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# *In vitro* propagation of human hepatitis B virus in a rat hepatoma cell line

(Morris hepatoma/reverse transcription/Dane particles/animal model)

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ABSTRACT **A rat hepatoma cell line (Q7) of Morris hepatoma origin was transfected with a construct containing the tandem dimer genome of human hepatitis B virus (HBV) and the neomycin-resistant selection marker. The culture medium of several neomycin-resistant single-cell clones was found to accumulate high levels of secreted HBV surface antigen and core-related e antigen. HBV-specific replication intermediates, including relaxed circular and single-stranded DNA with a minus-strand polarity, could be found in both the intracellular fraction and the extracellular culture medium by the Southern blot procedure. One of these clones, designated Q7 HBV-21, was characterized in further detail. DNA polymerase activity was present in the virus particles produced by Q7 HBV-21 cells. Characteristic transcripts of HBV, including the 3.5-, 2.5-, and 2.1-kilobase mRNA as well as a core-gene-related transcript of 2.2 kilobases could be detected. Electron microscopic examination of the conditioned medium from Q7 HBV-21 cells identified 42-nm Dane-like particles as well as 22-nm subviral particles with a spherical or filamentous shape. This Q7 HBV-21 cell line has been maintained in the absence of neomycin for 1 year without losing the properties of HBV DNA replication and Dane-like particle production. Our results strongly suggest that the species barrier of HBV infection is at an early step of viral absorption onto or penetration into the target hepatocytes. This nonhuman system for HBV production in culture could be used to complement the human HepG2 system.**

Research on human hepatitis B virus (HBV) has been hampered by the slow development of a tissue culture system for HBV propagation (1–3). It is possible to study HBV production in culture by using human hepatoma cell lines HepG2 (4, 5) or Huh6-c15 (6) and stable transfection techniques. In addition, the human hepatoma cell line Huh7 was used as a recipient for production of HBV through the transient transfection technique (7, 8). In all these studies, HBV-specific DNA replication intermediates were identified. Moreover, characteristic subviral and matured viral particles (Dane particles) were observed by electron microscopy. These viral particles produced by HepG2 cells were later shown to be infectious in chimpanzees (9, 10).

HBV is a member of the so-called hepadnavirus family including hepatitis B viruses of woodchucks, ground squirrels, tree squirrels, Pekin ducks, etc. (1–3). Members of the hepadnavirus family in general exhibit a rather strict host range. For example, HBV is known to replicate only in humans and chimpanzees. It has been reported that infectious particles of duck hepatitis B virus can be generated by transient gene transfer to the established human hepatoma cell lines HepG2 (11, 12) and Huh7 (12, 13). Although these experiments demonstrated that duck hepatitis B virus can replicate and mature in certain human hepatoma cell lines, it remains unclear if the converse is true—i.e., if HBV can propagate and mature into infectious Dane particles in a nonhuman hepatoma system. For example, although ground squirrel hepatitis virus can grow in eastern woodchucks, it has not been possible to grow woodchuck hepatitis virus in ground squirrels (14). In fact, attempts to grow HBV in mouse fibroblasts were made but no mature virions were observed (15). It is unclear if this failure was due to the species barrier, the tissue-type barrier, or both. However, successful replication of the complete genome HBV was observed in a transgenic mouse obtained after injection of head-to-tail dimers of the HBV genome (16). However, no completely matured Dane particles were observed in this transgenic mouse system (16).

To address the issues of cellular permissiveness and host range determination and to establish a nonhuman HBV-producing system in culture, we have screened, by a transient transfection assay, various HBV recipients of nonhuman origin for the ability to support HBV replication. Q7, a rat hepatoma cell line, was found to be competent to support HBV replication and maturation. Subsequently, stable transfection techniques have allowed us to establish a series of HBV-producing Q7 rat hepatoma cell lines.

