Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 1005–1009, February 1987
Cell Biology

Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA

(episomal DNA/electron microscopy/hepatitis B surface antigen/hepatitis B e antigen)

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Communicated by Hans Popper, October 20, 1986 (received for review October 6, 1986)

ABSTRACT **The hepatoblastoma cell line Hep G2 was transfected with a plasmid carrying the gene that confers resistance to G418 and four 5′–3′ tandem copies of the hepatitis B virus (HBV) genome positioned such that two dimers of the genomic DNA are 3′–3′ with respect to one another. Cells of one clone that grew in the presence of G418 produce high levels of hepatitis B e antigen and of hepatitis B surface antigen. HBV DNA is carried by these cells as chromosomally integrated sequences and episomally as relaxed circular, covalently closed, and incomplete copies of the HBV genome. Viral DNA was detected also in conditioned growth medium at the buoyant densities characteristic for infectious Dane and immature core particles. Finally, HBV-specific components morphologically identical to the 22-nm spherical and filamentous hepatitis B surface antigen particles as well as 42-nm Dane particles were visualized by immunoelectron microscopic analysis. Therefore, we have demonstrated that the Hep G2 cell line can support the assembly and secretion not only of several of the replicative intermediates of HBV DNA but also of Dane-like particles. This *in vitro* system can now be used to study the life cycle of HBV and the reaction of immunocompetent cells with cells carrying HBV.**

The human pathogen hepatitis B virus (HBV) is one of a family of small DNA hepadnaviruses that share the ability to cause liver damage but differ completely in their host range specificity. The genome of HBV (as well as those of all of the hepadnaviruses) is a relaxed circular, partially double-stranded DNA molecule that is held together by hydrogen bonding of the ≈300-base-pair (bp) 5′ cohesive termini (1). The (−) strand has an invariable length of ≈3.2 kilobases (kb), whereas the (+) strand is ≥20% of this length (2).

Investigation of the expression and replication of the HBV genome as well as the full viral life cycle has been hampered by the lack of an *in vitro* tissue culture system in which HBV is propagated. In numerous attempts to rectify this situation, several mammalian cell lines have been transfected with cloned HBV DNA (3–10). Thus far, these experiments have yielded cells that are able to synthesize and secrete sizable amounts of two of the viral proteins, hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg). However, neither the replicative DNA intermediates similar to those identified in the livers of animals infected with other hepadnaviruses (11–15) nor Dane-like viral particles were obtained by the transfection experiments. Why these attempts to obtain viral replication by transfecting mammalian cells have failed has not been ascertained. Whatever the reason, it does not appear to be due to the inability of cloned HBV DNA to be infectious, since this DNA has been successfully used to cause the complete spectrum of clinical manifestations in chimpanzees (16).

The results reported here indicate that Hep G2 cells, hepatoblastoma cells of human origin, which have been transfected with cloned HBV DNA, support the replication of HBV DNA and intact virus particles.

MATERIALS AND METHODS

Construction of the Plasmid Vector. A recombinant vector, pDolTHBV-1, was prepared by insertion of the HBV DNA-containing fragments from a *Ban* I digest of pTHBV-1 (a pBR322 plasmid carrying two head-to-tail copies of the HBV genome; ref. 5) into the *Xho* I site between the long terminal repeats (LTRs) of Moloney murine leukemia virus in pDolmp10, a plasmid that is carrying the *neo* gene. Ligation of the fragment to the vector was performed after the staggered ends of both components were filled in by polymerization using the Klenow fragment of *Escherichia coli* polymerase I. We are grateful to R. Mulligan for providing the cloning vector, pDolmp10.

Cells, Culture Conditions, and Transfection. A human hepatoblastoma cell line, Hep G2, was obtained from B. Knowles. These cells were transfected with pDolTHBV-1, a vector that contains two head-to-tail dimers of HBV in a tail-to-tail orientation. Transformation with the plasmid was mediated by exposure of the cells to pDolTHBV-1 in the presence of 5 μg of Polybrene per ml (17, 18). The cultures were incubated at 37°C for 6 hr, and the cells then were shocked for 4 min with 30% dimethyl sulfoxide in minimal essential medium (MEM), washed several times with phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+}, and maintained thereafter in MEM supplemented with 10% fetal bovine serum and 380 μg of G418 per ml. Clones of cells that grew in the presence of G418 were isolated, allowed to grow to confluence, and tested for their ability to synthesize and secrete HBsAg and HBeAg. One of the clones obtained in this manner, designated 2.2.15, has been maintained for >6 months and was analyzed as described below. All cultures were maintained at 37°C in a moist atmosphere containing 5% CO_2 in air.

