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In Vitro Experimental Infection of Primary Duck Hepatocyte Cultures with Duck Hepatitis B Virus

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Duck hepatitis B virus (DHBV) obtained from the serum of congenitally infected ducks was used to infect primary duck hepatocyte cultures 1 to 4 days after plating. Virus replication was demonstrated by the appearance, beginning at 2 days after infection, of intracellular covalently closed-circular and single-stranded DHBV DNA replicative intermediates which were not present in the inoculating virus preparation. With increasing time after infection there was further amplification of intracellular relaxed circular, covalently closed-circular, and single-stranded DHBV DNA. Cultures of primary duck hepatocytes are competent for infection with DHBV only during the first 4 days of culture. Synthesis of DHBV core antigen and DHBV surface antigen was detected by immunofluorescence in 10% of the hepatocytes in culture. De novo synthesis and release of infectious virus was also demonstrated. Therefore, all stages of viral replication were carried out by these experimentally infected primary hepatocyte cultures. This system makes it possible to study DHBV replication in vitro.

Hepadnaviruses are a family of DNA-containing viruses that include human hepatitis B virus (HBV), woodchuck hepatitis virus (30), ground squirrel hepatitis virus (14), and duck hepatitis B virus (DHBV) (18, 31). These viruses replicate primarily in hepatocytes in vivo and can establish chronic infections (13, 15, 18, 22, 26). Although experimental infection of animals with hepadnaviruses can be achieved (3, 12, 17), one of the major technical problems in hepadnavirus research has been the lack of an in vitro system for productive viral infection and replication. Attempts to infect primary human adult and fetal hepatocyte cultures (2, 7, 9, 21, 25), fetal hepatocyte organ cultures (32), and HeLa cells (7) with human HBV have been described. Although intracellular virus-specific proteins were detected after exposure to virus (2, 9, 23, 27) and extracellular virus was found in the culture medium (9), there was no unequivocal evidence for human HBV replication and de novo viral synthesis.

In this paper we describe a reproducible system for infecting primary duck hepatocytes in vitro with DHBV. DHBV was chosen to study in vitro hepadnavirus replication because of both the availability of duck hepatocytes and the high titer of virus obtained from young congenitally infected ducklings. In vitro infection of primary duck hepatocyte cultures with DHBV resulted in de novo viral DNA synthesis, virus-specific protein production, and release of infectious virus into the medium.

MATERIALS AND METHODS

Experimental animals, virus, and cell culture. Uninfected 1-week-old Pekin ducks were used to obtain primary hepatocyte cultures. DHBV-free Pekin ducks shown previously not to transmit DHBV to their progeny were used as a source of eggs (17) (generous gift of William Mason, Fox Chase Cancer Center). DHBV was obtained from the serum of 2- to 3-week-old Pekin ducks obtained from a flock of congenitally infected ducks (generously provided by Anna O'Connell, Fox Chase Cancer Center). All ducklings were hatched at the Fox Chase Cancer Center, and infected animals were housed separately from uninfected animals. Sera from these animals were tested for DHBV DNA by dot hybridization (17).

Hepatocytes were obtained by a modification of the methods of Seglen and Berry and Friend (1, 24). After the ducks were anesthetized with approximately 0.4 ml of pentobarbital sodium (Nembutal), the livers were perfused via the portal vein with 200 ml of 0.5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] in Swimms 77 medium (GIBCO Laboratories, Grand Island, N.Y.) that was buffered with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) followed by 200 ml of 0.5 mg of collagenase type 1 (Sigma Chemical Co., St. Louis, Mo.) per ml–2.5 mM $CaCl_2$ in Swimms 77 medium. To maintain the perfusion at 37°C, all solutions were kept at 39°C, and livers were covered with plastic wrap. After the perfusion, livers were removed and cells were dispersed in L-15 medium (GIBCO) containing 5% fetal bovine serum (FBS), 15 mM HEPES, 300 mg of penicillin per liter, 100 mg of streptomycin per liter, 1 mg of insulin (Sigma) per liter, 1.5 mg of glucose per liter, 10^{-5} M hydrocortisone-hemisuccinate (Sigma), and nystatin (10 U/ml). Cells were filtered through gauze and centrifuged at $50 \times g$ for 4 min. The cell pellet was washed three times with L-15 medium containing 5% FBS. Cells were counted in a hemacytometer, and 60-mm dishes (Falcon; Becton Dickinson Labware, Oxnard, Calif.) were seeded with circa 5×10^6 cells per dish in L-15 medium containing 5% FBS. Cultures were incubated at 37°C, and the medium was changed every day for the first week and every other day for the next 2 weeks.

Infection of cell cultures with DHBV. Plates (diameter, 60 mm) of primary duck hepatocytes were infected 1 to 7 days after plating with 0.3 to 300 μl of DHBV-positive duck sera adjusted to a final volume of 0.3 ml with L-15 medium containing 1% FBS. The cell monolayer was washed with L-15 medium containing 1% FBS and was incubated with the virus suspension for 1 h at room temperature. The inoculum was removed, and L-15 medium containing 5% FBS was added to the cells. When infections were performed in the presence of rabbit anti-DHBV surface antigen (anti-DHBsAg) antibodies, rabbit anti-DHBV core antigen (anti-

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DHBcAg) antibodies (5, 6) (generous gifts of William Mason, Fox Chase Cancer Center), or normal rabbit serum, a 1:10 dilution of these antibody preparations was incubated with an appropriate dilution of DHBV-positive duck serum at 37°C for 1 h followed by incubation at 4°C for 18 h.

Preparation and analysis of DHBV DNA from hepatocyte cultures and purified virus. To obtain total intracellular viral DNA, cells were lysed in a solution containing 0.2% sodium dodecyl sulfate (SDS), 20 mM Tris hydrochloride (pH 7.4), 10 mM EDTA, 5 mM EGTA, and 150 mM NaCl. To obtain DNA from virus in the cell culture medium and from gradient-purified virus, virus preparations were treated with the above cell lysis solution. Extrachromosomal DNA was obtained essentially by the method of Hirt (8). The cells were lysed in 1 M NaCl–20 mM Tris hydrochloride (pH 7.4)–10 mM EDTA–5 mM EGTA–1% SDS and stored at 4°C overnight. Cellular DNA was pelleted at 16,000 × *g* for 40 min, and the extrachromosomal DNA was extracted from the supernatant (Hirt supernatant). DNA was extracted from all samples by digestion with 500 μg of pronase (Calbiochem-Behring, La Jolla, Calif.) at 37°C for 2 h and deproteinized by extraction with an equal volume of phenol:chloroform (1:1) followed by chloroform. The aqueous phase was brought to 0.3 M sodium acetate (pH 5.2), and nucleic acids were precipitated with 2 volumes of 100% ethanol. The DNA was dissolved in 5 mM Tris hydrochloride (pH 7.4)–1 mM EDTA.

