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Rapid Resolution of Duck Hepatitis B Virus Infections Occurs after Massive Hepatocellular Involvement

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A study was carried out to determine some of the factors that might distinguish transient from chronic hepadnavirus infection. First, to better characterize chronic infection, Pekin ducks, congenitally infected with the duck hepatitis B virus (DHBV), were used to assess age-dependent variations in viremia, percentage of DHBV-infected hepatocytes, and average levels of DNA replication intermediates in the cytoplasm and of covalently closed circular DNA in the nuclei of infected hepatocytes. Levels of viremia and viral DNA were found to peak at about the time of hatching but persisted at relatively constant levels in chronically infected birds up to 2 years of age. The percentage of infected hepatocytes was also constant, with DHBV replication in virtually 100% of hepatocytes in all birds. Next, we found that adolescent ducks inoculated intravenously with a large dose of DHBV also developed massive infection of hepatocytes with an early but low-level viremia, followed by rapid development of a neutralizing antibody response. No obvious quantitative or qualitative differences between transiently and chronically infected liver tissue were detected in the intracellular markers of viral replication examined. However, in the adolescent duck experiment, DHBV infection was rapidly cleared from the liver even when up to 80% of hepatocytes were initially infected. In all of these ducks, clearance of infection was accompanied by only a mild hepatitis, with no evidence that massive cell death contributed to the clearance. This finding suggested that mechanisms in addition to immune-mediated destruction of hepatocytes might make major contributions to clearance of infections, including physiological turnover of hepatocytes in the presence of a neutralizing antibody response and/or spontaneous loss of the capacity of hepatocytes to support virus replication.

Hepadnaviruses have the capacity to cause chronic, productive infections of susceptible cells, especially hepatocytes. Recent studies, particularly with the duck hepatitis B virus (DHBV), have led to a fairly refined model of how this is achieved. When a hepadnavirus infects a susceptible cell, the 3-kbp relaxed circular DNA genome is converted in the nucleus to a 3-kbp covalently closed circular (CCC) DNA which functions as a template for the production of a number of species of viral RNA, including the greater-than-unit-length pregenomic RNA. Progeny relaxed circular viral DNA is subsequently synthesized by reverse transcription in immature viral cores localized to the cytoplasm of the infected cell, and mature viral cores are enveloped and exported from the cell as complete infectious virus. Studies of virus replication in cultures of primary hepatocytes have suggested that a small percentage of viral cores containing progeny relaxed circular DNA cycle to the nucleus to amplify and maintain CCC DNA at a modest level (16, 19, 23); moreover, CCC DNA levels can increase in response to changes in the differentiated state of a cell, and CCC DNA amplification is regulated by the pre-S protein of the viral envelope gene (16, 17).

Although few longitudinal studies have been performed on hepadnavirus infection, in vivo, it is generally assumed that the model developed in cell culture is relevant to understanding the progression of natural infections; however, the in vivo infections may have additional features not seen in vitro. In a majority of immunocompetent individuals, infection with hepatitis B virus (HBV), the prototype member of the hepadnavirus family, usually causes a transient viremia, followed by rapid clearance of the virus and subsequent immunity to reinfection, and in only 5 to 10% of patients is a chronic infection established. The immune system clearly has a decisive role in mediating a recovery, and it might be assumed that all infected cells are killed by immune effector cells. However, though the assumption that all infected hepatocytes are destroyed may be correct, it is difficult to understand, in view of early evidence that transient HBV infections in chimpanzees, for example, can involve, at the peak, virtually every hepatocyte in the liver, but with only very modest liver damage (2, 3, 6).

Likewise, recovery in a woodchuck hepatitis virus-infected woodchuck has been reported following 75% involvement of the liver (12). In fact, since transient HBV infections often entail a viremia of several weeks' duration and since hepatocytes are believed, in humans, to have a half-life of many months, it may be that virtually 100% hepatocellular involvement is a common feature of transient infections. These observations, together with the observations that CCC DNA levels are regulated to maintain chronic, productive, cellular infections, seem to be incompatible with either cell killing or cell curing as the primary mechanism for recovery from transient infections, suggesting that some key

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features of an infection and the host antiviral response, as well as the natural history of hepatocytes, are still far from being characterized and understood.

As a first step in characterizing the molecular biology of in vivo infections, we decided to compare chronic and transient DHBV infections in the domestic duck. Chronic infections are generally caused by vertical transmission of DHBV, with hepatocyte infection being evident with the first appearance of the liver, at about 4 to 5 days of embryogenesis (20), well before the appearance of an immune system. These congenitally infected ducks, as expected, seem to be immunotolerant to the viral infection (5). Such early exposure with lack of immune reactivity is not normally encountered in mammalian hepadnavirus infections. In the present study, chronic infection was studied in congenitally infected birds from 14 days of embryogenesis to 2 years posthatch, while transient infections were induced by inoculation of adolescent ducks with a large dose of DHBV at ca. 5 months posthatch. In both systems, we have determined the percentage of infected hepatocytes and the average amount of CCC DNA and replicative intermediate DNA per infected cell, as well as the titer of circulating virus and, where appropriate, the time course of appearance of neutralizing antibodies. Our results reveal, in agreement with earlier reports on HBV and woodchuck hepatitis virus (2, 3, 6, 12), that DHBV infections can also resolve after massive hepatocellular involvement. We have also obtained evidence for the maintenance of multiple copies of CCC DNA per cell in chronic infections and for early CCC DNA amplification in transient infections, as previously reported in hepatocyte cultures (19). The only obvious determinants distinguishing chronic from transient infections were low levels of circulating virus even at the peak of transient infection of the liver, accompanied by the early development of a neutralizing humoral immune response. The very rapid subsequent clearance of virus from the liver, with apparently minimal histologic changes, may be compatible with a model involving both destruction of some infected cells and their replacement by liver regeneration and loss of other hepatocytes through a normal, programmed turnover, with newly formed hepatocytes that arise to replace senescent cells being protected from infection by the presence of neutralizing antibodies. The results also indicate that failure to establish a chronic infection is not due to a failure to amplify CCC DNA.

MATERIALS AND METHODS

Animals. Congenitally DHBV-infected duck embryos and ducklings were obtained from the eggs produced by a flock of congenitally infected Pekin ducks (*Anas domesticus platyrhynkos*) maintained by the Fox Chase Cancer Center. Serum and liver tissue from groups of three ducks sacrificed at 14 and 26 days of embryogenesis and at 1 and 14 days, 6 weeks, 3, 6, and 9 months, and 2 years posthatch were quantitatively monitored for markers of DHBV infection.

In the transient infection experiment, a group of seven 5-month-old Pekin ducks were obtained from the eggs produced by a flock of DHBV-negative ducks also maintained by the Fox Chase Cancer Center. Each duck first was bled by venipuncture and then underwent open-wedge biopsy of the liver (4), using the injectable anesthetic Telazol (A. H. Robins), to provide preinoculation serum and liver samples. Several weeks following surgery, each of the seven ducks was inoculated intravenously with 20 ml of a pool of DHBV-positive serum obtained from 3-week-old congenitally DHBV-infected Pekin ducks. Following inoculation, ducks were bled at weekly intervals to obtain serum samples for analysis of DHBV DNA and anti-DHBV antibodies, and liver biopsy samples were obtained at 6, 40, 77, 154, and 224 days postinoculation to provide liver tissue for analysis of DHBV DNA and antigens and for liver histology.