Assay of HBV-Specific Proteins. HBsAg was identified by solid-phase radioimmunoassay (RIA, Travenol-Genentech Diagnostics, Cambridge, MA). Various dilutions of culture medium conditioned by the 2.2.15 cells were assayed according to the manufacturer's specifications. The cpm obtained from the medium samples were compared to dilution from a standard preparation of HBsAg. HBeAg was detected in the medium by a RIA kit obtained from Abbott. A signal-to-noise ratio of >2 was considered positive.

Isolation and Analysis of DNA. Total cellular DNA was isolated from cells by the method of Jeffreys and Flavell (19). Episomal DNA was isolated by lysing the cells in 20 mM Tris·HCl, pH 7.5/10 mM EDTA/5 mM EGTA/1% NaDod-

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; ccc, covalently closed circular.

SO_4 following the method described by Hirt (20). Extracellular DNA was prepared from medium samples conditioned by growing 2.2.15 cells. The medium was clarified by low-speed centrifugation, incubated for 1 hr at 4°C in the presence of 10% (wt/wt) polyethylene glycol (M_r 8000), and centrifuged at 10,000 × *g* for 10 min. The resulting pellet was resuspended in 10 mM Tris·HCl, pH 7.5/10 mM EDTA. The DNA was purified by treatment with 400 μg of proteinase K per ml for 2 hr at 37°C and deproteinized by two extractions with equal volumes of phenol/chloroform.

One and one-half grams of cesium chloride was added to 4.5-ml samples of medium from either Hep G2 or 2.2.15 cells and was centrifuged at 235,000 × *g* for 60 hr at 4°C. The gradients were fractionated into 250-μl samples, the density of each fraction was determined by refractive index, and then the samples were dialyzed against H_2O. DNA contained within fractions corresponding to densities between 1.14 and 1.41 g/ml from cesium chloride equilibrium gradients was purified by direct deproteinization and extraction as described above.

All DNA samples were precipitated with ammonium acetate and ethanol. Some samples were resuspended in 10 mM Tris·HCl at pH 7.5 and then incubated with 50 μg of RNase A per ml for 2 hr at 37°C. The extraction and precipitation procedures were then repeated. All purified DNA samples were electrophoresed through 1.0% agarose gels and transferred to Zeta Probe nylon (Bio-Rad) according to Reed and Mann (21). Hybridization was performed at 42°C with HBV DNA radiolabeled by nick-translation with [α-^{32}P]dCTP. Molecular weight markers consist of a mixture of *Eco*RI- and *Eco*RI/*Bam*HI-digested pHBV-1 (a pBR322 plasmid with a single copy of the HBV genome cloned into the unique *Eco*RI site; ref. 5).

Examination of HBV-Specific Particles by Electron Microscopy. Pellets prepared from cesium chloride gradient fractions corresponding to densities of 1.18–1.40 g/ml were examined with transmission electron microscopy after negative staining with 1% phosphotungstic acid. Additionally, 50 μl of the same fractions were incubated with an equal volume of a 1:50 dilution of goat anti-HBsAg (DAKO, Santa Barbara, CA) for 30 min at 37°C and then incubated overnight at 4°C. The precipitates were washed with 1 ml of PBS and centrifuged at 190,000 × *g* for 2 hr. The resulting pellets were examined as described above.

RESULTS

Detection of HBV-Specific Proteins. The G418-resistant clone 2.2.15 was selected for analysis based on its ability to synthesize and secrete HBsAg and HBeAg. These cells produced HBsAg over an ≈10-day period (from subculture to confluence) at levels that increase from 4.2 to 94.3 ng/ml of culture medium during a 5-month period (Table 1). The amounts varied slightly from plate to plate of cells but, on the average, increased 10-fold between the first and second month after cloning and another 2.5-fold during the next month. The cells also consistently synthesized and secreted levels of HBeAg that gave signal-to-noise ratios of ≥114 (data not shown). This level is considerably higher than those obtained from testing conditioned medium samples from any of our clonal lines of HBV-transfected murine fibroblasts (5, 22).

Table 1. HBsAg secretion by 2.2.15 cells

Month*	ng/ml†
1	4.2
2	42.5
3	89.2
4	94.3
5	94.2

Assays were performed by using the HBsAg RIA from Travenol-Genentech.

