A Molecularly Cloned Hepatitis B Virus Produced In Vitro Is Infectious in a Chimpanzee

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The infectivity of hepatitis B virus (HBV) produced in vitro by HepG2 cells transfected with HBV DNA (HepG2T14) has been assayed in a chimpanzee. Following inoculation, the chimpanzee underwent a typical course of type B hepatitis infection, characterized by elevation of serum aminotransferases and by histological identification of hepatic damage. Hepatitis B surface antigen and core-related antigen appeared in the serum at weeks 5 and 7, respectively, after infection. HBV DNA was detected in serum samples, and replicative forms of the HBV genome were identified in liver biopsies. Subtype identification of hepatitis B surface antigen and restriction enzyme analysis of HBV DNA in both the inoculum and the serum of the infected chimpanzee confirmed that the hepatitis B infection observed in this animal was caused by viral particles produced by HepG2T14 cells. These findings indicate that, although HepG2 cells do not seem to be susceptible to infection by HBV in vitro, they can produce biologically active infectious virions after transfection with cloned HBV DNA.

Hepatitis B virus (HBV) shares structural, molecular, and biological properties with other members of the hepadnavirus family (9). HBV, woodchuck hepatitis virus (13), ground squirrel hepatitis virus (7), and Pekin duck hepatitis B virus (8) are mainly hepatotropic and may lead to persistent infection (15). Although animal models for the study of woodchuck, ground squirrel, and Pekin duck hepatitis B viruses have played a major role in our understanding of the biology of HBV (10, 12, 15), important aspects of the study of HBV have been hampered by failure to establish an in vitro culture system in which the entire replication cycle could be examined. Using HepG2 cells (5), we recently reported the production of HBV particles after transfection with cloned HBV DNA (14). Complete virions (Dane particles) containing viral DNA and DNA polymerase activity were found in the culture medium of transfected HepG2 cells (HepG2T14), and intracellular replicative intermediates of the HBV genome were identified. However, DNA analysis suggested that HBV production in HepG2T14 cells was initiated by transcription from integrated HBV DNA sequences and, thus, that it would not result from a complete cycle of infection. Consequently, it was not possible to predict whether virions produced by the HepG2T14 cells would be infectious.

We assessed the infectivity of hepatitis B virions produced in vitro by inoculating these particles into a chimpanzee. Viral particles were purified from 200 ml of culture medium from HepG2T14 cells harvested at the stationary phase of the cell growth cycle. Particles were subjected to isopycnic cesium chloride density gradient centrifugation (14). Fractions containing Dane-like virions were pooled, and the particles were sedimented and suspended in 3 ml of 3-day-old culture medium from HepG2T14 cells. Of this mixture, 1 ml, containing approximately 10^7 virions (quantified by

dot-blot analysis) (14), was inoculated intravenously into a healthy anti-hepatitis B surface antigen (HBsAg) antibody-negative chimpanzee (PTT X186). One week before inoculation, and weekly thereafter, the animal was bled and serum samples were examined for the liver-specific enzymes alanine aminotransferase and aspartate aminotransferase. The markers of HBV infection, HBsAg, hepatitis B envelope antigen (HBeAg), and anti-hepatitis B core antigen (HBcAg), were tested with the enzyme-linked immunosorbent assays AUSZYME II, HBe, and CORZYME, respectively (Abbott Laboratories) (Fig. 1). The presence of HBV DNA was assayed for by spotting 5 μ l of plasma on a nitrocellulose filter, denaturing with an alkali, and hybridizing to a ³²P-labeled HBV probe, as previously described (14). Liver biopsies performed before inoculation and every other week thereafter were done with a Menghini needle.