DNA was electrophoresed in a horizontal slab gel of 1.5% agarose in 40 mM Tris hydrochloride (pH 6.9)–20 mM sodium acetate–1 mM EDTA (E buffer). The gel was soaked in 0.05 M sodium acetate (pH 4.2) for 1 h at room temperature and for 1 h at 5°C; the gel was denatured, and the DNA was transferred to nitrocellulose by the method of Southern (27). Hybridization was performed at 52°C with a ^{32}P-labeled plasmid pSP6-derived RNA probe transcribed as described by Melton et al. (19) from DHBV cloned into a plasmid pSP65 vector (19) at the *Eco*RI site (pSP6DHBV 5.1). The DHBV DNA used for cloning was obtained from a pBR subclone of pBR322-DHBV 2.3 which was originally derived from supercoiled DHBV DNA cloned at the *Eco*RI site (20). The DHBV DNA was inserted into the pSP65 vector in a direction which yields transcripts of plus-strand polarity. For molecular weight markers, bacteriophage λ DNA was digested with *Hin*dIII, and the fragments were labeled with [^{32}P]TTP. Autoradiography was done at −80°C with film (XAR; Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen.

Purification of virus from cell culture medium and sucrose gradients. Cell culture medium (30 ml) was clarified at 16,000 × *g* for 10 min. The medium was then layered onto a 4-ml 10 to 20% (wt/vol) sucrose gradient in 150 mM NaCl–20 mM Tris hydrochloride (pH 7.4) and subjected to centrifugation at 113,000 × *g* for 19 h at 4°C. The supernatant was then discarded, and the virus pellet was suspended in L-15 medium. Virus was stored at 4°C.

Isopycnic centrifugation of pelleted virus was done in gradients of 20 to 60% (wt/vol) sucrose containing 150 mM NaCl–20 mM Tris hydrochloride (pH 7.4) with a 70% sucrose (wt/vol) cushion in D_2O containing 150 mM NaCl–20 mM Tris hydrochloride (pH 7.4). After centrifugation at 157,000 × *g* for 16 h at 4°C, fractions were collected from the bottom of the centrifuge tube. The density of the fractions was determined with a refractometer. DNA was isolated from these gradient fractions, and viral DNA was detected by using the technique of Southern blotting (27) as described above.

Immunofluorescent staining of cultured primary duck hepatocytes. Cells were grown on glass cover slips in 35-mm petri dishes. After the culture medium was removed, cells were fixed in 95% ethanol–5% glacial acetic acid for at least 30 min at −20°C. Samples could be left in this fixative for several days at −20°C. Cells were washed in phosphate-buffered saline (PBS) and incubated for 30 min at 37°C with rabbit antisera directed against either viral surface antigen or immature core antigen. The production and characterization of these antisera is described by Halpern et al. (5, 6). Both antisera were preadsorbed with an equal volume of a homogenate of uninfected duck liver. The antisera were clarified by centrifugation and diluted 1:20 in DHBV-seronegative duck serum.

Cells were next washed four times in PBS and were incubated for 30 min at 37°C with a 1:100 dilution of affinity-purified goat anti-rabbit immunoglobulin G conjugated to either fluorescein or rhodamine (Cooper Biomedical, Inc., West Chester, Pa.). Cells were washed five times in PBS, and cover slips were inverted on a drop of 90% (vol/vol) glycerol–PBS–1 mM EDTA on a microscope slide and viewed with a microscope (Universal; Carl Zeiss, Inc., New York, N.Y.) with epi-illumination. Photographs were taken (Tri-Xpan film; Kodak). The ASA rating of this film when developed (Diafine; Acufine Inc.) was 1600.

Detection of DHBV surface antigen by immunoblotting. Fractions from the sucrose gradient of DHBV pelleted from cell culture medium were applied directly to nitrocellulose prewet in 10 mM Tris hydrochloride (pH 7.4)–150 mM NaCl (TN) with a slot-blot apparatus (Schleicher & Schuell, Inc., Keene, N.H.). The filter was washed briefly in TN and blocked for 1 h with 5% (wt/vol) ovalbumin in TN at room temperature. After washing for 5 min in TN, the filter was incubated for 4 to 24 h at 4°C with a 1:50 dilution of monoclonal antibody directed against DHBV surface antigen obtained from tissue culture fluid supernatants (generous gift of Anna O'Connell and Kathy Molnar-Kimber, Fox Chase Cancer Center). The filter was washed extensively in TN and incubated with 10 μg of rabbit anti-mouse immunoglobulin G (Cooper) per ml in ovalbumin in TN for 1 h at room temperature. After washing in TN, the filter was incubated for 1 h with ^{125}I-labeled protein A from *Staphylococcus aureus* (2 × 10^5 cpm/ml) in ovalbumin in TN. The filter was washed four times in TN and once in 0.05% Nonidet P-40 in TN. After drying, the filter was subjected to autoradiography.

Assay of endogenous DNA polymerase activity. Virus pelleted from 4 ml of culture medium was incubated with a mixture of 0.5 mM dATP–0.5 mM dGTP–0.5 mM dCTP–1 μCi of [α-^{32}P]TTP (400 to 800 Ci/mmole)–10 mM $MgSO_4$–10 mM Tris hydrochloride (pH 7.5)–0.1% (wt/vol) Triton X-100–25 mM NaCl. Reactions were incubated for 1 h at 37°C. Pronase was then added to a final concentration of 200 μg/ml, SDS was added to a final concentration of 0.2%, and the reactions were incubated for 2 h at 37°C. The samples were electrophoresed on a 1.5% agarose gel in E buffer containing 0.1% SDS. The gel was dried and subjected to autoradiography.

RESULTS

Intracellular DHBV DNA replication during an experimental infection. To determine the ability of DHBV to infect cells in vitro, we exposed uninfected primary hepatocytes 1 day after plating to 30 μl of duck serum containing approximately 5 × 10^9 virions/ml (17). To determine if DHBV was replicating in hepatocyte cultures, cells were harvested from 1 h