To our knowledge, this is the first demonstration that Dane-like particles of HBV can be produced in a nonhuman cell culture system. These results have been presented previously.§

## MATERIALS AND METHODS

**Construction of Donor DNA.** An HBV-containing plasmid, pcp10, was a generous gift from P. Tiollais (Pasteur Institute) (17). This plasmid DNA was linearized with *Eco*RI. The resulting 3.1-kilobase (kb) HBV DNA fragment was electroeluted from the gel and ligated with *Eco*RI-digested pSV232ANeo vector DNA (18). This ligated DNA mixture was transformed into TG1 *Escherichia coli*. A test for PSV2ANeo–HBV monomer was done by *Eco*RI digestion of miniprep DNA. A partial digestion of a pSV2ANeo–HBV monomer DNA with *Eco*RI was then conducted. The linearized 9.3-kb band was electroeluted from a gel and ligated to an *Eco*RI-cleaved 3.1-kb HBV DNA insert. Preparations of plasmid DNA from transformed colonies were screened for head-to-tail dimers by digestion with *Xho* I, which should give the 3.1-kb HBV DNA insert upon cleavage of HBV dimer DNA.

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Abbreviations: HBV, human hepatitis B virus; ccc, covalently closed circular; rc, relaxed circular; ss, single-stranded; e/core, HBV core or e antigen; Neo$^r$, neomycin resistance(t).

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§Li, L.-S. & Shih, C., Molecular Biology of Hepatitis B Viruses, Aug. 7–11, 1988, University of California at San Diego, La Jolla, CA, p. 37.

**Transfection and Cell Culture.** The rat hepatoma cell line Q7 was kindly provided by J. Chou (National Institutes of Health, Bethesda, MD) and maintained in 5–10% (vol/vol) fetal bovine serum in Dulbecco's modified Eagle's medium, at 37°C in an atmosphere of 5.5% $CO_2$/94.5% air. The calcium phosphate transfection procedure was used as described (19). Briefly, $7 \times 10^5$ cells per 10-cm dish were transfected with 5–10 μg of pSV2ANeo–$(HBV)_2$ DNA plus 20 μg of human genomic DNA as carrier. Donor DNA was removed 4 hr after transfection. Neomycin-resistant ($Neo^r$) colonies were isolated 4 weeks later. G418 was removed from medium 3 months after transfection.

**Detection of e or Core (e/core) and Surface Antigens.** Conditioned medium from $Neo^r$ Q7 cell lines was assayed according to suppliers' recommendations [Abbott enzyme immunoassay (EIA) kit for e/core antigen and RIA kit for surface antigen].

**Fractionation of Extracellular HBV Particles.** Conditioned medium (80 ml) from each sample was precleared at 2500 rpm (Sorvall SS34 rotor) for 10 min at 4°C. Particles from 27 ml of cell-free medium were pelleted through an 11-ml 20% (wt/vol) sucrose cushion in TNE buffer (150 mM NaCl/20 mM Tris·HCl/1 mM EDTA) in an SW 28 rotor at 25,000 rpm for 16 hr at 4°C. Each pellet was resuspended in 0.5 ml of TNE buffer, loaded onto a 20–50% (wt/vol) cesium chloride isopycnic gradient in TNE buffer and centrifuged using an SW 50.1 rotor at 35,000 rpm at 4°C for 16 hr. After centrifugation, 0.3-ml fractions were collected from the bottom of the tube. The density of the fractions was monitored with a refractometer and ranged from ≈1.130 g/ml to 1.386 g/ml.

**Electron Microscopy.** Putative Dane particle-containing fractions from a cesium chloride gradient were pooled and dialyzed into TNE buffer using a Centricon-10 concentrator (Amicon). Starting with 80 ml of conditioned medium, 50–100 μl of a concentrated particle fraction was produced. About 10 μl was applied to each Formvar-coated grid, followed by washing with six drops of 0.1 M ammonium acetate (pH 7.2) and staining with freshly filtered 1% uranyl acetate. The JEOL model 4000EX electron microscope was used for photography.

**DNA and RNA Analysis.** Genomic DNA was prepared as described (20). The Hirt extraction protocol was adopted from Hirt (21). RNA was prepared according to Chirgwin *et al.* (22). Southern and Northern blot analyses followed standard procedures (23).