*Period of time after initial cloning of G418-resistant cells.

†Aliquots of culture medium were assayed by removing samples when cells reached confluence (≈10 days after subculturing).

Identification of Extracellular and Intracellular HBV DNA. Southern blotting of undigested total cellular DNA indicated that HBV DNA sequences integrated chromosomally and were also present in a low molecular weight, episomal form (data not shown). To examine the nature of this intracellular, episomal HBV DNA, low molecular weight DNA was isolated and ≈20 μg was Southern blotted (Fig. 1, lane a) along with DNA purified from ≈40 ml of medium that had been conditioned by the cells for 10 days (Fig. 1, lane b). The majority of HBV DNA in both samples appears to be in the form of covalently closed circular (ccc) and nicked recircularized forms, together with incomplete copies of the genome.

Characterization of HBV DNA in Particles. DNA isolated from fractions of cesium chloride gradients was analyzed by Southern blotting to determine the precise nature of the HBV DNA that was carried within assembled viral particles (Fig. 2). The hybridization pattern obtained indicated that at densities similar to those of infectious Dane particles (23), between 1.22 and 1.25 g/ml, HBV DNA was present primarily in the form of full-length, nicked, and recircularized molecules (Fig. 2*B*, fractions 10–12). Most of the HBV DNA identified within the cesium chloride gradient at densities

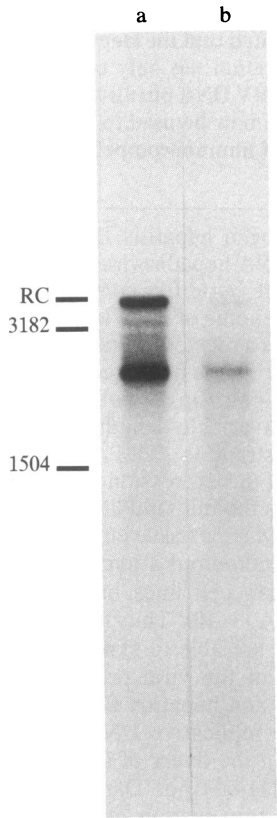

FIG. 1. Southern blot analysis of episomal and extracellular DNA from cultures of 2.2.15 cells. The autoradiograph shows a 20-μg sample of low molecular DNA isolated from the cells (lane a) and the DNA isolated from ≈40 ml of conditioned medium (lane b). Both DNA samples had been treated with RNase A prior to electrophoresis. The procedures for DNA purification and probing the blot with HBV DNA sequences are described in the text. Marker fragments (molecular weights indicated) were prepared by combining a mixture of 20 pg of *Eco*RI-digested/100 pg of *Eco*RI/*Bam*HI-digested pHBV-1 DNA and nicked, recircularized HBV DNA of unit length, 3.2 kb (RC).