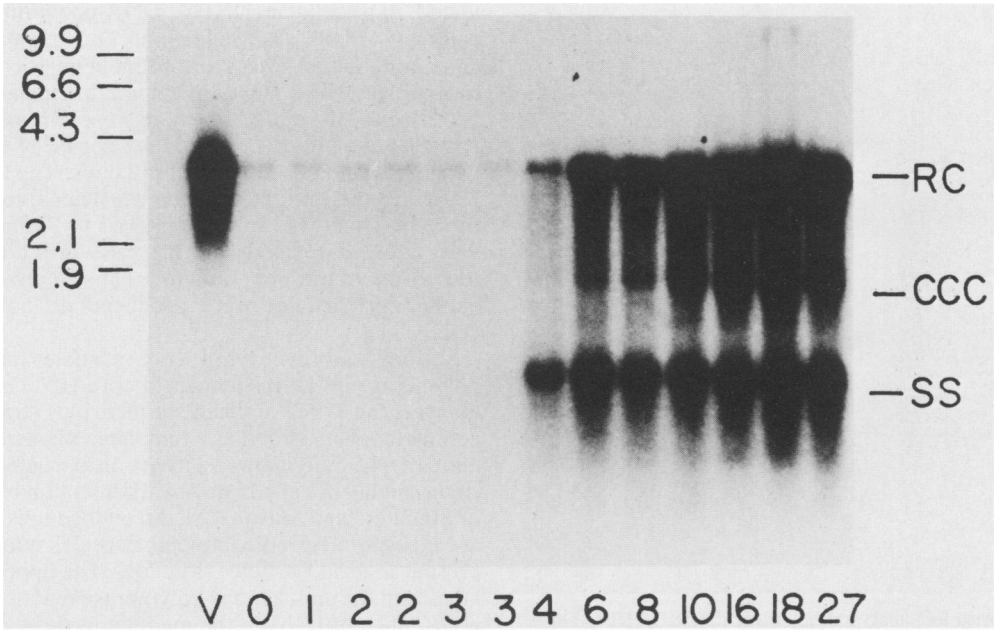

FIG. 1. Southern blot analysis of intracellular DHBV DNA separated on a 1.5% agarose gel. DNA was isolated at various times after an infection of primary duck hepatocyte cultures with serum-derived DHBV. Intracellular DNA was extracted, after pronase digestion, from cells harvested at 0 (1 h), 1, 2, 3, 4, 6, 8, 10, 16, 18, and 27 days after infection. Lanes: five times the amount of virus used to infect one plate of cells was loaded onto lane V, and DNA from one-fourth of a 60-mm dish of cells was loaded onto the other lanes of the gel. Days 2 and 3 after infection are duplicate samples. RC, CCC, and SS DNA species are indicated. The size markers (in kilobases) are *Hin*dIII-digested bacteriophage λ DNA.

(day 0) to 27 days after infection, and DNA from the cell monolayers was analyzed for relaxed-circular (RC), single-stranded minus strand (SS), and covalently closed-circular (CCC) virus-specific DNA replicative intermediates (16, 17, 28) by agarose gel electrophoresis and Southern blot hybridization. These intracellular DNA species are found in DHBV-infected hepatocytes in vivo. CCC DNA is formed at an early step in the initiation of an in vivo infection, and it has been suggested that the CCC DNA may serve as a template for DNA transcription (17). SS DNA is synthesized by reverse transcription of a genome-length viral RNA within immature core particles. The RNA is then degraded, leaving the SS DNA free to serve as a template for plus-strand DNA synthesis. Completion of the plus strand results in the formation of the RC virion DNA (28).

Intracellular DHBV, RC, SS, and CCC DNA molecules were detected 4 days after the experimental infection (Fig. 1). Most of the inoculating virus did not adsorb to the cell monolayer; therefore, only a small amount of inoculating virus remained associated with the cells at 1 h to 3 days after infection. DNA replicative intermediates differed from the inoculating virus (Fig. 1, lane V) which contained only RC DNA. In other experiments and in a longer exposure of the blot in Fig. 1, relatively small amounts of CCC and SS DNA were detected as early as 2 days after the infection. The quantity of replicative intermediates continued to increase during 3 weeks in culture. By using this Southern blot assay we were able to detect virus replication in plates infected with as little as 0.3 μl of infectious serum (data not shown).

Susceptibility of hepatocyte cultures to infection. To determine the time in which cells propagated in vitro were susceptible to DHBV infection, cells were infected at 4 (Fig. 2A) and 7 (Fig. 2B) days after plating. DNA was isolated at various times after the infection and was analyzed by Southern blot hybridization. Intracellular viral replicative intermediates accumulated 5 through 14 days after infection when cells were exposed to virus 4 days after plating. However, as illustrated in Fig. 2B, at 7 days after plating cells were no longer susceptible to infection with the virus. Although a portion of the inoculum remained associated with the cell layer (Fig. 2B, day 2), no intracellular replicative intermediates could be detected as late as 14 days after infection.

Reduction of infectivity by anti-DHBsAg antibody. To characterize the infectious agent in the serum, we determined whether antibodies specific for viral proteins would block infectivity. Serum containing DHBV was incubated with rabbit antibodies directed against DHBV surface antigen (anti-DHBsAg), the viral coat protein, and against DHBV core antigen (anti-DHBcAg), the nucleocapsid protein. The serum was then assayed for infectivity on primary hepatocyte cultures by using Southern blot hybridization. The anti-DHBsAg antibodies (Fig. 3, lane b) reduced the infectivity of DHBV to undetectable levels, by at least 100-fold compared with DHBV not treated with antisera (Fig. 3, lane d). Virus infectivity was not reduced in serum pretreated with anti-DHBcAg antibodies (Fig. 3, lane c) or with normal rabbit serum (Fig. 3, lane a). These results suggest that the infectious agent in serum is a particle coated with surface antigen.

Production of viral antigens in infected cells. Polyclonal rabbit antisera directed against DHBsAg and against DHBcAg were used to detect viral antigens at various intervals after infection (5, 6). Cells were fixed at 1-day intervals after infection and stained with the appropriate antiserum. Core antigen was detected in hepatocytes approximately 5 days after infection (Fig. 4B) and was detected in nearly 10% of the cells 8 days after infection. The percentage of positive cells did not appear to increase after this time, but the intensity of staining did increase between 5 and 10 days (data not shown). Core antigen was detected in

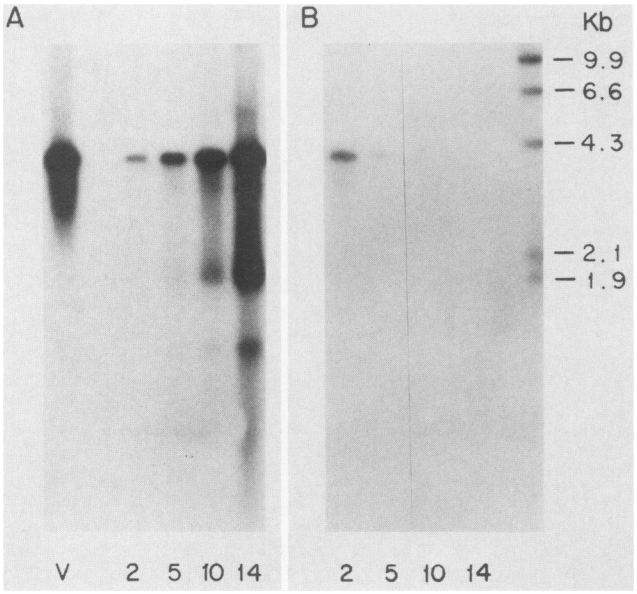

FIG. 2. Southern blot analysis of intracellular DHBV DNA separated on a 1.5% agarose gel. DNA was obtained after pronase digestion from Hirt supernatants of hepatocyte cultures infected at (A) 4 days and (B) 7 days after plating. The inoculating virus was loaded onto lane V (panel A). Cells were harvested from both sets at 2, 5, 10, and 14 days after infection. DNA from one-fourth of a 60-mm plate was loaded onto each lane. The size markers (in kilobases [kb]) are *Hin*dIII-digested bacteriophage λ DNA.

both the cytoplasm and nuclei of infected cells; in the nucleus, staining appeared to be associated with the nucleolus. Viral surface antigen reached detectable levels in the cytoplasm of 10% of infected cells approximately 8 days after infection (Fig. 4D). The intensity of staining increased between 8 to 10 days after infection (data not shown). The percentage of positive cells did not increase after this time. Double staining of cells for DHBsAg and DHBcAg with the rabbit antisera indicated that cells positive for DHBsAg were also positive for DHBcAg (data not shown).