**Polymerase Assay.** Polymerase activity was analyzed by standard procedures (5).

## RESULTS

**e/core Antigen Screening of Transiently Transfected Rat Hepatoma Cell Lines.** To investigate cellular permissiveness of HBV propagation, it was important to find cell lines in addition to HepG2 that were also competent to produce HBV. We, therefore, screened ≈10 hepatoma cell lines by transfecting with a tandem dimer of HBV genome and then assaying for secreted e/core antigen in the medium (Abbott EIA kit for e/core antigen). These cell lines include rodent hepatoma NI-SI (ATCC CRL 1604), H4-II-E (ATCC CRL 1548), MHICI (ATCC CCL 144), McA-RH7777 (ATCC CRL 1601), MCA-RH 8994 (ATCC CRL 1602), H4-II-E-C3 (ATCC CRL-1600), HTC (24), and Q7 (a clonal derivative of McA-RH7777) as well as human hepatoma lines FOCUS (25) and HepG2 (26). Among these cell lines, only McA-RH-7777 (27), Q7, and HepG2 exhibited detectable level of e/core antigen ≈24 hr after transfection (data not shown). We attempted, therefore, to establish a stably transfected Q7 cell line that supported HBV production.

**Stable Transfection of the Rat Hepatoma Cell Line Q7.** By using the calcium phosphate protocol (19), ≈$10^6$ Q7 cells were transfected with a tandem dimer of the HBV genome in a simian virus 40 vector that also contains a neomycin-resistance marker. Transfected cultures were selected with G418 30 hr later. To obtain a more effective killing, the dosage of G418 was increased from Geneticin (GIBCO) at 1 mg/ml to 1.2 mg/ml at the beginning of the third week after transfection. Colonies were subcultured into 35-mm dishes during the fifth week. Geneticin was removed from culture ≈3 months later. A transfection efficiency of $4 \times 10^{-5}$ colonies per cell per 10 μg of donor DNA was observed in this Q7 rat hepatoma system.

**Enzyme Immunoassay of Secreted e/core Antigen and Surface Antigen.** The conditioned medium of $Neo^r$ Q7 clones was collected and assayed for HBV-specific sero markers, such as e/core and surface antigens. When the enzyme immunoassay kits of Abbott were used, significant amounts of antigen were detected (Table 1). Since the e/core antigen is a marker often associated with active viral replication (1–3), we chose to characterize those clones with higher titer of e/core antigen production or secretion. According to our rough estimate using the Abbott Auszyme monoclonal EIA kit, cell line Q7 HBV-21 was able to produce surface antigen at ≈5.25 μg per $10^7$ cells per 24 hr.

**Characterization of HBV-Specific DNA Replication Intermediates.** The synthesis of hepadnavirus DNA is known to involve reverse transcription of an RNA intermediate (28). During the replication cycle, characteristic intermediates in DNA can be identified within infected cells, including the molecular forms of relaxed circular (rc) and the single-stranded (ss) DNA with minus-strand polarity (1–3). Low molecular weight, unintegrated DNA was extracted from our Q7 lines (21) and analyzed by the Southern blotting procedure

Table 1. Serological markers of the surface and e/core antigens of HBV assayed by enzyme immunoassay using conditioned medium from HBV DNA-transfected Q7 rat hepatoma cell lines

| Hepatoma cell lines | HBV antigen, S/N ratio | | |
|---|---|---|---|
| | e/core | Surface | DNA intermediates |
| Q7 HBV-1 | 4.6 | >37.0 | − |
| Q7 HBV-2 | 7.5 | >37.0 | + |
| Q7 HBV-4 | 14.9 | >37.0 | + |
| Q7 HBV-5 | 6.8 | 28.6 | + |
| Q7 HBV-6 | 0.3 | 0.1 | − |
| Q7 HBV-7 | 14.1 | >37.0 | − |
| Q7 HBV-9 | 13.0 | >37.0 | − |
| Q7 HBV-12 | 4.8 | >37.0 | − |
| Q7 HBV-14 | 16.6 | >37.0 | + |
| Q7 HBV-18 | 9.2 | 15.8 | − |
| Q7 HBV-19 | 12.7 | >37.0 | + |
| Q7 HBV-21 | 5.4 | >37.0 | ++ |
| Q7 HBV-23 | 8.4 | <0.1 | − |
| Q7 HBV-25 | 0.8 | >37.0 | − |
| Q7 parental | 0.4 | <0.1 | − |