Following inoculation, the chimpanzee developed signs of HBV infection, which was identified by the appearance of HBsAg and HBeAg in the serum at weeks 5 and 7, respectively, after inoculation (Fig. 1). Anti-HBcAg antibodies were detected by week 9. The appearance of viral DNA in the serum occurred at week 8 after inoculation and persisted for 10 weeks afterward. Hepatic injury was detected by an increase of the liver enzymes alanine aminotransferase and aspartate aminotransferase in the serum between weeks 10 and 25 and by typical lesions of hepatic tissue. Histological examination of the liver biopsy specimens revealed normal tissue for up to 11 weeks after inoculation (Fig. 2A); lesions characteristic of acute hepatitis began to appear between weeks 11 and 13. At this time, infiltration of lymphocytes, plasma cells, macrophages, and neutrophils was observed in the portal tract (Fig. 2B). Degeneration of hepatocytes was indicated by contraction of the cells with eosinophilic cytoplasm and basophilic nuclei. Other indications of acute viral hepatitis included marked cellular ballooning, disruption of hepatic cords, and focal necrosis.

To determine if the signs of hepatitis B correlated with replication of HBV DNA and production of the complete virus, we analyzed DNA samples from either liver biopsy

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FIG. 1. Profile of serological HBV markers and viral DNA detected in the serum of chimpanzee PTT X186. HBsAg, HBeAg and anti-HBeAg were measured by the enzyme-linked immunoassays AUSZYME II, Hbe, and COREZYME diagnostic kits, respectively. HBV DNA was detected by dot-blot analysis. Alanine aminotransferase and aspartate aminotransferase are expressed in International Units per liter.

specimens or blood by Southern blotting and hybridization with an HBV-specific probe. Total DNA was isolated from liver biopsy specimens as described elsewhere (14), and samples were then subjected to electrophoresis, transfer to nitrocellulose, and hybridization to ^{32}P -labeled HBV probe. Relaxed circular, linear, and single-stranded viral DNA forms, which represent replication intermediates of the HBV genome (10, 12), were detected at the acute phase of infection starting by weeks 6 to 8 and persisting for 8 to 10 weeks afterward (Fig. 3A). Total RNA was extracted from a biopsy performed during week 16 when the serum tested positive for HBV DNA. Analysis of viral RNA by Northern (RNA) blotting (Fig. 3B) indicated the presence of HBV transcripts of 2.3 and 3.5 kilobases similar to those described previously from the livers of infected chimpanzees (2). The quality of RNA recovered from a single biopsy did not permit us to clearly observe a 2.7-kilobase mRNA as was seen in the RNA extracted from HepG2T14 cells.

HBV particles contained in the serum of chimpanzee PTT X186 were analyzed by centrifugation in a CsCl density gradient and by Southern blotting. Unlike what was seen in the culture medium of HepG2T14 cells (14), DNA-containing, nonenveloped core particles were not observed. Only mature viral DNA-containing (Dane) particles with a density of 1.24 g/cm^3 were present (Fig. 4). Furthermore, unlike particles from HepG2T14 cells, virions contained DNA molecules of the mature type only (relaxed circular double-stranded and partially single-stranded forms), without any immature single-stranded molecules. Endogenous DNA polymerase assays were performed in the presence of radiolabeled nucleotides as described elsewhere (16), and the product was electrophoresed on a 1.5% agarose gel and autoradiographed. Radiolabeled viral DNAs of both relaxed circular and linear forms were observed (Fig. 5A).

The antigenic subtype of serum HBsAg proved to be identical to that of the inoculum (ayw), as determined by using subtype-specific monoclonal antisera. Analysis of viral DNA purified from serum was performed after the single-stranded regions were filled in with the Klenow fragment of DNA polymerase I in the presence of all four nucleotides (6). Repaired DNA was digested with the restriction enzymes *EcoRI*, *BamHI*, *MspI*, *SphI*, and *PstI* and was hybridized with a ^{32}P -labeled HBV DNA probe after agarose gel elec-

FIG. 2. Histological examination of liver biopsies from chimpanzee PTT X186. (A) Normal liver before inoculation. (B) Liver biopsy performed 14 weeks after inoculation. Note the inflammatory cell collection in the portal area. Hematoxylin-eosin ($\times 258$).