The intensity of staining for both DHBcAg and DHBsAg varied among cells, reflecting differential accumulation of viral proteins. Cells producing viral antigens did not appear to be morphologically different from neighboring cells not producing antigen. There was no evidence of foci of infected cells; most positive cells were well isolated from one another. Viral antigens were detected in infected cells for 3 weeks after infection. Increasing the multiplicity of infection (based on the number of DNA-containing particles) from 70:1 to 700:1 resulted in an increase in the proportion of infected cells from about 1 to 10%. Increasing the multiplicity of infection to 7,000:1 did not result in the appearance of substantially more infected cells.

Characterization of extracellular DHBV and DHBsAg released during experimental infection. To determine if virus was released from the in vitro-infected hepatocytes, medium from cultures experimentally infected with DHBV was analyzed for particles containing DHBV DNA at 3, 4, 8, 10, 15, 18, and 27 days after infection. Particles were pelleted from the total medium of one plate of cells, and viral DNA was extracted, separated on a 1.5% agarose gel, and analyzed by Southern blot hybridization (Fig. 5A). Particles containing RC viral DNA were detected in the culture medium 10 days after infection. In similar experiments (see Fig. 7) we detected extracellular DHBV DNA-containing particles as early as 4 days after infection. The quantity of particles containing DHBV DNA in the medium was determined by comparison with a standard amount of cloned viral DNA. At 2 to 3 weeks after infection there were approximately 3×10^7 particles containing DHBV DNA per ml of culture medium which had remained on cells for 2 days. The total quantity of DNA detected in extracellular particles over the course of the experiment was approximately 5 to 10 times that of input viral DNA associated with the cells 24 h after exposure to the virus (data not shown). Thus, extracellular DNA-containing particles were produced de novo by infected cells.

Virions and liver viral core particles contain a DNA polymerase in the nucleocapsid core (10, 11, 29) which can elongate the 3′ end of the incomplete plus strand by using the complete minus strand as a template. We assayed for endogenous DNA polymerase activity in particles pelleted from supernatants of cells 15 days after infection by the procedure of Summers and Mason (28). An endogenous DNA polymerase incorporated radioactive nucleotides into viral RC DNA present in virus particles (Fig. 5B). The appearance of DNA labeled in the endogenous polymerase reaction paralleled the appearance of DNA in the medium as detected by Southern blot hybridization (data not shown). These results indicate

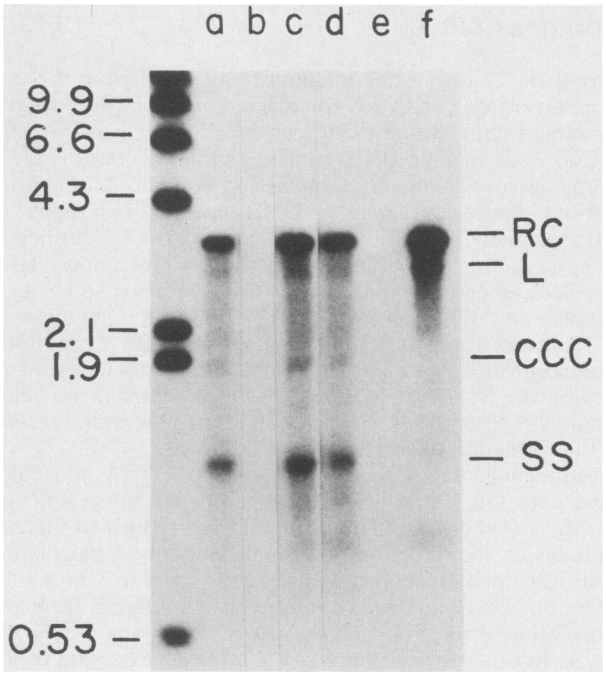

FIG. 3. Reduction of infectivity with anti-DHBsAg antibodies. Serum containing DHBV (lane f, DNA extracted from serum) was incubated for 1 h at 37°C with a 1:10 dilution of normal rabbit serum (lane a), rabbit anti-DHBsAg antibody (lane b), and rabbit anti-DHBcAg antibody (lane c). The virus was then used to infect hepatocytes in culture. At 12 days after infection the cells were harvested, intracellular DNA was isolated and separated on a 1.5% agarose gel, and the DHBV DNA was detected by Southern blotting. Lanes: d, DNA from a culture infected with DHBV without preincubation with antibody; e, DNA from an uninfected culture. L, Linear viral DNA molecules. The RC, CCC, and SS DNA species are described in Fig. 1. The size markers (in kilobases) are *Hin*dIII-digested bacteriophage λ DNA.