Stable HBV-producing and parental rat hepatoma cell lines were used. Three-day conditioned medium collected from plates with 20–100% confluency of cell density was analyzed with the Abbot enzyme immunoassay (EIA) kit. Procedure A was used for the measurement of secreted surface antigens. The results of surface antigen detection by enzyme immunoassay are consistent with those by radioimmunoassay (data not shown). The e/core antigen results represent two independent measurements at different times. The difference in the titer may reflect in part some difference in the cell density. The intracellular HBV-specific DNA was prepared as described by Hirt (21). S/N ratio, signal/noise ratio; +, DNA replication intermediate present; −, DNA replication intermediate not detected. For the S/N ratio for HBV e/core antigen, values of negative range from 0.030 to 0.090, and the cutoff was 0.120. For the S/N ratio for HBV surface antigen, values of negative range from 0.001 to 0.013, and the cutoff was 0.054.

(23). Intense banding around positions 4.0 kb (rc) and 1.5 kb (ss) was evident in several cell lines including Q7 HBV-2, -4, -5, -14, -19, and -21 (only part of the data is shown in Fig. 1*A*). The high molecular weight DNA band at the 23-kb position is due to the trace contamination of undigested chromosomal DNA containing integrated HBV in these Hirt DNA preparations (Fig. 1*A*). The ss nature of the 1.5-kb band was confirmed by direct transfer to a nitrocellulose membrane without prior alkaline denaturation (Fig. 1*B*). The ssDNA was shown to be of minus-strand polarity by hybridization to strand-specific probe of HBV DNA cloned in an M13 vector

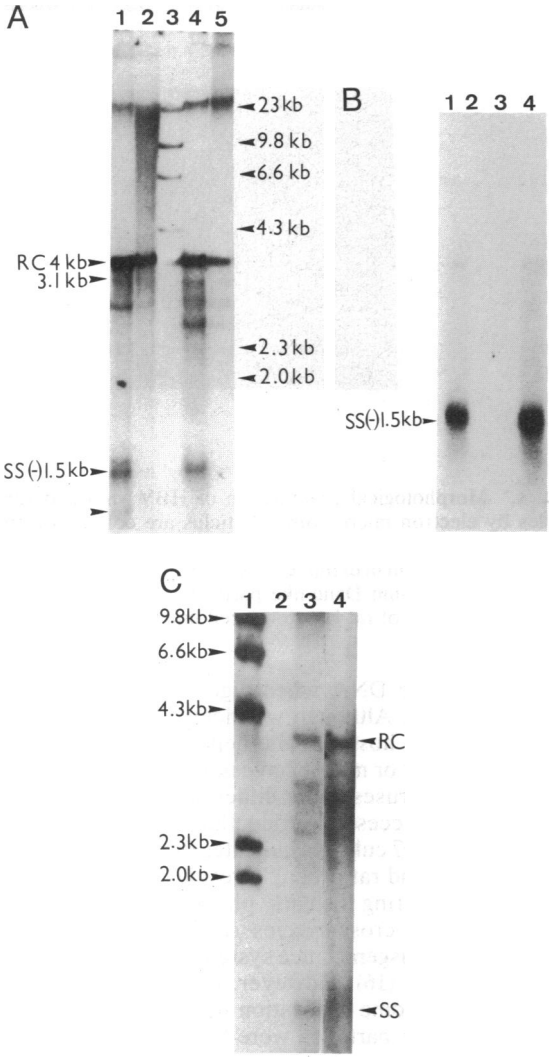


FIG. 1. Southern blot analysis of intracellular and extracellular HBV-specific DNAs prepared from several hepatoma cell lines producing Dane-like particles. (*A*) Intracellular, unintegrated HBV DNA replicative intermediates including rc- and minus-strand linear ssDNA as indicated. Vector-free HBV double-stranded DNAs were radiolabeled and used as probes. Lanes: 1, human HBV-producing HepG2 hepatoma cell line UP7-4; 2, human HBV-producing HepG2 hepatoma cell line UP7-7; 3, λ phage *Hin*dIII size marker; 4, rat hepatoma cell line Q7 HBV-21; 5, rat hepatoma cell line Q7 HBV-5. (*B*) Intracellular, unintegrated minus-strand ssDNA replicative intermediates were characterized by Southern transfer without prior alkaline denaturation. Strand-specific probes of HBV DNA in M13 plasmid were used. Only the radiolabeled plus-strand probe could cross react with the 1.5-kb minus-strand intermediate. Lanes 1–4 correspond to lanes 1–4 in *A*. (*C*) Extracellular, virion-associated DNAs prepared from 10 ml of medium from Q7 HBV-21 cells were analyzed by Southern procedures. Lanes: 1, λ phage *Hin*dIII size markers; 2, Q7 parental cell line; 3, Q7 HBV-21 intracellular DNA; 4, Q7 HBV-21 extracellular DNA.