Analysis of serum samples for DHBV. To detect viremia, serum samples were assayed for the presence of viral DNA. Twenty-microliter serum samples from each congenitally DHBV-infected duck were digested with 4 mg of Pronase per ml, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 0.01 M Tris-HCl (pH 7.5), and 0.01 M EDTA in a total volume of 400 μl at 37°C for 2 h. The mixture was then extracted twice with equal volumes of a mixture of phenol and chloroform (1:1), the nucleic acids were precipitated with ethanol, and an aliquot extracted from the equivalent of 2.5 μl of serum was then subjected to electrophoresis in a slab gel of 1.5% agarose containing 0.04 M Tris-HCl (pH 7.2), 0.02 M sodium acetate, and 0.001 M EDTA. DHBV DNA was detected by Southern blot hybridization (22) using a ^{32}P-labelled DNA probe containing the entire DHBV genome. The amounts of radioactive probe binding to specific regions of DNA blots were determined by comparison with an internal DHBV standard derived from cloned genomic DNA included in each gel. In the same manner, nucleic acids extracted from the equivalent of 12.5 μl of serum taken from each of the seven adolescent ducks at various times after DHBV inoculation were analyzed for viral DNA by blot hybridization. In addition, 5-μl serum samples taken at weekly intervals from each of the adolescent ducks from days 0 to 63 postinoculation were spotted in duplicate onto nitrocellulose and hybridized to detect DHBV DNA, as previously described (8).

Detection of antibodies to DHBV. The presence of serum antibodies capable of neutralizing DHBV was determined by preincubation of 100 μl of heat-inactivated (56°C, 60 min) duck serum with a 0.01-ml aliquot of DHBV-positive duck serum at 37°C for 1 h and then overnight at 4°C. Surviving DHBV was then measured by infection of primary duck hepatocyte cultures, as described by Pugh and Summers (12a), with addition to the culture medium from 1 day postinfection of Suramin (100 μg/ml) to prevent secondary rounds of infection (23). Eight days following inoculation, the monolayers were rinsed with phosphate-buffered saline (PBS), and DHBV replication was detected by extraction and analysis of virus-specific nucleic acids as described by Wu et al. (23).

DHBV antigen staining and detection of DHBV DNA by in situ hybridization. Following euthanasia, two 3- to 5-mm pieces of liver tissue were removed from each congenitally DHBV-infected duck and duck embryo, fixed in 10% formalin or in ethanol-acetic acid (3:1) at room temperature for 30 min and then overnight in 70% ethanol at 4°C, processed into paraffin wax, and sectioned at 6 μm onto gelatin-coated slides (13). In the same manner, yolk sac tissues dissected from 14- and 26-day-old duck embryos were washed in sterile PBS to remove yolk, fixed in ethanol-acetic acid (3:1), paraffin wax embedded, and sectioned as described above.

Samples (200 to 300 mg) of liver biopsy tissue taken under direct vision from each adolescent duck before inoculation and on days 6, 40, 77, and 154 and from two ducks on day 224 postinoculation were each divided in two. One half was snap-frozen in liquid nitrogen and stored at −80°C for extraction of non-protein-bound nucleic acid and total DNA and for in situ hybridization as described below. The other half was further divided into two pieces. One piece was fixed in ethanol-acetic acid (3:1) and the other piece was fixed in

10% formalin before being processed into paraffin wax and sectioned as described above. DHBV surface antigen (DHBsAg) and DHBV core antigen (DHBcAg) were detected in ethanol-acetic acid-fixed tissues by standard immunoperoxidase techniques (8), using rabbit anti-DHBsAg (a generous gift of John Newbold) and rabbit anti-recombinant DHBcAg (rDHBcAg), raised by immunizing rabbits with rDHBcAg. The rDHBcAg was extracted from *Escherichia coli* cultures transformed with plasmid pKKcore (a generous gift of J. Summers and R. Lenhoff), which contained the DHBV core protein gene (nucleotides 2647 to 677 [10]) cloned into plasmid pKK233-2 (Clonetech Laboratories, Palo Alto, Calif.), under the control of the isopropylthiogalactopyranoside (IPTG)-inducible *trc* promoter. The DHBV DNA insert in pKKcore had been prepared by purification of the *Xba*I-*Ssp*I fragment of DHBV DNA coupled to a synthetic DNA linker (nucleotides 2647 to 2662) into *Nco*I-*Hin*dIII-digested pKK233-2. rDHBcAg expression was induced by treatment with 2 mM IPTG for 16 h at 37°C. Following lysis of bacteria by sonication in 0.025 M glucose–0.05 M Tris-HCl (pH 8)–0.001 M EDTA–1 mg of lysozyme per ml, proteins were precipitated with 0.5 M NaCl–5% polyethylene glycol 8000 at 4°C for 30 min, pelleted at 3,000 × *g*, and redissolved in 0.01 M Tris-HCl (pH 8)–0.001 M EDTA. rDHBcAg particles were then subjected to rate-zonal centrifugation in a linear, 15 to 30% sucrose gradient at 25,000 rpm in an SW27 rotor at 4°C for 5 h; core protein-containing fractions were identified by Western immunoblotting, pooled, banded to equilibrium in cesium chloride (1.36 g/ml), diluted, pelleted, and used to hyperimmunize rabbits. The final yield of purified rDHBcAg was 5.4 mg/liter of the original *E. coli* culture.

DHBV DNA was detected by in situ hybridization in both RNase A-digested frozen and ethanol-acetic acid-fixed liver tissue sections, using ^{125}I-labelled DHBV DNA probes, as previously described (8). Plasmid pBR322 DNA labelled to the same specific activity as DHBV DNA was used to confirm the specificity of hybridization. Histologic analysis of the liver biopsy tissues included blind assessment by each of two pathologists (J.M.E. and P.M.H.) by analysis of formalin-fixed liver biopsy tissues stained with hematoxylin and eosin, or with Congo red for amyloid, and by trichrome for collagen detection. Quantitation of DHBcAg and DHBV nucleic acid-positive hepatocytes was performed by light microscopy on sections of ethanol-acetic acid-fixed liver tissue. In each case, an average of 25 fields was examined with a 40× objective, and the number of positive hepatocytes was expressed as a percentage of the total hepatocytes examined. The same method of quantitation was used to determine the percentage of necrotic hepatocytes in formalin-fixed liver tissues.

Quantitation of CCC DNA and total DHBV DNA in liver and yolk sac tissue. Liver tissue was removed from each congenitally DHBV-infected duck, and duplicate 300-mg samples, made up of pools of tissue from four different sites in both lobes, were finely minced and then homogenized in 3 ml of 0.01 M Tris-HCl (pH 7.5)–0.01 M EDTA, using a ground-glass homogenizer. Nuclei were then stained with ethidium bromide (1 μg/ml) and counted on a hemocytometer under illumination at 580 nm. Each homogenate was divided into two 1.5-ml samples. One sample was diluted to 7.5 ml in 0.01 M Tris-HCl (pH 7.5)–0.01 M EDTA and used to purify non-protein-bound nucleic acid containing the viral CCC DNA, which, unlike the bulk of intracellular viral DNA, is not covalently attached to protein. Non-protein-bound nucleic acid was purified essentially as described by Wu et al. (23) except that the CCC DNA-containing supernatant was extracted two times with an equal volume of phenol buffered with 0.05 M Tris-HCl (pH 8) and then extracted with an equal volume of phenol and chloroform (1:1) buffered with 0.5 M Tris-HCl (pH 8) before ethanol precipitation at room temperature. The non-protein-bound nucleic acids extracted from each 150-mg sample of liver were redissolved in 400 μl of 0.01 M Tris-HCl (pH 7.5)–0.002 M EDTA, and 20-μl samples of each preparation, containing non-protein-bound nucleic acid extracted from on average 5.2×10^6 cells, were analyzed for DHBV DNA by blot hybridization. The second 150-mg liver sample, used to purify total DNA, was digested for at least 2 h in pronase-SDS as described above and then subjected to one phenol and one phenol-chloroform extraction, precipitation with 2 volumes of ethanol in the presence of 0.3 M sodium acetate (pH 5.2), and finally digestion with 100 μg of RNase A per ml in 800 μl of 0.01 M Tris-HCl (pH 7.5)–0.002 M EDTA. Samples of each preparation, containing total cellular DNA extracted on average from 1.3×10^6 cells, were then analyzed by blot hybridization. In addition to direct nuclei counts by ethidium bromide staining as described above, quantitation of total cellular DNA was performed by fluorimetric measurements (9).