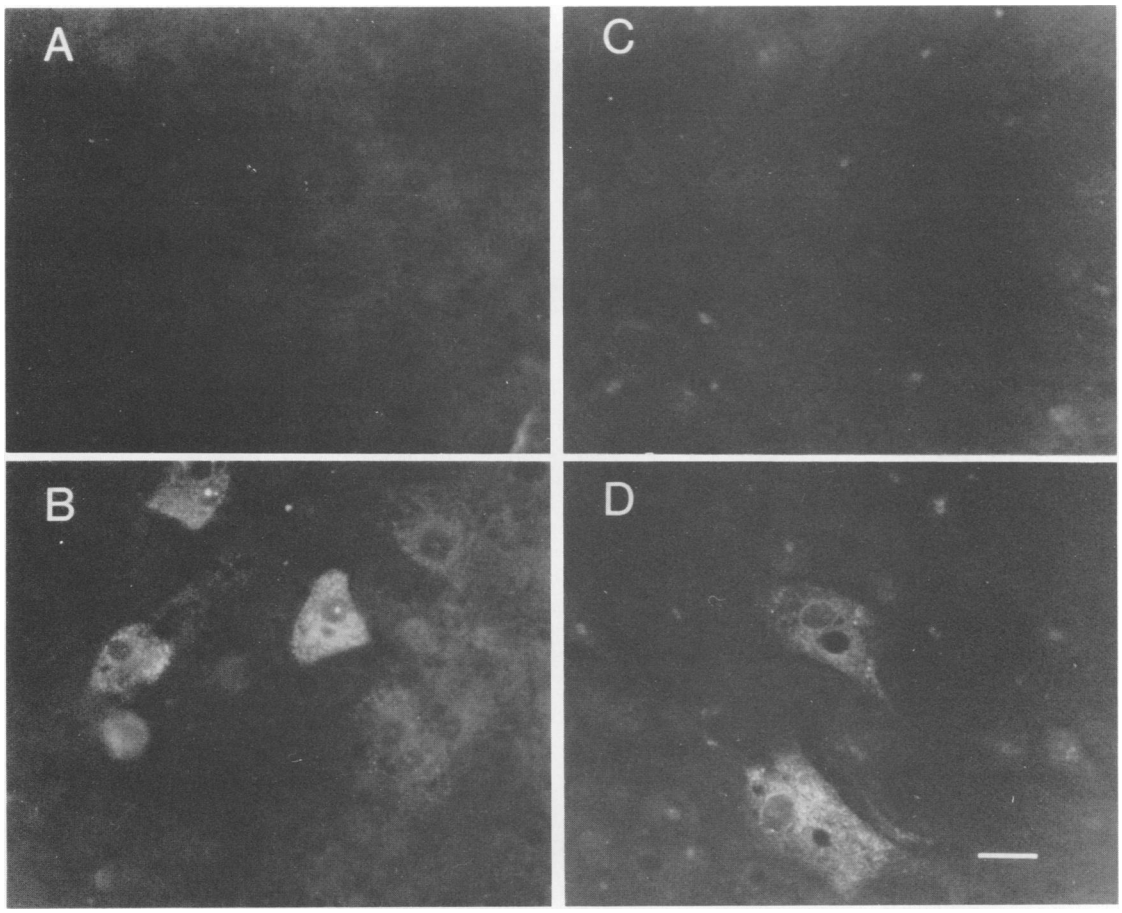

FIG. 4. Indirect immunofluorescence of cultured hepatocytes. Panels A and B are uninfected and infected cells, respectively, fixed 9 days postplating and stained for the presence of DHBcAg. Panels C and D are uninfected and infected cells, respectively, fixed 12 days after plating and stained for DHBsAg. Exposure times were approximately 30 s. Bar, 15 μm.

that virions released into the medium contain the DNA polymerase activity found in serum-derived virus.

At 10 and 15 days after infection, extracellular particles contained only RC DNA (Fig. 5A). This differed from intracellular viral DNA molecules (RC, CCC, and SS DNA replication intermediates) found during the same interval after infection (Fig. 1), indicating that there was a selective release of more mature virus particles containing only RC DNA. This is consistent with the normal maturation and release of mature virus particles in vivo. Later, at days 18 through 27 after infection (Fig. 5A), RC and SS DNA were found in particles in the culture medium. The RC and SS DNA could be packaged into virions containing DHBsAg. Alternatively, the particles containing RC and SS DNA could result either from the selective release of immature viral core particles from viable infected cells or from release caused by cell death. Evidence for cell death, i.e., loss of cellular DNA and detection of floating cells in the medium, began 2 weeks after plating the hepatocytes whether or not the cultures were infected with DHBV.

To determine which, if any, of the DNA-containing extracellular particles had the physical properties of mature virions, particles obtained from culture medium 18 days after infection were fractionated on isopycnic density gradients. DNA was extracted from each fraction, subjected to agarose gel electrophoresis, and analyzed by Southern blot hybridization. Parallel gradients were used to determine the banding position of authentic virus (1.14 g/cm^3) and cores (1.42 g/cm^3). Particles banding at the density of complete virus (fractions 10 through 15) contained only mature DNA, whereas immature forms of viral DNA were found exclusively in particles banding in fractions 2 through 6, a density characteristic of immature cores (Fig. 6A).

To determine if infected cells released DHBsAg into the medium, fractions of the gradient were assayed for DHBsAg by immunoblotting (Fig. 6B). DHBsAg reactivity was detected in fractions containing mature viral DNA (fractions 10 through 15) but not in fractions containing immature core particles. The combined presence of DHBsAg and mature viral DNA in the same fractions of the sucrose gradient is consistent with the interpretation that infected cells released virus particles with DHBsAg envelopes. When extracellular particles were analyzed by density gradient centrifugation in CsCl, we were able to separate virions containing DHBV DNA and DHBsAg from surface antigen particles lacking DHBV DNA (data not shown), indicating that infected cells released noninfectious surface antigen particles as well as virions.

Infectivity of extracellular virus. To determine if infectious virus was released into the medium of experimentally infected cells and if infectivity was related to the appearance of particles containing viral DNA, particles pelleted from culture medium at 2 and 18 days after infection were assayed for infectivity on primary hepatocyte cultures. Medium ob-

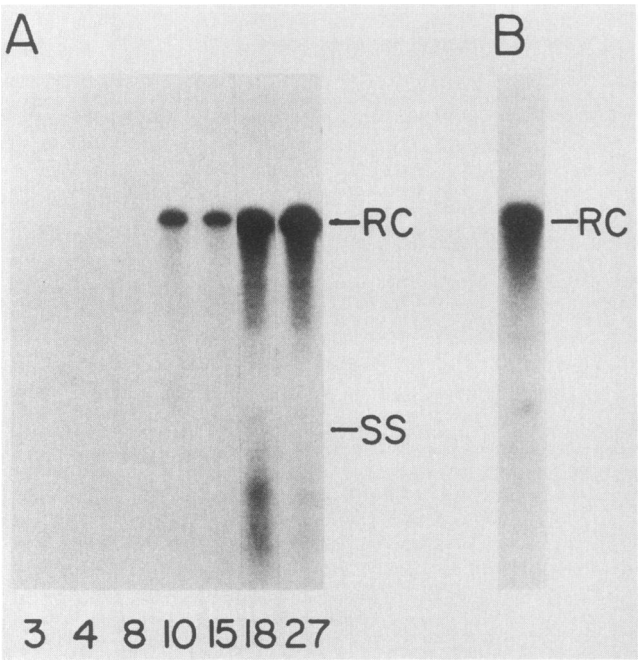

FIG. 5. (A) Southern blot analysis of extracellular DHBV DNA separated on a 1.5% agarose gel. Extracellular virus was isolated by pelleting particles from medium harvested at 3, 4, 8, 10, 15, 18, and 27 days after infection from DHBV-infected primary hepatocytes. DNA was extracted from the pellet, and the entire harvest from one plate of cells was loaded onto each lane. (B) Products of the endogenous polymerase assay of extracellular DHBV separated on a 1.5% agarose gel. Virus was pelleted from culture medium 18 days after infection and was incubated for 1 h at 37°C with dATP, dGTP, dCTP, and [α-^{32}P]TTP. Pronase and SDS were added to final concentrations of 200 μg/ml and 0.2%, respectively, and the reaction was incubated for 2 h at 37°C. The DNA was directly run on a 1.5% agarose gel. The gel was dried and autoradiographed. RC and SS DHBV DNA species are indicated.

