral genes on viral gene expression and assembly. However, the ability to test the biological activities of mutant HBVs that may be assembled and secreted from such transfected cells is severely limited by the lack of a cell culture system that can be infected in vitro by any mammalian hepadnavirus. Such an in vitro infection system has only been described for duck HBV (DHBV) with primary cultures of duck hepatocytes (13).

A recent report revealed that a human hepatoma cell line, HepG2, previously shown to be competent for HBV replication after transfection with cloned HBV DNA (2, 12) can support high levels of expression of the DHBV core antigen (DHBcAg) gene products under control of DHBcAg gene promoter sequences (9). Moreover, this cell line can produce infectious DHBV when transfected with closed circular DHBV DNA (3). In the present study, we used the in vitro system for DHBV infection of duck hepatocytes (13) to test the capacity of an independently isolated human hepatoma cell line, HuH7 (8), to produce infectious DHBV particles after introduction of cloned DHBV DNA. This cell line has been shown to support HBV replication after transfection with cloned HBV DNA (2, 14).

The source of DHBV DNA used in all of our transfection assays was plasmid pSP.DHBV5.2Galx2 (p5.2Galx2), which consists of a tandem *EcoRI* dimer of the DHBV genome cloned into the unique *EcoRI* site of pSP65 (7). The DHBV sequences in p5.2Galx2 were derived from a virion DNA clone used by Mandart and co-workers for the determination of the DHBV DNA nucleotide sequence (5). A cloned DHBV dimer was used, as it should allow transcription of an RNA containing all the nucleotide sequence information present in the terminally repetitious DHBV RNA pregenome

(1). p5.2Galx2 DNA had previously been shown to result in the production of infectious virus following inoculation into the livers of young ducks (data not shown).

To investigate transient replication of DHBV in HuH7 cells, we performed a time course experiment in which several dishes of cells were transfected with equivalent amounts of plasmid DNA, harvested at intervals after transfection, and analyzed for the presence of replicative DHBV DNA intermediates (13). HuH7 cells maintained in Dulbecco minimum essential medium-Ham's nutrient mixture F12 (DMEM:F12) (1:1) (GIBCO Laboratories) medium plus 10% (vol/vol) fetal bovine serum were plated at 2×10^6 cells per 60-mm dish 24 h before transfection. Each dish was transfected with 3.5 μ g of p5.2Galx2 by the calcium phosphate precipitation procedure (4). At various intervals after transfection (see Fig. 1), cells were washed with HBS (2 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 0.15 M NaCl [pH 7.45]), and 1 ml of lysis buffer (10 mM Tris hydrochloride [pH 7.9], 1 mM EDTA, 0.1% [wt/vol] Nonidet P-40, 50 mM NaCl, 8% [wt/vol] sucrose) was added directly to the cells. After incubation for 10 min at 37°C, the lysate was removed and centrifuged at 13,000 rpm for 2 min in a model B Microfuge (Beckman Instruments, Inc.) to pellet nuclei and other insoluble material. Viral core particles, which contain all the intermediates in DHBV DNA synthesis, were then isolated by the following procedure. The supernatant was removed, and magnesium acetate was added to a final concentration of 6 mM. DNase I (type II; Sigma Chemical Co.) was added to 100 μ g/ml, and the supernatant was incubated at 37°C for 15 min. Polyethylene glycol (6.5% [wt/vol]; PEG 8000; Baker Biochemicals), 0.35 M NaCl, and 10 mM EDTA were then added, followed by incubation for 30 min at 4°C. The precipitate that formed was pelleted in a Microfuge at 13,000 rpm for 4 min and suspended in 100 μ l of 10 mM Tris hydrochloride (pH 7.9)-6 mM magnesium acetate-100 μ g of DNase I per ml. After digestion of the suspension for 10 min at 37°C, 300 μ l of sodium dodecyl sulfate-pronase lysis buffer (25 mM Tris hydrochloride [pH 7.5], 10 mM EDTA, 0.1 M NaCl, 0.5% [wt/vol] sodium dodecyl sulfate, 0.5 mg of pronase per ml) was added, and the mixture was incubated for 30 min at 37°C before extraction with an equal volume of phenol. Nucleic acids were precipitated from the aqueous fraction with ethanol, dissolved in 10 mM Tris hydrochloride (pH 7.5)-1 mM EDTA, and subjected to electrophoresis in a