Duplicate 100-mg samples of yolk sac tissue were removed from each 14- and 26-day old embryo, washed in sterile PBS, extracted for non-protein-bound nucleic acid and total DNA as described above, and redissolved in 400 μl of 0.01 M Tris-HCl (pH 7.5)–0.002 M EDTA, and 20-μl samples of each preparation were analyzed by blot hybridization for DHBV DNA.

In the same manner as described above, 100- to 150-mg samples of snap-frozen liver biopsy tissue were homogenized in 2 ml of 0.01 M Tris-HCl (pH 7.5)–0.01 M EDTA. Each homogenate was then divided into two 1-ml aliquots; one sample was used to purify the non-protein-bound nucleic acid, and the second sample was used to purify total DNA. The non-protein-bound nucleic acid extracted from 1.2×10^7 liver cells and total cellular DNA extracted from 2×10^6 liver cells were analyzed for DHBV DNA by blot hybridization as described above.

Viral DNA quantitation. Amounts of DNA on Southern blots was estimated following hybridization with a ^{32}P-labelled probe representing the entire DHBV genome, using an AMBIS scanning image analyzer. In the case of DNA replicative intermediates, which are present as a heterogeneous-sized population, the entire population of viral species was scanned, and the copy number is given as double-stranded genome equivalents. This estimate is therefore a measure of the total mass of viral DNA per cell, not the number of molecules of viral DNA. In contrast, because most CCC DNA is present as a single electrophoretic species, a copy number estimate was feasible.

RESULTS

Chronic infection following congenital transmission of DHBV. To understand events during transient DHBV infections or even to use the infected duck as a model for evaluating antiviral therapies, it is important to know how parameters of viral replication vary as a function of age. This can best be done in congenitally infected ducks, which do not immunologically respond, as far as is known, to viral infection. The parameters we particularly wanted to assess were age-dependent variations in viremia, percentage of DHBV-infected hepatocytes, and average levels of DNA

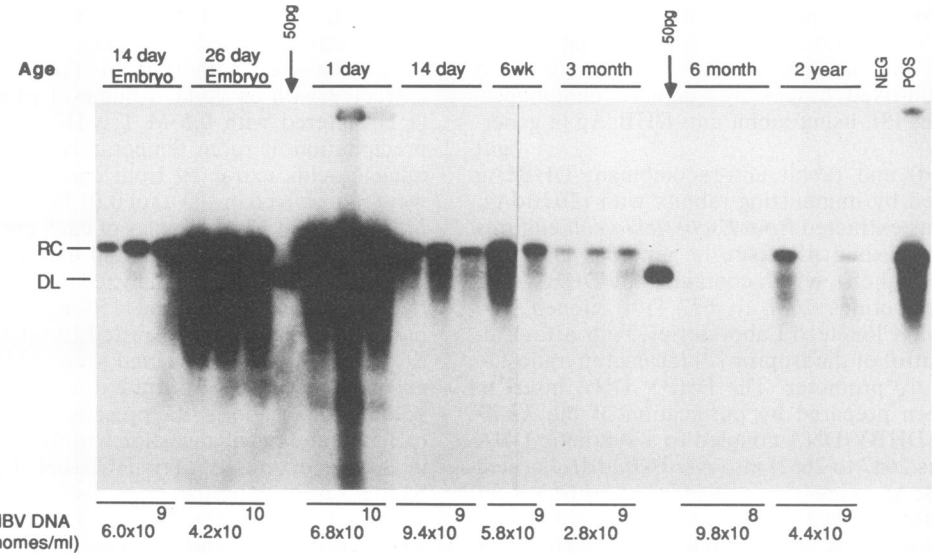

FIG. 1. Viremia in congenitally DHBV-infected ducks. Nucleic acids extracted from the equivalent of 2.5 μl of serum from groups of three congenitally DHBV-infected ducks, from 14 days of embryogenesis to 2 years posthatch, were analyzed by Southern blot hybridization. Hybridization was with a DHBV-specific genomic probe. DHBV DNA genomic equivalents per milliliter of serum were estimated by comparison with the amount of radioactive probe binding to a 50-pg DHBV DNA standard, using an AMBIS scanning image analyzer. NEG, negative control; POS, positive control; RC, relaxed circular 3-kbp DHBV DNA; DL, double-stranded, linear 3-kbp DHBV DNA. Autoradiographic exposure was for 18.5 h. Two separate blots are shown.

replication intermediates in the cytoplasm and CCC DNA in the nuclei of the hepatocytes that were infected.

The first parameter that we investigated was age-related variations in viremia, as assessed by measuring viral DNA in circulating virions. As shown in Fig. 1, serum DHBV levels were unusually high at about the time of hatching, as previously reported by Tagawa et al. (18), with a peak of 6.8×10^{10} virions per ml at 1 day of age. However, the level quickly dropped about 10-fold and, with the exception of birds just reaching maturity (6 months old), was in the range of 0.2×10^{10} to 1.0×10^{10} virions per ml for ducks up to 2 years posthatch. A slightly lower viremia (9.8×10^{8}) was seen in the two 6-month-old ducks that were examined; in fact, intrahepatic levels of total DHBV DNA were also lower in these two ducks.

We found, in agreement with the earlier work of Tagawa et al. (18), that the elevated viremia at hatching could reflect a contribution from the yolk sac. Yolk sac tissues from 14- and 26-day-old embryos were analyzed for CCC and total DHBV DNA and found to contain high levels of all the replicative intermediates of DHBV (Fig. 2). Direct quantitation of DHBV DNA levels per cell was not possible because of the extreme fragility of the yolk sac cell nuclei, which prevented accurate nucleus counts. When cell numbers were calculated from total DNA recovery (2.5 pg per diploid nucleus [21]), CCC DHBV DNA per cell was ca. 5 at 14 days and 70 at 26 days of embryogenesis, while total DHBV DNA copies per cell were estimated at ca. 180 and 4,050, respectively. In situ hybridization, used to detect replicative levels of DHBV DNA in the cytoplasm, and immunoperoxidase staining of cytoplasmic DHBcAg also suggested that a majority of yolk sac cells contained high levels of replicating DHBV at both 14 and 26 days of embryogenesis (data not shown).