tained 2 days after infection did not contain detectable DNA-containing particles, whereas medium collected 18 days after infection contained virus-like particles as well as immature cores (Fig. 5A). Cells exposed to particles from these two samples were harvested 2, 8, and 12 days after infection and were subsequently analyzed for intracellular DHBV DNA replicative intermediates as evidence of infection (Fig. 7A and B). No evidence of infectivity was detected in medium obtained 2 days after infection (Fig. 7A). Medium obtained 18 days after infection did contain infectious virus as evidenced by the de novo appearance of intracellular DHBV DNA replicative intermediates in the exposed cultures (Fig. 7B). To determine the kinetics of the production of infectious virus in the culture supernatants, media obtained 2, 4, 6, 11, and 17 days after infection were assayed on uninfected hepatocytes (Fig. 7C). Viral DNA-containing particles were first detected in the media at 4 days after infection and increased in amount through 17 days. The relative infectivity of these culture supernatants was determined by Southern blot hybridization analysis of intracellular DHBV DNA 8 days after infection and was found to be proportional to the amount of viral DNA-containing particles (Fig. 7C).

DISCUSSION

Virus replication in primary duck hepatocytes was demonstrated 2 days after infection by the appearance and subsequent amplification of intracellular CCC and SS DHBV replicative intermediates which were not present in the inoculating virus, by the synthesis of DHBcAg and DHBsAg by infected hepatocytes, and by the release of whole infectious virus. Therefore, a full cycle of viral replication occurred after an in vitro infection of primary hepatocyte cultures.

The first forms of intracellular viral DNA to appear after infection were the SS and CCC DNA followed by the RC DNA. The ratio of SS to RC DNA was higher earlier in infection than at later times. These results are in agreement with the model of DHBV DNA replication proposed by Summers and Mason (28); minus strands (SS) are synthesized before plus strands in the formation of an RC molecule.

Viral DNA-containing particles were found in culture fluids 6 to 12 days after intracellular viral DNA replicative intermediates were detected. These particles had a buoyant density similar to that of serum-derived virus and contained an active DNA polymerase. Virus-like particles contained mainly mature forms of viral DNA (RC molecules) rather than the immature forms (incomplete RC and SS molecules) which are found in intracellular viral core particles. These results indicate that there is a selective release of virus-like particles from the cells. In older cultures, immature forms of viral DNA were detected in extracellular particles which had a density similar to that of immature core particles. The amount of cell death occurring in the cultures at this later stage could easily account for the number of free immature cores in the culture fluids.

The rate of release of extracellular virus increased as the infection proceeded. Infectivity in the medium also increased during this time and was approximately proportional to the amount of virus-like particles, indicating that the DNA-containing particles were infectious. Since the medium contained immature core particles as well as virions, we cannot distinguish between the infectivity of the virions and the possible infectivity of the immature core particles. Our data indicate that additional cycles of infection are not significant in this experimental system. At the time when infectious virus is released into the medium, the cultures are no longer susceptible to infection with serum-derived virus. Also, since focal staining for viral antigens was not detected in the infected cultures, we conclude that spread of virus to adjacent cells was not occurring.

Via immunofluorescence analysis we were able to observe infection of only 10% of the hepatocytes in the cultures. Increasing the multiplicity of infection 10-fold did not result in substantially more infected cells. We have previously observed (unpublished observations) that all of the hepatocytes in primary hepatocyte cultures from a duck congenitally infected with DHBV appear to carry out virus replication, and therefore we do not know the reason for the limited infection of hepatocytes in vitro. The perfusion procedure could have destroyed the capacity for infection of all but 10% of the cells. Alternatively, a greater percentage of cells were susceptible to infection, yet surface antigen particles in the inoculating virus preparation could have competed with the virus for cell surface receptors.

We have found that polyclonal rabbit anti-DHBsAg antibody reduces the infectivity of DHBV in vitro. This indicates that the infectious agent in the serum contains an envelope that shares determinants with the surface antigen particles. At least two mechanisms could account for the reduction of infectivity. Antibodies could bind to viral receptors for cell attachment, thereby blocking attachment of the virus to the cells. Alternatively, the anti-surface antigen