FIG. 3. (A) Southern blot analysis of DNA extracted from liver biopsies taken 0, 6, 8, 10, 12, and 26 weeks after inoculation. DNA (2 μ g) was isolated from each biopsy, separated on a 1.5% agarose gel, and analyzed for the presence of HBV DNA by Southern blotting. Radiolabeled fragments from *Hind*III-digested bacteriophage lambda DNA were used as markers (lane M). (B) Northern blot analysis of total cellular RNA extracted from a liver biopsy performed on chimpanzee PTT X186 at 16 weeks after inoculation. Sizes of the major HBV transcripts are indicated in kilobases.

trophoresis and transfer to nitrocellulose. The restriction map of circular HBV DNA derived from the serum of chimpanzee PTT X186 was identical to that of DNA extracted from viral particles produced by HepG2T14 cells; it corresponded to the genome derived from plasmid pCP10 (3,

FIG. 5. (A) Products of the endogenous DNA polymerase assay of HBV purified from chimpanzee PTT X186 serum. Radiolabeled fragments from *Hind*III-digested bacteriophage lambda DNA were used as markers. (B) Southern blot analysis of HBV DNA purified from particles isolated from the serum of chimpanzee PTT X186 after reaction with the Klenow fragment of DNA polymerase I in the presence of all four nucleotides. Uncut *Bam*HI-, *Eco*RI-, *Msp*I-, *Sph*I-, and *Pst*I-digested DNA samples were separated on a 1.5% agarose gel. Radiolabeled fragments from *Hind*III-digested bacteriophage lambda DNA were used as markers (lanes M). Sizes are given in kilobases at the left of each gel.

4) used for transfection of HepG2 cells (Fig. 5B). Digestion with *Eco*RI or *Sph*I converted the relaxed circular HBV DNA to a linear form of 3.2 kilobases in size. Both enzymes cut at a single site in the cloned genome of HBV (ayw). As expected, digestion with *Pst*I, which does not cut within the cloned genome, did not linearize the viral DNA. Digestion with *Bam*HI and *Msp*I generated subgenomic fragments of sizes predicted from the sequence of the cloned genome used for transfection. These fragments (Fig. 5) have the predicted sizes of 1,504, 912, and 766 base pairs after digestion with *Bam*HI and of 1,369, 760, 638, and 415 base pairs after digestion with *Msp*I. The presence of additional fragments in each DNA digest is due to an incomplete DNA polymerase fill-in reaction and to partial restriction enzyme digestion. These results identify the molecularly cloned virus produced

FIG. 4. Southern blot analysis of a CsCl gradient of HBV particles obtained 8 weeks after inoculation from the serum of chimpanzee PTT X186. Viral particles from 100 μ l of serum were subjected to centrifugation in a 20 to 50% (wt/vol) CsCl gradient for 16 h at 35,000 rpm in an SW41 rotor. Fractions were collected from the bottom of the tube, and the DNA was isolated. The DNA was separated on a 1.5% agarose gel and analyzed for the presence of HBV sequences by Southern blotting. The number (1 to 12) of each fraction and its density are indicated. Radiolabeled fragments from *Hind*III-digested bacteriophage lambda DNA were used as markers (lane M).

by HepG2T14 cells as the causative agent for the resulting HBV infection of chimpanzee PTT X186 and thus demonstrate its infectivity after intravenous injection. The possibility that free HBV DNA in the inoculum was responsible for the infection seems highly unlikely in view of previous experiments in which intravenous injection of purified cloned HBV DNA into chimpanzees failed to induce infection (17). Recently, Acs et al., using their own source of HBV production (1, 11), have reported results similar to ours. Particles produced in vitro initiated infection in chimpanzees, but analysis of viral DNA revealed the presence of free high-molecular-weight viral DNA in the particles produced by the chimpanzees. This observation suggests that an unusual mode of viral DNA replication occurred in that case.