The fact that viremia stayed at a relatively constant level after 14 days posthatch suggested that the number of infected hepatocytes also remained constant. This, in fact, turned out to be the case. High levels of both cytoplasmic DHBV nucleic acids, detected by in situ hybridization, and cytoplasmic DHBcAg, detected by immunoperoxidase staining of liver tissue (Fig. 3), were used as markers of DHBV replication, and both suggested that virtually 100% of hepatocytes in birds of all ages, from 14 days of embryogenesis to

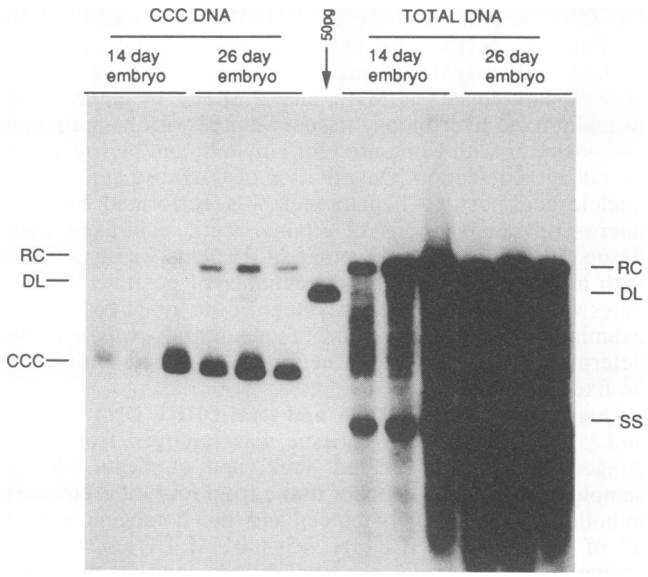

FIG. 2. Evidence that DHBV replication in yolk sac tissues from congenitally infected embryos may contribute to the high viremia at hatching. Yolk sac tissues from groups of three 14- and 26-day-old embryos were extracted for non-protein-bound and total nucleic acids, and the nucleic acids extracted from the equivalents of 5- and 2.5-mg samples, respectively, of yolk sac tissue were analyzed by Southern blot hybridization. SS, single-stranded DNA. RC and DL are defined in the legend to Fig. 1. Autoradiographic exposure was for 19 h.

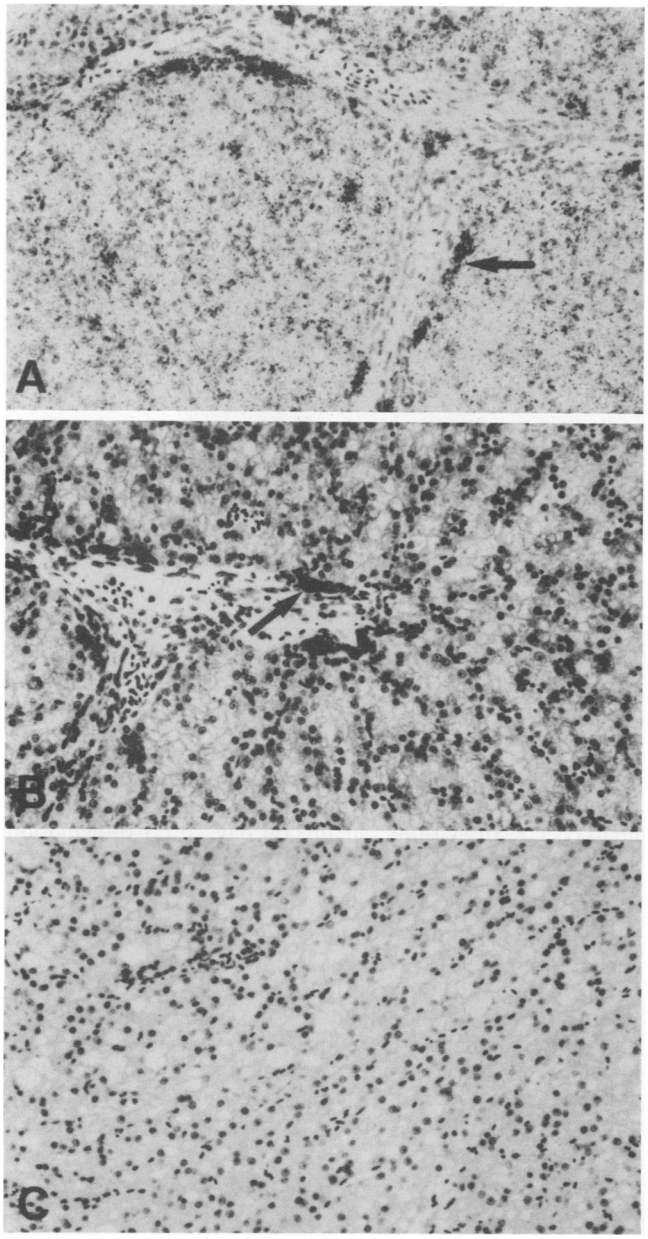

FIG. 3. Evidence that virtually 100% of hepatocytes in congenitally infected ducks are infected by DHBV. Shown is ethanol-acetic acid-fixed liver tissue from a 3-month-old congenitally infected duck. (A) Detection of cytoplasmic DHBV nucleic acids in hepatocytes and bile ductular cells (arrow) by in situ hybridization using a ^{125}I-labelled DHBV DNA probe (autoradiographic exposure, 165 h; counterstained with hematoxylin and eosin). (B and C) Detection of cytoplasmic DHBcAg in hepatocytes and bile ductular cells (arrow) by immunoperoxidase staining with rabbit-anti-rDHBcAg (B) and control staining with preimmune rabbit serum (C) (counterstained by hematoxylin). Magnification, ×165.

2 years posthatch, supported DHBV replication. This result does not reveal whether all hepatocytes of an uninfected duck would remain susceptible, with age, to de novo infection, but it does suggest that all cells, once infected, might produce DHBV.

Hepatocytes are the major infected cell population in the liver, bile duct epithelium representing only a few percent of the hepatocyte population. Knowledge of the percentage of infected hepatocytes facilitates, therefore, an estimate of the average amount of total, and especially of CCC, DHBV DNA per infected cell. Southern blot analysis of both non-protein-bound nucleic acid and total cellular DNA extracted from liver tissue was used to quantitate the copy number of both CCC and total DHBV DNA in virus genomic equivalents per cell at all time points. Two different methods were used to determine the number of cells in the liver samples. The first was to count nuclei in liver homogenates (assuming the cells to be mononuclear) following ethidium bromide staining. The second was to determine the amount of DNA recovered from the samples by fluorimetric measurements and to then estimate the number of cells in the original tissue. The estimates of cell numbers by the two methods were in close agreement, and the viral DNA copy number calculations were found to be in agreement within a factor of 1.5 (Table 1). As can be seen in Fig. 4, the CCC DHBV DNA copy number ranged from a peak of around 15 at the time of hatching to 2.9 at 3 weeks of age. Since erythrocytes, which have nuclei in birds, were estimated to account for ca. 20% of the nucleated cells in our tissue sections, and since hepatocytes represented ca. 60% of the nucleated cells, the actual copy numbers of viral DNA per infected hepatocyte may be up to twofold higher than those shown here. Therefore, the average range per hepatocyte is probably from 6 to 30 copies. Although CCC DNA copy numbers decreased to a low at 6 weeks posthatch, they remained relatively constant throughout the study period, with around 10 copies per cell (20 per infected hepatocyte) in both 6-month-old and 2-year-old birds. Similarly, total DHBV DNA levels per cell were maximal at the time of hatching (816 viral genome equivalents per cell [1,632 per infected hepatocyte] in 1-day-old birds) and remained at lower but relatively constant levels thereafter, with 178 (356 per infected hepatocyte) in 2-year-old birds.

As can be seen in Fig. 4, non-protein-bound nucleic acid preparations also included DHBV DNA forms that migrated with the same mobility as did relaxed circular and double-stranded linear DHBV DNA seen in total cellular DNA preparations. It is notable that they were most prominent in the preparations of non-protein-bound DNA from very young birds. Preliminary analysis (22a) suggested that the non-protein-bound relaxed circular DNA is not randomly

TABLE 1. Maintenance of virus production as a function of age in congenitally DHBV-infected ducks and duck embryos

Age	DHBV DNA		
	Viremia (genomes/ml)	In liver (genomes/cell)	
		CCC	Total
14-day embryo	6.0×10^9	5.7 (11.2)[a]	335 (662)
26-day embryo	4.2×10^{10}	15.8 (23.8)	468 (699)
1 day	6.8×10^{10}	14.9 (16.5)	816 (904)
14 day	9.4×10^9	7.1 (10.3)	281 (410)
6 wk	5.8×10^9	2.9 (2.9)	204 (204)
3 mo	2.8×10^9	4.3 (5.4)	176 (219)
6 mo	9.8×10^8	9.4 (15.9)	82 (147)
9 mo	7.4×10^9	7.8 (2.7)	228 (123)
2 yr	4.4×10^9	10.6 (18.2)	178 (306)

[a] DHBV DNA copy number per cell calculated by liver homogenate nucleus counts (and, in parentheses, by fluorimetric measurement of total cellular DNA).