(Fig. 1*B*). Depending on the DNA preparations, another signal of ssDNA intermediates could sometimes be seen at ≈1.3 kb (data not shown).

The covalently closed circular (ccc) form of HBV DNA was not detected in these HBV-producing cell lines, either alone or in the presence of proteinase K during the DNA preparation procedure (see *Discussion*). The bands that were detected represent HBV replicative intermediates of various length. Although we have noted the presence of one or two extra bands in these zebra patterns in HBV-producing rat hepatoma cell lines (Fig. 1*A*, lanes 4 and 5), these bands (at ≈2.6 kb and ≈2.3 kb) can sometimes be found as well in the HBV-producing human HepG2-derived cell lines UP7-4 or UP7-7 (unpublished results). According to our rough estimate, each cell of Q7 HBV-21 contains ≈50 copies of unintegrated HBV DNA.

**Extracellular DNA.** Ten milliliters of 3-day conditioned medium from Q7 HBV-21 cells was collected and passed through a filter to remove any cellular contamination. The particles in the cell-free filtrate were pelleted by centrifugation and resuspended in TNE buffer. The 4.0-kb rcDNA and the 1.5-kb ssDNA can be seen by Southern blot analysis (Fig. 1*C*), indicating that the complete virion of Dane particles and probably the less matured core particles were also present in the medium. The banding pattern of the extracellular DNA appears to be identical to the intracellular, unintegrated HBV DNA (Fig. 1 *A* and *C*).

**Q7-Produced HBV e/core Particles Contain Polymerase Activity.** The enzymatic activity of the HBV core-associated DNA polymerase can be detected when radioactive deoxynucleotides are provided (28, 29). The core-particle fractions with density ≈1.34 g/ml purified from the Q7 HBV-21 conditioned medium by using a CsCl gradient incorporated deoxynucleotides into viral DNAs (Fig. 2, lane 2). The smearing of signals started at position 4.0 kb of the gel containing full-length rcDNA. The control culture exhibited no detectable polymerase activity (Fig. 2, lane 3). Intracellular core particles purified through a sucrose gradient (28)

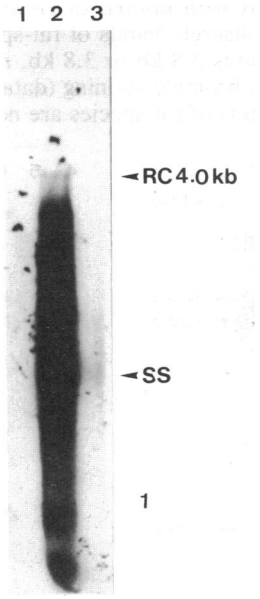


FIG. 2. Enzymatic assay of HBV endogenous polymerase activity. Core particles enriched from a CsCl gradient were incubated with radiolabeled dNTPs. HBV-specific incorporation of radioactivity was monitored by gel electrophoresis (28, 29). Lanes: 1, λ phage *Hin*dIII size markers; 2, core particles from the medium of Q7 HBV-21 cells; 3, negative control medium of the untransfected parental cell line Q7.

also exhibited significant polymerase activity (data not shown).

**HBV-Specific RNA Transcripts.** Transcription products of the HBV genome include three major size classes: 3.5, 2.5, and 2.1 kb (1–3). The 2.1-kb transcript had the strongest intensity, and the 2.5-kb transcript had the weakest intensity (Fig. 3, lanes 1–3). This intensity pattern was virtually indistinguishable from that of a HBV-producing system of human HepG2 hepatoblastoma cell line (data not shown). No HBV-specific transcripts were detected in the untransfected parental cell line Q7 (Fig. 3, lanes 1–3).