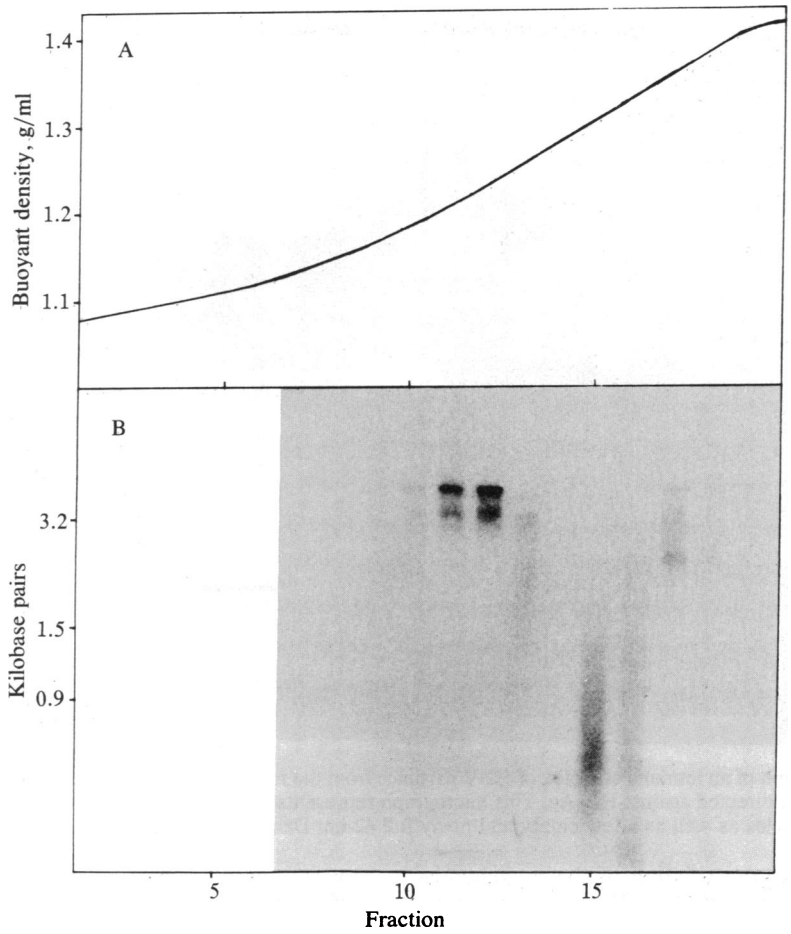

FIG. 2. Cesium chloride equilibration of replicative intermediates of HBV DNA in the medium from the 2.2.15 cells. DNA was isolated from each 250-μl cesium chloride gradient fraction, electrophoresed, and blotted. (*A*) Density of each of the fractions. (*B*) Autoradiograph of the DNA from fractions 7–19 that have been probed for HBV DNA sequences. The markers are those used in the analysis described in Fig. 1.

between 1.25 and 1.36 g/ml contained DNA with electrophoretic mobilities indicative of either partially double-stranded molecules or single-stranded HBV DNA (Fig. 2*B*, fractions 13–16). At densities typical for immature core particles (23, 24), the overall pattern of hybridization (Fig. 2*B*, fractions 17–19) was strikingly similar to that obtained in the low molecular weight intracellular DNA (Fig. 1, lane a). Whether the DNA identified at high densities is secreted as part of immature core particles or is a consequence of cell lysis remains to be determined.

Identification of HBV Particles by Immunoelectron Microscopic Examination. When gradient fractions of density ≥1.22 g/ml containing medium from the 2.2.15 cells were examined, several individual 42- to 46-nm virus particles as well as many 22-nm spherical particles were identified (data not shown). In samples that had been immunoprecipitated with antiserum directed against HBsAg, immune complexes were obtained, some of which include many HBsAg 22-nm spherical and filamentous particles and several 42-nm double-shelled Dane-like particles (Fig. 3). Interestingly, these particles were detected not only at a density of 1.22 g/ml but also at a density of 1.38 g/ml. However, no HBV-associated particles were found when similar fractions were examined from gradients containing medium from control, Hep G2 cells.

DISCUSSION

The establishment of an *in vitro* system in which the replication, pathobiology, and oncogenic potential of HBV, a member of the family of hepadnaviruses, can be investigated has proven to be elusive. Summers and his co-workers (25) showed that Peking duck liver cells in culture are capable of supporting duck hepatitis virus replication. The advantage of this system over the system reported here is that the penetration and the uncoating of the virus can be studied. On the other hand, we confirm the data of Will and his collaborators (16) that DNA of the human HBV is infectious. Furthermore, the replication in our system occurs continuously for an extended time period, which is not feasible using primary liver cells.

The Hep G2 cells have been shown to maintain a substantial number of liver-specific functions (26). Our investigations indicate that they also are capable of supporting some phases of HBV replication. Since the cells are derived from a hepatoblastoma, the oncogenic potential of HBV cannot be investigated by using this system. However, the transfected Hep G2 cells are amenable to the study, in detail, of the enzymatic reactions involved in the replication of HBV and can serve to test the effect of drugs that could interfere with viral replication. They can also serve as an *in vitro* model system for analysis of involvement of the immune system in the disease process and in recovery from the disease.

The medium of transfected Hep G2 cells contains not only all of the particles (spherical and filamentous HBsAg particles and empty and full Dane particles) present in the serum of infected individuals but also a number of replicative intermediates that probably represent recircularized, ccc, and single-stranded HBV DNA. In the replication scheme proposed for other hepadnaviruses (25), the ccc form of viral DNA that is found in the 2.2.15 cells in high concentrations