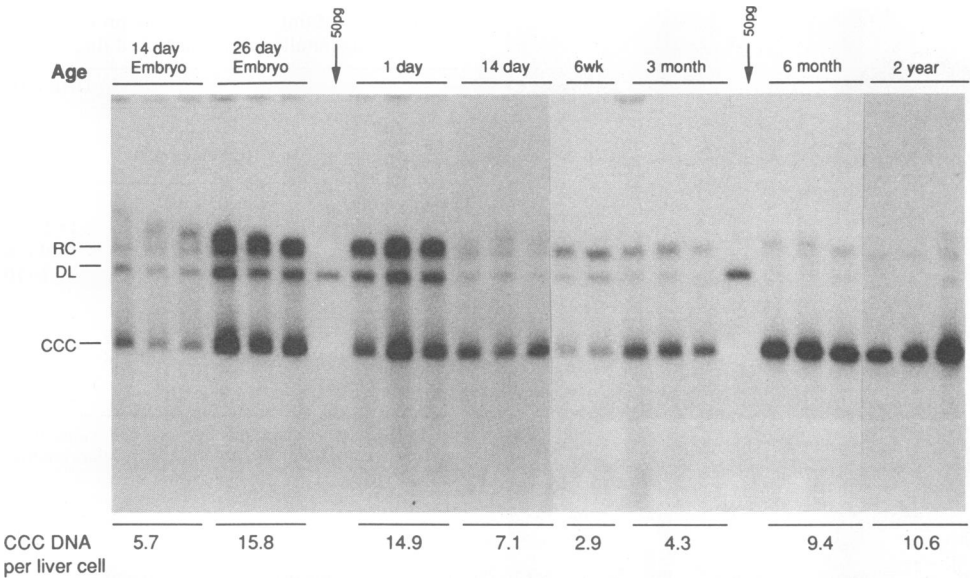

FIG. 4. Evidence that CCC DNA levels in liver tissue from congenitally infected ducks remained at relatively constant levels throughout the 2-year study period. Duplicate 150-mg samples of liver tissue were extracted for non-protein-bound nucleic acid, and nucleic acid extracted from on average 5.2×10^6 cells was analyzed by Southern blot hybridization. Cloned DHBV DNA (50 pg) served as an internal marker to allow quantitation of CCC DNA. Autoradiographic exposure was for 2 h. Two separate blots are shown.

nicked but instead is similar or identical to relaxed circular virion DNA and is therefore unlikely to have been derived just from nicking of CCC DNA during the purification procedure.

A transient infection was obtained when DHBV was inoculated into adolescent ducks. Following inoculation of seven adolescent ducks (5 months old) with a large dose of DHBV-positive serum, weekly serum samples were collected and assayed for the presence of viremia by spot blot hybridization to detect DHBV DNA. Spot blot analysis of serum samples up to 63 days postinoculation showed that following inoculation, adolescent ducks had very low or undectable viremia (data not shown). This result was confirmed by Southern blot hybridization of serum DHBV DNA. As can be seen in Fig. 5, five of the seven ducks had detectable levels of DHBV DNA in the serum on day 4 postinoculation, but DHBV DNA levels had dropped by 8 days postinoculation, and only duck 7 had detectable DHBV DNA (approximately 5×10^7 virions per ml) on day 40 postinoculation. The Southern blot assay for DHBV DNA was estimated to be able to detect $\geq 1 \times 10^7$ virions per ml. It is not known whether circulating virus present on day 4 postinoculation represented residual inoculum or newly formed virus released following virus replication.

Further analysis showed that the absence of a prolonged viremia could be explained by the rapid appearance of virus-specific immunoglobulins. Weekly serum samples collected from selected ducks were assayed in vitro for their ability to block DHBV infection of primary hepatocyte cultures (Table 2). As can be seen, serum samples collected

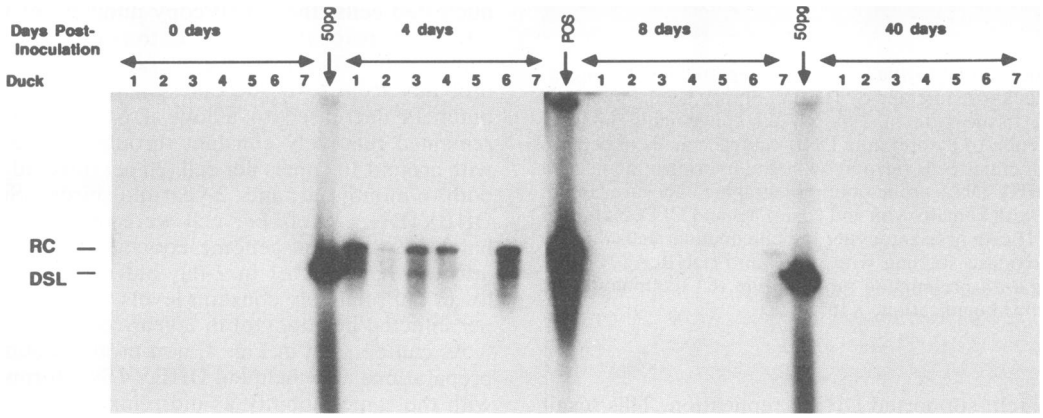

FIG. 5. Evidence that inoculation of adolescent ducks does not result in a prolonged viremia. Nucleic acids extracted from the equivalent of 12.5 μl of serum from each of a group of seven adolescent ducks, both before inoculation and on days 4, 8, and 40 postinoculation, were analyzed by Southern blot hybridization. Nucleic acid extracted from 1.25 μl of serum from a 14-day-old congenitally infected duck served as positive control (POS). DHBV DNA was detected only in the sera of duck 7 (*) on day 40 postinoculation. Autoradiographic exposure was for 16 h. RC, relaxed circular DNA; DSL, double-stranded linear DNA. Two separate blots are shown.

TABLE 2. Neutralization of DHBV infection of primary hepatocyte cultures by preincubation of positive control sera with heat-inactivated sera from adolescent ducks

Duck	Blocking of DHBV replication in primary hepatocyte cultures[a] at day postinoculation:																
	0	8	15	22	29	37	47	50	55	62	78	115	160	205	259	275	295
2	4+	2+	+	+	+	+	2+	2+	2+	4+	4+	4+	4+[b]	NA[c]	NA	NA	NA
3	4+	3+	2+	+	+	+	+	+	+	+	NA	3+	4+	NA	NA	NA	NA
4	4+	3+	2+	+	+	+	NA	+	+	+	+	2+	NA	4+	4+	4+	4+
5	4+	4+	3+	3+	3+	2+	3+	NA	3+	2+	+	+	NA	4+	NA	NA	4+
7	4+	4+	4+	4+	4+	NA	4+	4+	4+	4+	4+	4+	NA	+	+	+	NA

[a] Neutralizing activity was assessed by assaying for surviving DHBV following incubation of the virus with the indicated duck serum samples as described in Materials and Methods. The quantitation of viral infectivity was carried out by assaying for replicative forms of viral DNA in hepatocyte cultures compared with the infectivity of virus incubated with preinoculation sera (≡100%). Relative amounts of viral DNA on Southern blots were estimated by densitometry or by visual comparison of autoradiograms. 4+, 50 to 100% of control; 3+, 25 to 50%; 2+, 10 to 25%; +, <10%.