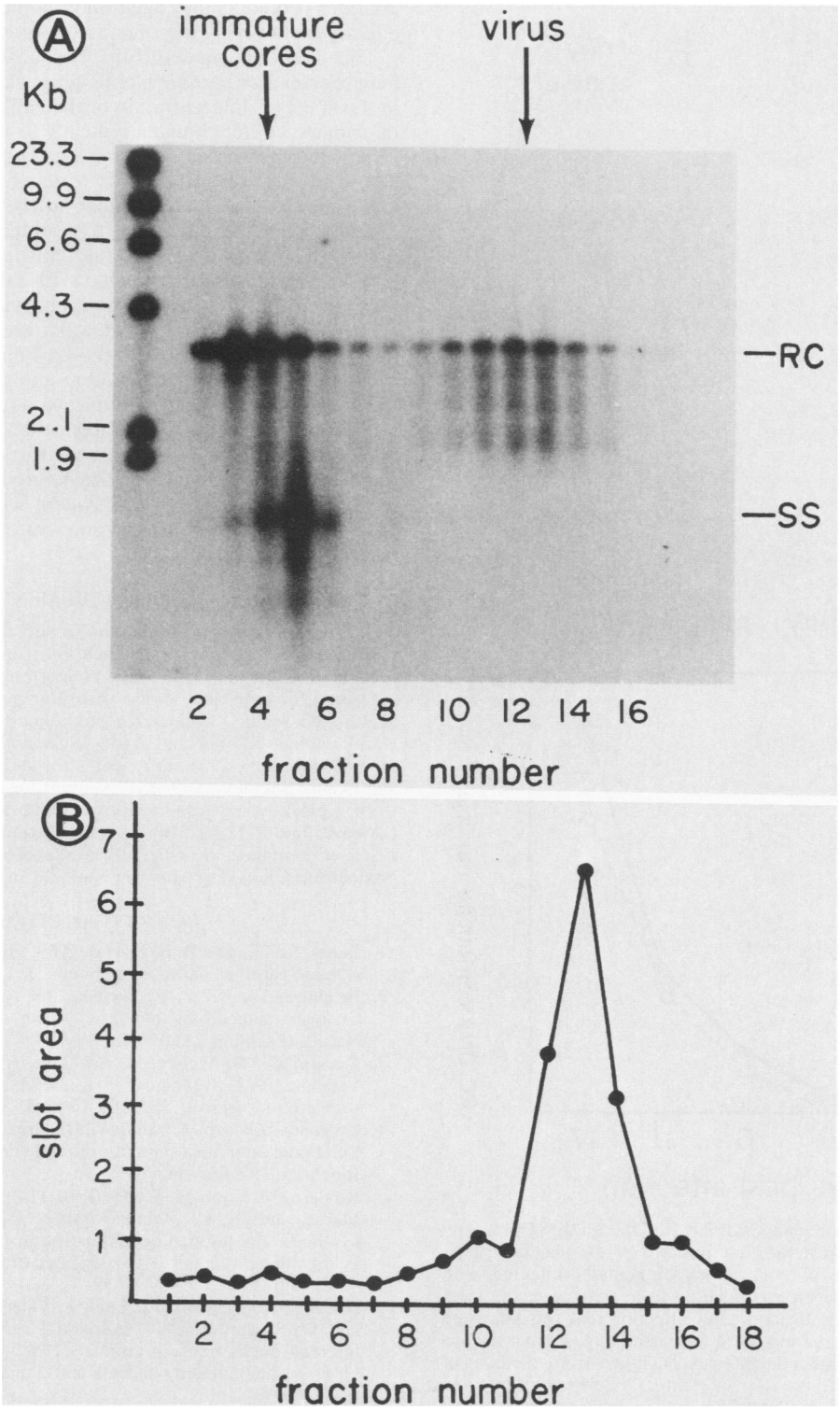

FIG. 6. (A) Southern blot analysis of a sucrose gradient of DHBV particles obtained from medium of cultures 18 days after infection. Virus was pelleted over a 10 to 20% sucrose gradient at 113,000 × *g*. The pellet was subjected to isopynic centrifugation in a 20 to 60% sucrose gradient (wt/vol) with a 70% sucrose (wt/vol) cushion in D_2O. The gradients were centrifuged for 16 h at 157,000 × *g* and were fractionated from the bottom of the centrifuge tubes. The DNA was isolated from each fraction and separated on a 1.5% agarose gel, and the DHBV DNA was detected by Southern blotting. Virus, position of serum-derived DHBV; immature cores, position of Triton-treated cytoplasmic cores from a DHBV-infected liver. RC and SS DNA species are indicated. The size markers (in kilobases [kb]) are *Hin*dIII-digested bacteriophage λ DNA. (B) Immunoblot analysis of DHBV surface antigen (DHBsAg) in the sucrose gradient. To detect DHBsAg, gradient fractions were dotted onto a nitrocellulose filter, and the filter was incubated with a monoclonal rabbit anti-DHBsAg antibody followed by rabbit anti-mouse immunoglobulin G and ^{125}I-labeled protein A. The filter was autoradiographed, and the autoradiogram was scanned with a densitometer. ^{125}I signals (slot area) versus fraction numbers are plotted.

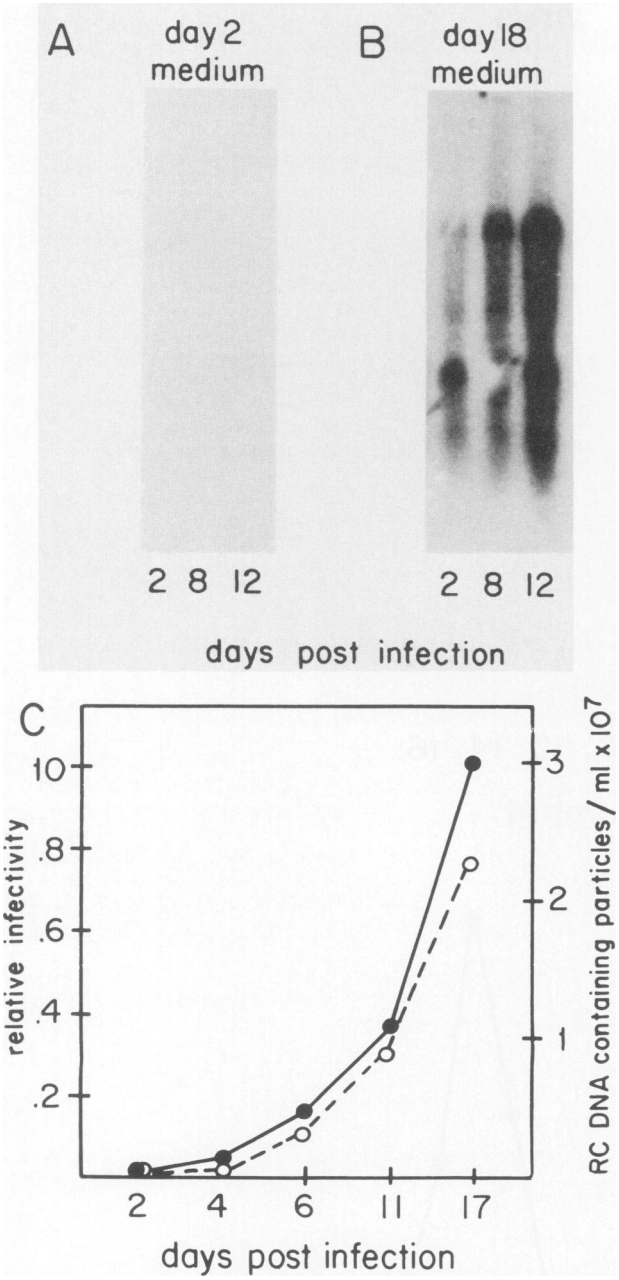

FIG. 7. Southern blot analysis of intracellular DHBV DNA from hepatocytes infected with the first passage of extracellular virus pelleted from medium of cultures experimentally infected with DHBV. Extracellular virus was pelleted from 8 ml of media from cells (A) 2 days and (B) 18 days after infection and was passaged onto uninfected cultures. Cells were harvested at 2, 8, and 12 days after infection. (C) Extracellular virus passaged from medium of cells 2, 4, 6, 11, and 17 days after infection. Cultures were infected with pelleted material from 8 ml of medium and were harvested at 8 days after infection. The intracellular DNA was isolated and separated on a 1.5% agarose gel, and the DHBV DNA was detected by Southern blotting. Relative infectivity (●) was determined by comparing the amount of the intracellular RC DHBV DNA to RC DHBV DNA obtained at 8 days postinfection from a culture infected with 30 μl of serum-derived virus (1.0 units). The amount of DHBV DNA-containing particles in the medium (○) used for the infection (before concentrating) was determined by comparison with a standard amount of cloned DHBV DNA.

antibody could cause agglutination of the virus, thereby effectively lowering the titer of virus available for infection.