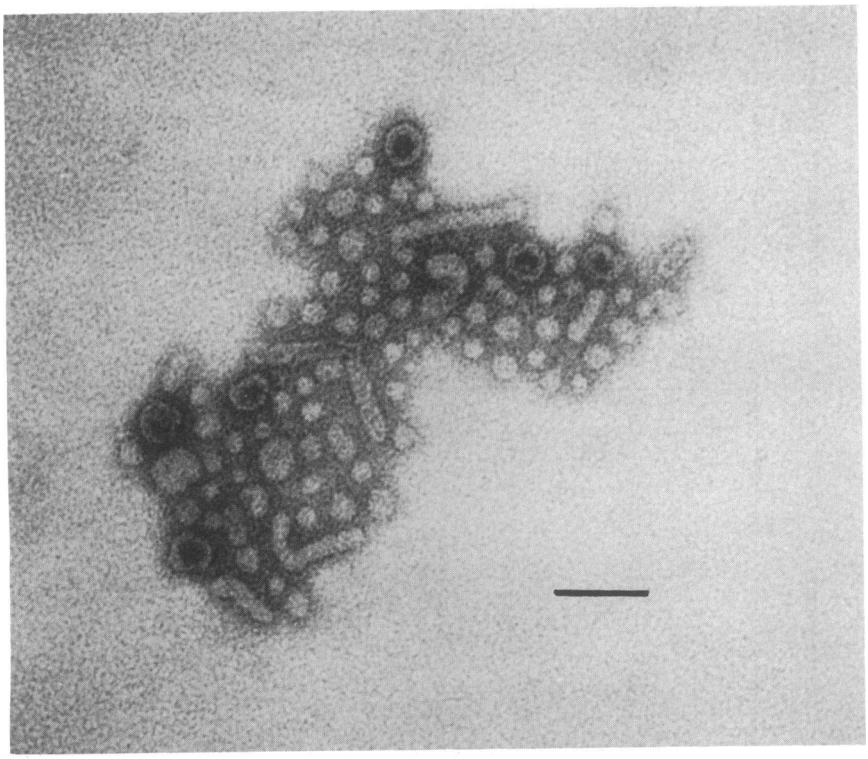

FIG. 3. Electron micrograph of an immune complex of HBV particles from the medium of 2.2.15 cells. A 50-μl sample of a gradient fraction was incubated with antiserum directed against HBsAg. This micrograph represents a typical complex that includes numerous 22-nm spherical and filamentous HBsAg particles as well as a few empty and many full 42-nm Dane-like particle (×125,000; bar = 100 nm).

acts as a template for the transcription of the pregenomic RNA. Furthermore, the forms of HBV DNA isolated from cesium chloride equilibrium density gradients at densities equivalent to those of Dane and immature core particles possess electrophoretic mobilities similar to the DNA isolated from authentic viral components (23, 24, 27, 28). In addition, the medium contains much higher amounts of HBeAg than any medium of other transfected cells. Rutter and his co-workers (29) proved that the HBeAg arises by processing core antigen (HBcAg) in the endoplasmic reticulum. Moreover, Roossinck *et al.* (30) confirmed this finding by showing that HBcAg is not converted to HBeAg if a portion of the precore region is not translated. Thus, these results indicate that transfected Hep G2 cells are very effective in synthesizing HBcAg. Considering that HBsAg and empty particles are overproduced by several logarithmic units in infected individuals, it can be assumed that the synthesis of HBcAg represents the rate-limiting factor in the production of infectious particles.

Various parameters of the replicative cycle can be quantitated in the transfected Hep G2 cells—for example, the secretion of HBsAg or HBeAg and the amount of episomal HBV DNA. Thus, this *in vitro* system can be employed not only to test existing antiviral drugs but also to design new drugs.

The involvement of the immune system in the pathogenesis of hepatitis is well established but the mechanism for this interaction has not yet been clarified. The transfected Hep G2 cells have the potential to serve as an *in vitro* model system for the investigation of the interaction of specific cytotoxic T lymphocytes and cytolytic antibodies with infected liver cells. Our preliminary results, which will be published elsewhere, show that lymphocytes derived from infected patients form rosettes around the 2.2.15 cells but not surrounding their normal counterparts.

Thus, we have created conditions that permit a penetrating analysis of HBV replication. However, it remains to be seen whether this system produces particles that have the capacity to cause hepatitis in chimpanzees and/or can be propagated by infecting cells in tissue culture. In the latter case, the penetration and the uncoating of the virus can also be investigated.

Note Added in Proof. Since communication of this manuscript, Sureau *et al.* (31) reported results that are similar to those we have obtained.

We acknowledge the substantial contribution of Marina Shvartsman for her technical assistance. This research was supported, in part, by National Institutes of Health Grants CA-34818 and ADDKD 30854.

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