[b] Autopsy sample.

[c] NA, not analyzed.

from duck 7 up to 115 days postinoculation failed to significantly reduce DHBV infectivity, suggesting the absence of excess neutralizing antibodies in the serum from that duck, which was the only one with a prolonged viremia. Surprisingly, by 205 days postinoculation, even duck 7 developed a high-level neutralizing activity. Serum samples from the other ducks (ducks 2 to 5) that were tested markedly reduced DHBV infectivity from as early as 8 days postinoculation. A humoral immune response to the large amount of virus in the inoculum may explain the rapid appearance of neutralizing activity (by 8 days postinoculation). The identification of this early activity as antibody to viral antigens was supported by the observation that sera taken at 8 days postinfection but not preinoculation sera stained DHBV-infected pancreatic cells, as detected by immunofluorescence microscopy (5). This neutralizing activity declined or was no longer detectable following clearance of DHBV from the liver. Ducks 1 and 6 were not tested for the production of neutralizing activity.

To determine whether the failure of ducks 1 to 6 to develop a chronic infection was due to resistance of hepatocytes to DHBV, biopsies taken at various times postinoculation were first examined for signs of virus infection. De novo replication of DHBV DNA in these hepatocytes was readily demonstrated on day 6 postinoculation by in situ hybridization of frozen sections, which revealed the presence of many copies of largely single-stranded, cytoplasmic DHBV DNA in RNase A-digested, nondenatured tissue (data not shown). In situ hybridization and immunoperoxidase staining of DHBcAg in ethanol-acetic acid-fixed tissues showed that the percentage of DHBV-positive hepatocytes ranged in different birds from 27 to 80% (Table 3) and that infected hepatocytes were randomly distributed throughout the liver acini (Fig. 6C and D). Southern blot analysis of CCC and total DHBV DNA extracted from liver biopsy tissues at all time points is shown in Fig. 7. As can be seen, liver samples contained multiple copies of all of the expected species of DHBV DNA replicative intermediates, including DHBV CCC DNA. Following quantitation, average copy numbers of CCC and total DHBV DNA per cell were determined (Table 3), and when these values were corrected to represent the levels of total and CCC DNA copy number per infected hepatocyte (as shown in parentheses in Table 3), hepatocytes in transiently infected ducks were shown to contain levels of CCC and total DHBV DNA similar to those seen in chronically DHBV-infected liver tissue (Table 1). Taken together, the results indicated that the failure of ducks 1 to 6 to become viremic was not due to

TABLE 3. Transient DHBV infection of adolescent ducks

Duck	Days postinoculation	% Positive hepatocytes[a]	CCC DNA (genomes/cell)	Total DNA (genomes/cell)	Histology, acute hepatitis[b]
1	0	0	0	0	−
	6	40	4.1 (10.2)[c]	299 (573)	++
	40	0	0	0	+
	77	0	0	0	−
	160	0	0	0	−
2	0	0	0	0	−
	6	27	2.7 (9.8)	83.5 (309)	+
	40	0	0	0	−
	77	0	0	0	−
	160	0	0	0	−
3	0	0	0	0	−
	6	62	1.4 (2.3)	200 (322)	+
	40	0	0	0	−
	77	0	0	0	−
	160	0	0	0	−
4	0	0	0	0	−
	6	80	2.3 (2.8)	327 (408)	+
	40	9	0.3 (3.5)	16 (180)	++
	77	0	0	0	−
	154	0	0	0	−
	224	0	0	0	−
5	0	0	0	0	−
	6	51	2.3 (4.5)	188 (369)	+
	40	68	3.7 (5.5)	210 (310)	−
	77	0	0	0	−
	154	0	0	0	−
6	0	0	0	0	−
	6	68	5.5 (8.0)	269 (384)	++
	40	0	0	0	−
	77	0	0	0	−
	154	0	0	0	−
7	0	0	0	0	−
	6	54	1.8 (3.4)	206 (381)	+
	40	95–100	6.6	603	+
	77	95–100	4.9	910	+
	154	95–100	4.3	186	+
	224	10–95	3–2	298	+

[a] Derived from counts of DHBcAg- and DHBV nucleic acid-positive hepatocytes.

[b] Histologic features of acute hepatitis are described in the text.

[c] Numbers in parentheses have been corrected for percentage of DHBV-positive hepatocytes, assuming that the liver was 100% hepatocytes. Since erythorocytes and other nonparenchymal cells account for 40% of the hepatitic cell population, the actual DHBV DNA copy number per infected hepatocyte should be about twofold higher than shown.