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FIG. 1. Transient replication of DHBV following transfection of HuH7 cells with DHBV DNA. Nucleic acids were isolated from cores partially purified from the cytoplasm of transfected cells at 2 (lane a), 3 (lane b), 5 (lane c), 6 (lane d), 7 (lane e), 8 (lane f), and 10 (lane g) days following transfection (see the text). The amount of nucleic acid in each lane represents that recovered from one-fourth of the cells on a 60-mm dish. Lane h represents an equivalent amount of material extracted from HuH7 cells that received no DNA. Lane i contains 10 pg of full-length linear DHBV DNA (3.0 kilobases) generated by *Eco*RI digestion of p5.1Gal (see the text). The positions of relaxed circular (RC) and SS replicative DHBV DNA species are shown on the left. *Hind*III-digested bacteriophage λ DNA size markers (in kilobases) are shown on the right.

1.5% (wt/vol) agarose gel under conditions previously described (13). Following electrophoresis, the gel was soaked for 30 min in 0.2 N NaOH–1.5 M NaCl and transferred to a nylon membrane (Hybond N; Amersham Corp.) in 0.2 M Tris hydrochloride (pH 4.0)–1.5 M NaCl by the Southern

blotting procedure (10). After transfer, the filter was washed briefly in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), air dried, and exposed to a UV light source (240 to 390 nm) for 5 min to covalently cross-link DNA to the membrane. The filter was hybridized at 50°C with a ^{32}P -labeled DHBV ribonucleotide probe of plus-strand polarity produced by in vitro transcription with SP6 polymerase of plasmid pSP.DHBV5.1Gal (p5.1Gal), a pSP65-derived clone containing a single copy of the entire DHBV genome cloned at the *Eco*RI site. Autoradiography was performed at -80°C with Kodak X-OMAT AR film and an intensifying screen.

The results in Fig. 1 show that core particles isolated from the cytoplasm of HuH7 cells transfected with a DHBV plasmid dimer contained replicative DHBV DNA molecules. The appearance de novo of single-stranded (SS) DHBV DNA of minus-strand polarity, the product of reverse transcription of viral pregenome RNA (11), indicated that DHBV DNA replication in HuH7 cells was similar to that observed in normal permissive host cells. It appeared that the total amount of replicating DHBV DNA in HuH7 cells was maximal approximately 3 days after transfection. The amount of viral DNA remained at this level for a further 3 days, at which time the relative abundance of DHBV DNA declined (Fig. 1). However, SS DHBV DNA in intracellular particles could still be detected as late as 10 days after transfection.

To investigate the relative abundance and morphology of transfected HuH7 cells supporting DHBV replication, we stained cells for the presence of viral core antigen (DHBcAg) 5 days after transfection. Cells were fixed at -20°C in 95% (vol/vol) ethanol–5% (vol/vol) glacial acetic acid. DHBcAg was detected by indirect immunofluorescence with rabbit antiserum to DHBcAg (provided by W. Mason) and rhodamine-conjugated goat anti-rabbit immunoglobulin G (Organon Teknika) (13). DHBcAg was present in the cytoplasm of approximately 1% of the HuH7 cells (Fig. 2). Interestingly, many cells that stained positive for DHBcAg exhibited