In our experiment, chimpanzee PTT X186 produced a high titer of a molecularly cloned virus of known sequence that contains typical replicative forms of HBV DNA and DNA polymerase activity, as usually observed after infection with serum Dane particles. HBV recovered from the animal was grown first through a cycle of production in vitro by HepG2 cells after transfection and second through a cycle of replication in vivo in the liver of a chimpanzee after infection, without any major modification of its DNA sequence or biological properties.

Although we have demonstrated the ability of HepG2 cells to produce infectious HBV particles, our attempts to infect this cell line with virions produced by HepG2T14 cells have not been successful. HepG2 cells may differentiate to a stage sufficient to fulfill the requirements for transcriptional and posttranscriptional events leading to the expression of complete infectious HBV particles, but this stage may be insufficient under our culture conditions to support a full cycle of replication. This finding may imply that expression of receptors for HBV at the surface of the cell membrane or expression of other specific liver cell factors required in the initial steps of infection are not achieved in HepG2 cells.

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LITERATURE CITED

1. Acs, G., M. A. Sells, R. H. Purcell, P. Price, R. Engle, M. Shapiro, and H. Popper. 1987. Hepatitis B virus produced by transfected HepG2 cells causes hepatitis in chimpanzees. *Proc. Natl. Acad. Sci. USA* **84**:4641-4644.
2. Cattaneo, R., H. Will, and H. Schaller. 1984. Hepatitis B virus transcription in the infected liver. *EMBO J.* **3**:2191-2196.
3. Dubois, M. F., C. Pourcel, S. Rousset, C. Chany, and P. Tiollais. 1980. Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA. *Proc. Natl. Acad. Sci. USA* **77**:4549-4553.
4. Galibert, F., E. Mandart, F. Fitoussi, P. Tiollais, and P. Charnay. 1979. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) clone in *E. coli*. *Nature (London)* **281**:646-650.
5. Knowles, B. B., C. C. Howe, and D. P. Aden. 1980. Human hepatocellular carcinoma cell-lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* **209**:497-499.
6. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. Marion, P. L., L. S. Oshiro, D. C. Regnery, G. H. Scullard, and W. S. Robinson. 1980. A virus in beechey ground squirrels which is related to hepatitis B virus of man. *Proc. Natl. Acad. Sci. USA* **77**:2941-2945.
8. Mason, W. S., G. Seal, and J. Summers. 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. *J. Virol.* **36**:829-836.
9. Robinson, W. S., P. Marion, M. Feitelson, and A. Siddiqui. 1981. The Hepa-DNA-virus group: hepatitis B and related virus, p. 57-68. *In* W. Szmuness, J. Alter, and J. Maynard (ed.), *Viral hepatitis 1981*. International Symposium. Franklin Institute Press, Philadelphia, Pa.
10. Seeger, C., D. Ganem, and H. Varmus. 1986. Biochemical and genetic evidence for the hepatitis B virus replication strategy. *Science* **232**:477-484.
11. Sells, M. A., M. L. Chen, and G. Acs. 1987. Production of hepatitis B virus particles in HepG2 cells transfected with cloned hepatitis B virus DNA. *Proc. Natl. Acad. Sci. USA* **84**:1005-1009.
12. Summers, J., and W. S. Mason. 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell* **29**:403-415.
13. Summers, J., J. Smolec, and R. Snyder. 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proc. Natl. Acad. Sci. USA* **75**:4533-4537.
14. Sureau, C., J. L. Romet-Lemonne, J. Mullins, and M. Essex. 1986. Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. *Cell* **47**:37-47.
15. Tiollais, P., C. Pourcel, and A. Dejean. 1985. The hepatitis B virus. *Nature (London)* **317**:489-495.
16. Tuttleman, J. S., J. C. Pugh, and J. W. Summers. 1986. In vitro experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. *J. Virol.* **58**:17-25.
17. Will, H., R. Cattaneo, G. Darai, F. Deinhardt, H. Schellekens, and H. Schaller. 1985. Infectious hepatitis B virus from cloned DNA of known nucleotide sequence. *Proc. Natl. Acad. Sci. USA* **82**:891-895.