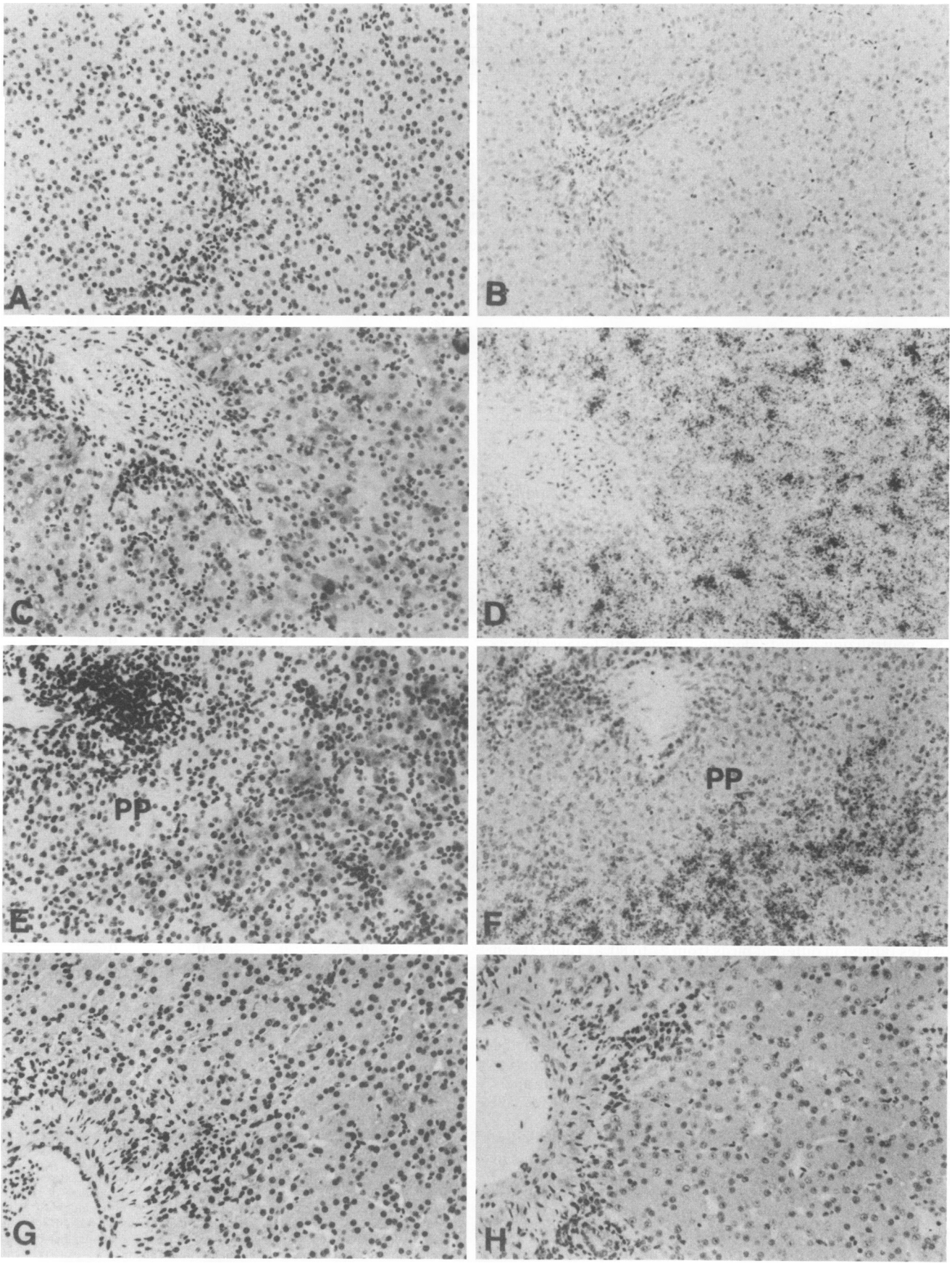
A
B
C
D
PP
E
PP
F
G
H

FIG. 6. Evidence that inoculation of adolescent ducks resulted in transient infection with initially widespread DHBV infection of hepatocytes. Shown is ethanol-acetic acid-fixed liver biopsy tissue sampled from adolescent duck 4, preinoculation (A and B) and on day 6 (C and D), day 40 (E and F), and day 77 (G and H) postinoculation. (A, C, E, and G) Detection of cytoplasmic DHBcAg by using rabbit-anti-rDHBcAg (counterstained with hematoxylin); (B, D, F, and H) detection of cytoplasmic DHBV nucleic acids by in situ hybridization using ^{125}I-labelled DHBV DNA (autoradiographic exposure, 156 h; counterstained with hematoxylin and eosin). Note that clearance of DHBV appeared to occur first in periportal (PP) regions. Magnification, ×165.

a widespread resistance of the hepatocytes in adolescent ducks to DHBV infection.

Analysis of liver biopsy tissues taken at later time points showed that, though initially massively infected, each of the adolescent ducks (with the possible exception of duck 7) showed only transient DHBV expression in the liver, with liver tissue from ducks 1, 2, 3, and 6 completely cleared of detectable DHBV DNA, DHBsAg, and DHBcAg by day 40 postinoculation. Similarly, ducks 4 and 5 appeared to have completely cleared DHBV infection by day 77 postinoculation, while duck 7, although demonstrating continued DHBV infection in the liver on day 224 postinoculation, had a markedly reduced percentage of DHBV-positive hepatocytes and reduced levels of CCC and total DHBV DNA (Table 3) in the presence of neutralizing antibodies and appeared to be in the process of clearing its infection. Continued analysis of the DHBV infection in this duck was not possible, as this duck subsequently died of unknown causes.

A major problem that remains is to understand how such massive infections are so rapidly cleared. As a first step, a histologic evaluation of viral expression was carried out to look for possible explanations. In contrast to the random distribution of DHBV-infected hepatocytes in liver biopsy tissue from all ducks on day 6 postinoculation, tissues from ducks 4 and 5 on day 40 postinoculation and from duck 7 on day 224 postinoculation had evidence of partial clearance of DHBV replication. For example, liver tissue from duck 4,

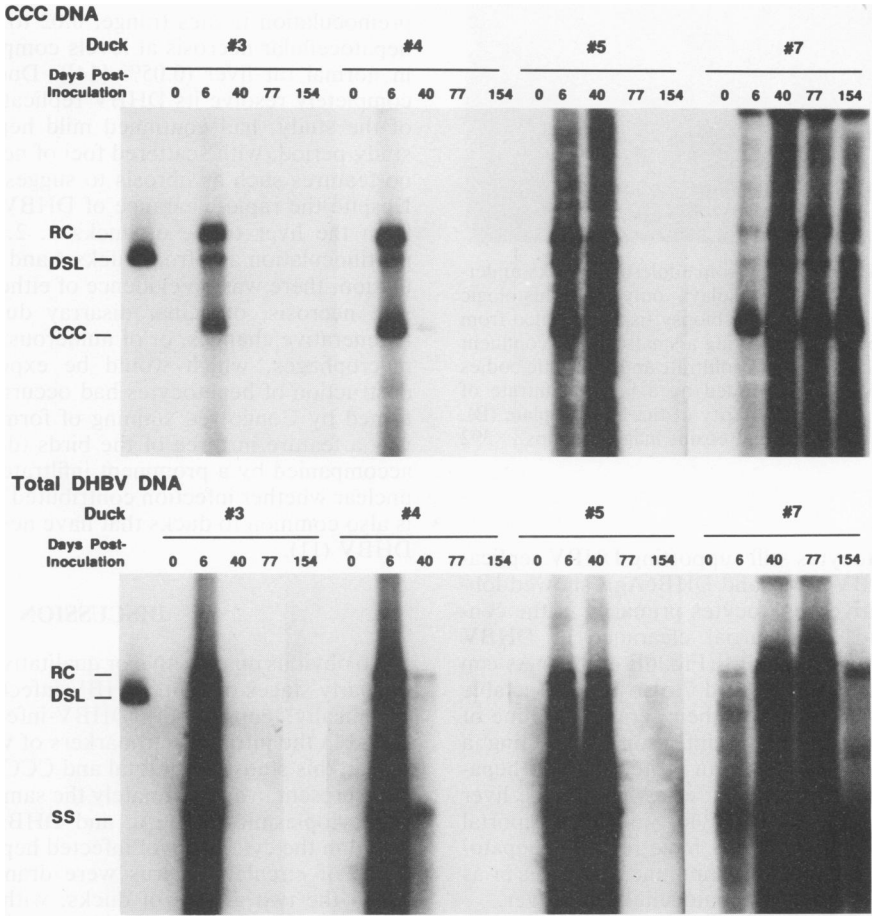

FIG. 7. Evidence that DHBV replicative intermediates seen in transient infection of liver tissue are the same as those seen in congenital infection. Snap-frozen liver biopsy tissues taken from ducks 3, 4, 5, and 7 on days 0, 6, 40, 77, and 154 days postinoculation were extracted for non-protein-bound and total nucleic acid. The non-protein-bound nucleic acid extracted from 1.2×10^7 cells from each duck and total nucleic acid from 2×10^6 cells from ducks 3, 4, and 5 and from 0.5×10^6 cells from duck 7 were analyzed by Southern blot hybridization. A 50-pg DHBV DNA marker was included in each gel (e.g., lane 1). Autoradiographic exposure was for 13 h. RC, relaxed circular DNA; DSL, double-stranded linear DNA.