FIG. 2. Transient expression of DHBcAg in HuH7 cells transfected with DHBV DNA. Cells were fixed 5 days after transfection and stained for DHBcAg by indirect immunofluorescence (see the text). Panels A and B represent the same field on a dish of cells transfected with the DHBV dimer plasmid p5.2Galx2. In panel A, the film was exposed simultaneously to UV light and white light to visualize all cells in the field in addition to those that expressed DHBcAg. Cells in panel C were transfected with plasmid pSP65, which contains no DHBV DNA. Bar in panel C, 60 μm . Photographs were taken with Polaroid 57 film and a Nikon HFX-II camera attached to a Nikon Diaphot inverted microscope.

an abnormal morphology, and many were either binucleate or contained abnormal lobulated nuclei (data not shown). Transfected cultures stained for DHBcAg 8 days after transfection with the same amount of DHBV DNA contained significantly less than 1% DHBcAg-positive cells (data not shown). The relationship between the abnormal morphology of the antigen-positive cells and the subsequent disappearance of staining cells is unknown but suggests cell death.

To test whether virus produced in the HuH7 cells was infectious in normal host cells, we assayed culture fluids from transfected cells for infectivity in primary duck hepatocyte cultures. Hepatocytes were isolated from the livers of 2-week-old Pekin ducks by collagenase perfusion (13). Tissue culture petri dishes (60 mm) were seeded with ca. 5×10^6 cells in serum-free L15 medium (GIBCO) containing 10 mM sodium bicarbonate and 1.5% (vol/vol) dimethyl sulfoxide in addition to the supplements previously described (13). Cells were incubated at 37°C in an atmosphere containing 5% CO₂ (vol/vol). The addition of 1.5% (vol/vol) dimethyl sulfoxide greatly improves the capacity of duck hepatocyte cultures to support DHBV replication over a period of 2 weeks (J. C. Pugh and J. Summers, manuscript in preparation).

A 100-mm dish was seeded with ca. 5×10^6 HuH7 cells and transfected after 24 h with 10 µg of p5.2Galx2 as described above. At 48 h after transfection, the medium (DMEM:F12 plus 10% fetal bovine serum) (vol/vol) was replaced; after incubation for a further 3 days, the culture medium was removed from the cells and distributed equally onto three 60-mm dishes of primary duck hepatocytes prepared 48 h earlier. The hepatocytes were infected for 20 h at 37°C with the HuH7 culture medium. Cells were harvested for DNA preparation (13) at 3, 5, and 9 days postinfection, and total intracellular DNA was analyzed for the presence of DHBV DNA replication intermediates by Southern blot hybridization (Fig. 3). The results showed that replicative DHBV DNA was produced in the hepatocyte cultures and that the amount of viral DNA increased during the course of the infection (Fig. 3a to c). Hepatocytes that were not infected with the HuH7 culture medium contained no DHBV DNA after 8 days of incubation (Fig. 3d). Thus, we conclude that HuH7 cells transfected with a DHBV dimer plasmid support transient replication of DHBV DNA and secrete infectious DHBV particles.

To better characterize the infectious viral particles produced by HuH7 cells, we subjected the culture fluids to isopycnic centrifugation in CsCl under conditions that will band serum-derived DHBV and assayed gradient fractions for infectivity in vitro. Culture medium (2.5 ml) removed from HuH7 cells 5 days after transfection with p5.2Galx2 (see above) was adjusted to a density of 1.2 g/cm³ by the addition of solid CsCl. The sample was overlaid with silicone oil and centrifuged in an SW56 rotor (Beckman) at 4°C for 20 h at 50,000 rpm. As a control, an identical gradient was prepared with 100 µl of DHBV-containing duck serum in 2.5 ml of DMEM:F12. Fractions (100 µl) were collected dropwise from the bottom of the tubes. Fractions were assayed directly for the presence of DHBV DNA by slot blot hybridization with a ³²P-labeled ribonucleotide probe of plus-strand polarity, as described for Fig. 1 (Fig. 4, top panel). To assay for infectivity across the gradients, we added 10 µl from each fraction directly to individual 60-mm dishes of primary duck hepatocytes. Cells were infected with the gradient fractions at 37°C for 24 h, at which time the inocula were replaced with fresh L15 medium. The final concentration of CsCl during infection was approximately 8 mM, a concentration which did not appear to result in any