The loss of susceptibility to infection of cultured hepatocytes 7 days after plating most likely reflects a change in the stage of differentiation of the cells in culture. Cultures of primary rat hepatocytes typically lose hepatocyte specific functions (such as albumin synthesis) soon after plating (4, 23). The cultures of primary duck hepatocytes in these experiments also lose albumin mRNA and change their morphology from polygonal to fibroblast-like 7 days after plating (Christine Pourcel, unpublished observation). Although cells cannot be infected with DHBV at 7 days after plating, viral DNA replicative intermediates continue to increase after this time in cells that are infected early after plating. Therefore, in cells infected 7 days after plating viral replication is probably blocked at an early step, i.e., before the synthesis of replicative intermediates.

This in vitro infection of primary duck hepatocyte cultures with DHBV will not be suitable for cloning and plaque-purifying virus because of the limited infectibility of the cells. However, this in vitro system will be very useful for studying early events in viral entry into cells as well as viral replication and maturation.

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LITERATURE CITED

1. **Berry, M. N., and D. S. Friend.** 1969. High-yield preparation of isolated rat liver parenchymal cells. J. Cell Biol. **43**:506–520.
2. **Brighton, W. D., P. E. Taylor, and A. J. Zuckerman.** 1971. Changes induced by hepatitis serum in cultured liver cells. Nature (London) **232**:57–58.
3. **Ganem, D., B. Weiser, A. Barchuk, R. J. Brown, and H. E. Varmus.** 1982. Biological characterization of acute infection with ground squirrel hepatitis virus. J. Virol. **44**:366–373.
4. **Guguen-Guillouzo, C., and A. Guillouzo.** 1983. Modulation of functional activities in cultured rat hepatocytes. Mol. Cell. Biochem. **53/54**:35–56.
5. **Halpern, M. S., J. M. England, D. T. Deery, D. J. Petcu, W. S. Mason, and K. L. Molnar-Kimber.** 1983. Viral nucleic acid synthesis and antigen accumulation in pancreas and kidney of Pekin ducks infected with duck hepatitis B virus. Proc. Natl. Acad. Sci. USA **80**:4865–4869.
6. **Halpern, M. S., J. M. England, L. Flores, J. Egan, J. Newbold, and W. S. Mason.** 1984. Individual cells in tissues of DHBV infected ducks express antigens cross reactive with those on virus surface antigen particles and immature viral cores. Virology **137**:408–413.
7. **Hirschman, S. Z., and E. Garfinkel.** 1980. Infection of human cells in culture with hepatitis B virus. Trans. Assoc. Am. Physicians **93**:60–71.
8. **Hirt, B.** 1976. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
9. **Ishida, N. M., M. Watanabe, and H. Ohori.** 1978. Further evidence for the growth of hepatitis B virus in oval cells originating from human embryonic liver, p. 85–103. *In* Japan Medical Research Foundation (ed.), Hepatitis viruses. University of Tokyo Press, Tokyo.

10. **Kaplan, P. M., R. L. Greenman, J. L. Gerin, R. H. Purcell, and W. S. Robinson.** 1973. DNA polymerase associated with human hepatitis B antigen. J. Virol. **12**:995–1005.
11. **Landers, T. A., H. B. Greenberg, and W. S. Robinson.** 1977. Structure of hepatitis B Dane particle DNA and nature of the endogenous DNA polymerase reaction. J. Virol. **23**:368–376.
12. **Lindberg, J., C. Pichoud, O. Hantz, L. Vitvitski, J. A. Grimaud, J. M. Gilbert, L. Joubert, D. Frommel, C. Trepo.** 1985. Woodchuck hepatitis virus infection: serologic and histopathologic course and outcome. Eur. J. Clin. Microbiol. **4**:59–61.
13. **Marion, P. L., S. S. Knight, B. K. Ho, Y. Y. Guo, W. S. Robinson, and H. Popper.** 1984. Liver disease associated with duck hepatitis B virus infection of domestic ducks. Proc. Natl. Acad. Sci. USA **81**:898–902.
14. **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.
15. **Marion, P. L., W. S. Robinson, C. E. Rogler, and J. Summers.** 1982. High molecular weight GSHV-specific DNA in chronically-infected ground squirrel liver. J. Cell. Biochem. Suppl. **6**: 203.
16. **Mason, W. S., C. Aldrich, J. Summers, and J. M. Taylor.** 1982. Asymmetric replication of duck hepatitis B virus DNA in liver cells: free minus strand DNA. Proc. Natl. Acad. Sci. USA **79**:3997–4001.
17. **Mason, W. S., M. S. Halpern, J. M. England, G. Seal, J. Egan, L. Coates, C. Aldrich, and J. Summers.** 1983. Experimental transmission of duck hepatitis B virus. Virology **131**:375–384.
18. **Mason, W. S., G. Seal, and J. Summers.** 1980. Virus of Pekin ducks with structure and biological relatedness to human hepatitis B virus. J. Virol. **36**:829–836.
19. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7057.
20. **Molnar-Kimber, K. L., J. Summers, and W. S. Mason.** 1984. Mapping of the cohesive overlap of duck hepatitis B virus DNA and of the site of initiation of reverse transcription. J. Virol. **51**:181–191.
21. **Noyes, F. N.** 1973. Hepatitis B virus antigen development in cultured human hepatocytes. Proc. Soc. Exp. Biol. Med. **144**:845–851.
22. **Omata, M., K. Uchiumi, Y. Ito, O. Yokosuka, J. Mori, K. Terao, Y. Wei-fa, A. P. O'Connell, W. T. London, and K. Okuda.** 1983. Duck hepatitis B virus and liver diseases. Gastroenterology **85**:260–267.
23. **Reid, L. M., and D. M. Jefferson.** 1984. Culturing hepatocytes and other differentiated cells. Hepatology **4**:548–559.
24. **Seglen, P. O.** 1976. Preparation of isolated rat liver cells. Methods Cell Biol. **13**:29–83.
25. **Smith, J. A., and T. I. Francis.** 1972. Immunoepidemiological and *in vitro* studies of possible relationships between Australia antigen and hepatocellular carcinoma. Cancer Res. **32**:1713–1720.
26. **Snyder, R. L., G. Tyler, and J. Summers.** 1982. Chronic hepatitis and hepatocellular carcinoma associated with woodchuck hepatitis virus. Am. J. Pathol. **107**:422–425.
27. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
28. **Summers, J., and Mason, W. S.** 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell **29**:403–415.
29. **Summers, J., A. O'Connell, and I. Millman.** 1975. Genome of hepatitis B virus: restriction enzyme cleavage and structure of the DNA extracted from Dane particles. Proc. Natl. Acad. Sci. USA **72**:4597–4601.
30. **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.
31. **Zhou, Y. Z.** 1980. A virus possibly associated with hepatitis and hepatoma in ducks. Shanghai Med. J. **3**:641–644.
32. **Zuckerman, A. J., P. M. Baines, and J. D. Almeida.** 1972. Australia antigen as a marker of propagation of the serum hepatitis virus in liver cultures. Nature (London) **236**:78–81.