In addition to the three transcripts mentioned above, we have found another transcript at 2.2 kb that cross reacts with a core-gene-specific probe (positions 1987–2840) (Fig. 3, lanes 4–6). This transcript was detected in all the HBV-producing human HepG2 or Q7 rat hepatoma cell lines examined (data not shown). When the pSV2ANeo vector was used as a probe on the same Northern blot, a 2.6-kb band specific to the Neo$^r$ gene was observed (data not shown).

**HBV Dane-like Particles and Subviral Particles.** To see if the maturation process of HBV virion could occur in the stably transfected cultures of Q7 cells, we examined conditioned medium from Q7 HBV-21 and Q7 HBV-5 cells by electron microscopy. The pellet collected from conditioned medium was fractionated by centrifugation through a cesium chloride density gradient. Dane-like particles with a double-shell concentric shape and an averaged diameter of 40–46 nm were observed in fractions at a density of ≈1.24 g/ml (Fig. 4*C*) (30). In addition, the 22-nm spherical and filamentous forms were observed as well in fractions at a density of ≈1.20 g/ml (Fig. 4 *A* and *B*). Southern blot analysis indicated that the Dane-particle fractions (1.34 g/ml density) contain predominantly a 4.0-kb rc form, and the core particle fractions contain predominantly a 1.5-kb ss form (data not shown).

## DISCUSSION

The species origin of these HBV-producing Q7 hepatoma cell lines was confirmed by the observation that these cells contain rat-specific repetitive sequences. Upon digestion of Q7 genomic DNA with *Bam*HI or *Hin*dIII, followed by gel electrophoresis, discrete bands of rat-specific repetitive sequences at positions 5.8 kb or 3.8 kb, respectively, can be seen by ethidium bromide staining (data not shown). These characteristic bands of rat species are not present in human

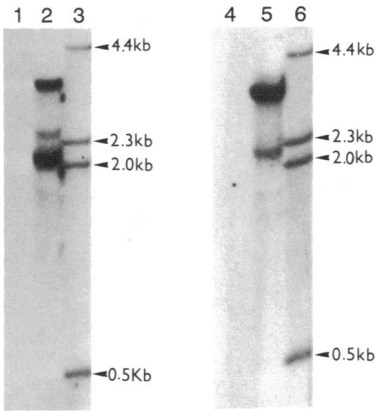


FIG. 3. Northern blot analysis of total RNAs prepared from HBV-producing rat hepatoma cell line Q7 HBV-21 and the untransfected parental hepatoma cell line Q7. Vector-free double-stranded DNA was radiolabeled and used as a probe. Lanes: 1–3, full-length HBV DNA (positions 1–3182) was used as a probe; 4–6, core-specific subgenomic DNA (positions 1987–2840) was used as a probe; 1 and 4, Q7 parental cells; 2 and 5, Q7 HBV-21 cells; 3 and 6, λ phage *Hin*dIII size markers.

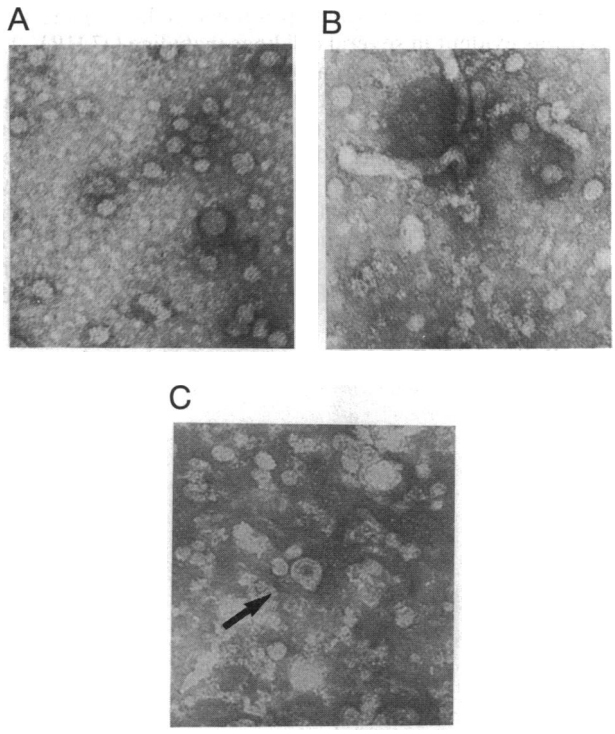


FIG. 4. Morphological examination of HBV viral and subviral particles by electron microscopy. Particles are collected from the conditioned medium of HBV-producing cell lines. HBV surface antigen particles 22 nm in diameter of spherical (*A*) or filamentous (*B*) shape and 38- to 42-nm Dane-like particles (*C*) enriched from the conditioned medium of rat hepatoma cell line Q7 HBV-21. (Bars = 100 nm.)