FIG. 3. In vitro assay for infectivity of viral particles released from transfected HuH7 cells. Hepatocytes were infected with culture medium from transfected HuH7 cells and harvested 3 (a), 5 (b), or 9 (c) days after infection for DNA analysis (see the text). Lane d represents DNA from uninfected hepatocytes. DNA isolated from one-fourth of a 60-mm dish of hepatocytes was loaded in each of the lanes. The positions of relaxed circular (RC) and SS forms of intracellular viral DNA are shown on the right.

obvious cytopathic effect. At 8 days after infection, cells were harvested for DNA preparation and Southern blot analysis to detect DHBV DNA (Fig. 4). Infectivity in the HuH7 cell culture medium was associated with particles exhibiting an average density of 1.22 g/cm³ in CsCl (Fig. 4B). Although this value is higher than that previously reported for DHBV (6), it corresponds closely in this experiment to the densities of both infectious DHBV particles (Fig. 4A) and DHBV DNA-containing particles (Fig. 4, top panel) derived from duck serum. Thus, the above results show that HuH7 cells transfected with a DHBV DNA plasmid dimer produce mature, infectious viral particles that exhibit physical properties similar to those of DHBV particles isolated from infected ducklings. Since the conditions used here for maintaining primary duck hepatocyte cultures allowed considerable amplification of infecting virus because of replication in individual cells and lateral spread of the virus through secondary cycles of infection (Pugh and Summers, in preparation), this assay was able to detect an amount of infectious DHBV that would be barely detectable by standard dot blot hybridization analysis. We estimated from the relative hybridization signals in Fig. 4A and 4B that 1 ml of the HuH7 cell culture medium removed from cells 5 days after transfection contained an equivalent of only approximately 0.3 µl of DHBV-containing serum. The titer of DHBV DNA-containing particles in this serum sample was determined to be 1.5×10^{10} /ml by hybridization analysis (data not shown), and we therefore estimated that 1 ml of HuH7 culture medium contained approximately 4.5×10^6 viral particles.

Both our results and those of Schlicht and co-workers (3, 9) show that at least two independently derived human hepatoma cell lines, HuH7 and HepG2, are competent in supporting DHBV gene expression and virus production.

FIG. 4. Characterization of infectious DHBV particles produced after transfection of HuH7 cells with DHBV DNA. Viral particles derived from duck serum (A) or culture medium from transfected HuH7 cells (B) was subjected to equilibrium density gradient centrifugation in CsCl, and samples from individual fractions were used to infect duck hepatocyte cultures (see the text). The top panel shows the density of CsCl in each fraction, and the arrow marks the position of DHBV DNA as determined by slot blot hybridization of fractions in panel A. Each lane in panel A received DNA from 5% of a 60-mm dish of hepatocytes. Each lane in panel B received DNA from 25% of a 60-mm dish of cells. The positions of relaxed circular (RC) and SS DHBV DNA are shown on the left.

One theory proposed to account for the very limited host range exhibited by hepadnaviruses has been that essential transcription factors may be lacking in nonpermissive host cells. Thus, the ability of the HuH7 cell line to support the replication of two distinctly different hepadnaviruses, HBV and DHBV, is rather a surprising observation. The failure of individual hepadnaviruses to infect a wider variety of hosts may thus more likely be caused by a block in some early step in the infection process.

In conclusion, we have described a cell culture system that supports the production of infectious DHBV particles

following transfection with DHBV DNA. This system may prove valuable in analyzing the effect of mutations in specific viral genes on viral assembly, secretion, or infectivity.

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