or mouse genomic DNA when digested with the same restriction enzymes. Although woodchucks and squirrels are among the natural hosts for the hepadnavirus family, other rodents such as rat or mouse have not been found to be hosts for any hepadnaviruses under either natural or experimental conditions. The successful production of Dane-like particles from transfected Q7 cultures indicates that the species barrier between human and rat probably resides at the penetration/absorption step during the early phase of the viral life cycle. Similar results of cross-species replication of HBV were reported in the transgenic mice system during the preparation of this manuscript (16). However, in that system, although complete viral genome replication was reported, completely matured Dane-like particles were not detected by electron microscopy. Our electron microscopic results of Q7 HBV-21 cells (Fig. 4) clearly indicate that even at the steps of viral morphogenesis or excretion, no species barrier exists between human and rat systems.

Among the many human and rat hepatoma cell lines being screened, HepG2, Huh6-c15, and Q7 are the only three cell lines that appear to be able to sustain the production of Dane-like particles (4–8). Although Huh7 seems to be a good host for the transient expression of HBV (7), to our knowledge, no establishment of stable HBV-producing Huh7 cell lines has yet been documented. Any factor(s) that is critical to the cellular permissiveness of HBV must be common to HepG2, Huh6-c15, and Q7 cell lines. The establishment of a stable culture with active HBV propagation in HepG2 has led to the speculation that a well-differentiated environment may be required for HBV production (4, 5). Although the Q7 rat hepatoma cell line retains the ability to produce serum albumin or α-fetoprotein, the degree of differentiation of Q7

cells is not comparable to that of HepG2 cells (26, 27). The relationship between cellular differentiation and viral propagation remains to be elucidated.

The ccc form of HBV DNA has been found in the liver of HBV-infected patients (31). This ccc form is believed to serve as a transcription template and will accumulate during the early phase of infection (32). Although we have detected extra bands of HBV-specific DNA in Q7 HBV-21 but not in HBV-producing HepG2 cells (Fig. 1*A*), these bands did not appear to be cccDNA upon further characterization (unpublished results). It is possible that the synthesis of the pregenomic RNA was from the multiple copies of integrated HBV DNA on the Q7 chromosome (data not shown), rather than from unintegrated cccDNA. Similar results were reported for the human HepG2 systems (5–8). The infectivity of the Dane-like particles produced from the Q7 HBV-21 system was confirmed by injection of conditioned medium into chimpanzee (unpublished results).

It is possible that the 2.2-kb transcript is derived from splicing the 3.5-kb pregenomic RNA (33). The fact that the vector probe alone will not hybridize with this transcript suggests very strongly that this transcript is not generated by initiation from or termination within the cloning vector (Fig. 3). The possibility that this transcript is produced as a virus/cell hybrid transcript from the integrated HBV DNA in Q7 HBV-21 cells also seems highly unlikely. Of four HBV-producing cell lines (either of human or rat origin) that were examined, all contained this transcript comigrating at 2.2 kb (data not shown). The biological significance of this transcript remains to be investigated.

The rat Q7 HBV-production system potentially will offer several major advantages over the human HepG2 system: (*i*) It makes possible a fair comparison of the tumor incidence between HBV-producing and nonproducing rat hepatoma cell lines with almost identical genetic backgrounds. (*ii*) It provides the potential of establishing an animal model to study the immunology of a tumor-bearing rat with active HBV production. The fact that the inbred rat is readily available will allow one to more conveniently study the immunological aspects of HBV-associated hepatocellular carcinoma. (*iii*) It provides a system to study the activities of viral integration and replication during the process of tumor progression in animals.

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