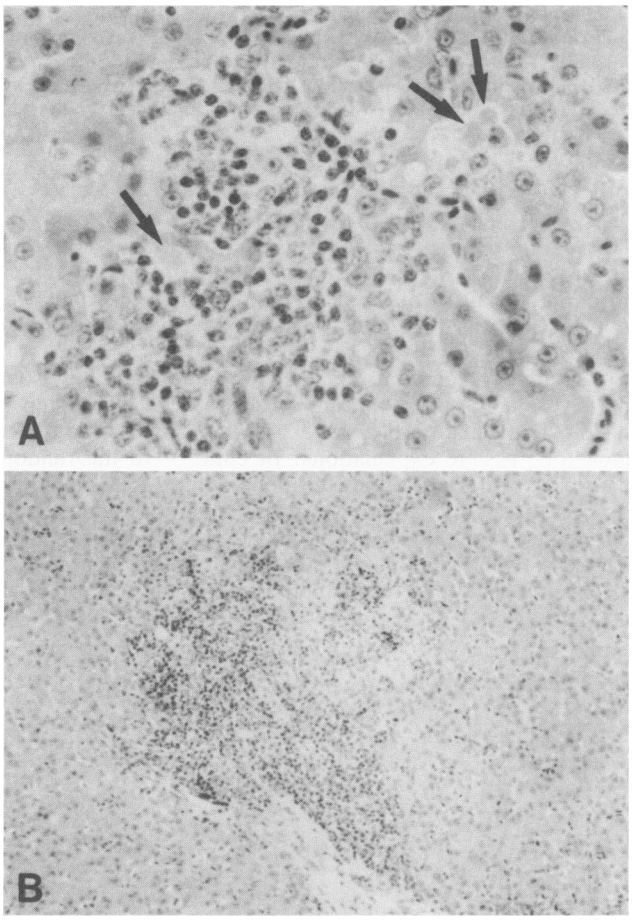

FIG. 8. Evidence that liver tissue from adolescent ducks undergoing transient DHBV infection displays only mild histologic changes. Shown is formalin-fixed liver biopsy tissue sampled from duck 6 on day 6 postinoculation showing a small area of confluent necrosis, with associated lymphocytic infiltrate and apoptotic bodies (arrows) (A), and portal tract expanded by a heavy infiltrate of mononuclear cells, with some irregularity of the limiting plate (B). Counterstained with hematoxylin and eosin; magnifications, ×392 (A) and ×117 (B).

with only 9% of hepatocytes still supporting DHBV replication (cytoplasmic DHBV DNA and DHBcAg), showed lobules with DHBV-positive hepatocytes primarily in the centrilobular regions and with partial clearance of DHBV replication in the periportal regions (Fig. 6E and F). As can be seen in Table 2, ducks 4, 5, and 7 also had detectable levels of neutralizing antibodies in their sera at the time of partial clearance of their DHBV infection, suggesting a possible role for humoral immunity in protecting new hepatocytes in the periportal regions and elsewhere in the liver from infection. Virus-free hepatocytes in the periportal region may represent new cells that have replaced hepatocytes that died as a result of cellular immune responses or as a result of a normal program of hepatocellular turnover.

As can also be seen in Table 3, by comparison with preinoculation liver biopsy samples, mild acute hepatitis was seen on day 6 postinoculation in all of the seven ducks. The features seen on day 6 included increased numbers of mononuclear cells in the portal tracts, some irregularity of the limiting plate due to the presence of mild piecemeal

TABLE 4. Necrotic hepatocytes during transient infection of adolescent ducks[a]

Duck	% Necrotic hepatocytes[b] at day postinoculation:			
	0	6	40	77
1	0.35	1.5	0.05	ND[c]
2	0.12	0.84	0.09	ND
3	0.16	0.64	0.19	ND
4	0.05	0.61	0.55	ND
5	0.07	0.43	0.34	ND
6	0.05	1.7	0.12	ND
7	0.02	0.34	0.17	0.33

[a] The necrotic hepatocytes included both cells with apoptotic features as well as heavily shrunken cells with fragmented nuclei.
[b] Differences between the percentage of necrotic hepatocytes between uninfected and DHBV-infected livers were statistically significant ($P < 0.005$).
[c] ND, not done.

necrosis in some livers, a few foci of liver cell necrosis scattered through the liver acini, occasional necrotic hepatocytes, and an accompanying focal infiltrate of mononuclear cells (Fig. 8). As can be seen in Table 4, the presence of DHBV infection on day 6 postinoculation coincided with an increase in the percentage of necrotic hepatocytes (range, 0.34 to 1.7%); the observation of necrotic hepatocytes in preinoculation tissues (range, 0.02 to 0.35%) demonstrated hepatocellular necrosis at levels comparable to those found in normal rat liver (0.05% [14]). Duck 7, which failed to completely resolve its DHBV replication during the course of the study, had continued mild hepatitis throughout the study period, with scattered foci of necrosis in the acini but no features such as fibrosis to suggest chronic liver injury. Despite the rapid clearance of DHBV-positive hepatocytes from the liver tissue of ducks 1, 2, 3, and 6 by day 40 postinoculation and from ducks 4 and 5 by day 77 postinoculation, there was no evidence of either massive or submassive necrosis, of acinar disarray due to degenerative or regenerative changes, or of numerous enlarged ceroid laden macrophages, which would be expected if a significant destruction of hepatocytes had occurred. Amyloidosis confirmed by Congo red staining of formalin-fixed liver tissue was a feature in three of the birds (ducks 3, 4, 7) and was accompanied by a prominent infiltrate of plasma cells. It is unclear whether infection contributed to amyloidosis, which is also common to ducks that have never been infected with DHBV (11).

DISCUSSION

No obvious quantitative or qualitative differences between the early stages of acute DHBV infection and those seen in chronically, congenitally DHBV-infected ducks were detected in the intracellular markers of viral replication examined in this study. Both total and CCC DHBV DNA species were present in approximately the same ratios to each other, and cytoplasmic DHBcAg and DHBsAg were readily detected in the cytoplasm of infected hepatocytes. In contrast, levels of circulating virus were dramatically different between the two groups of ducks, with low or undetectable DHBV DNA levels in the serum samples of transiently infected ducks from as early as 8 days postinoculation. Circulating virus in ducks 1 to 6 was apparently cleared by a humoral immune response, as reflected by the detection of neutralizing antibodies in the sera of all ducks tested. Even duck 7, which also failed to produce a detectable level of

neutralizing antibodies (until ca. 3 to 5 months postinoculation), had a consistently lower level of viremia early in infection compared with the levels detected in the sera of persistently infected ducks, despite the fact that the liver of duck 7 was completely infected. It is possible that antiviral immunoglobulins served to reduce the level of viremia in duck 7 even early in infection.

Variation in the number of infected hepatocytes in experimentally infected ducks at day 6 postinoculation (range, 27 to 80%) could represent variation in delivery of inoculum to liver, sample variability associated with examining just a small portion of the liver with each biopsy, or different stages in individual ducks between early rounds of DHBV production, release, and reinfection. Variations in the percentage of DHBV-positive hepatocytes among individual ducks have also been observed in early rounds of infection following intraperitoneal and intravenous inoculation of a large dose of virus to 1-day-old ducklings (7). The fact that CCC DNA and total DNA copy number estimates did not show any major variation between ducks (e.g., DNA copy numbers per viral antigen-positive hepatocyte were not lower in livers with higher estimates of the percentage of infected hepatocytes) suggests that the two measurements were internally consistent. Therefore, with the proviso that an entire liver could not be subjected to histologic analysis, the results indicate that DHBV infections can quickly resolve after extensive hepatocellular involvement, as previously reported following analysis of biopsy specimens for viral antigens in HBV-infected chimpanzees (2, 3, 6) and in a woodchuck hepatitis virus-infected woodchuck (12).

What factors might contribute to the resolution of these infections? At least four factors are conceivably involved: (i) immune-mediated destruction of infected hepatocytes, resulting, in this case, in mild acute hepatitis with increased necrotic hepatocytes postinfection; (ii) hypothetically, a spontaneous loss of the capacity of hepatocytes to support infection, perhaps mediated through the action on hepatocytes of components of the immune response; (iii) humoral immunity, to prevent spread to cells that were not initially infected and to newly regenerated normal hepatocytes formed in response to virally mediated liver damage; and (iv) physiological turnover of hepatocytes (1, 14, 15). The results, not unexpectedly, support the conclusion that in DHBV-infected ducks, rapid clearance depends, first, on the very rapid generation of neutralizing antibodies. Whether factor i, iii, or iv or all three then come into play is unknown, though the apparent absence of massive hepatic necrosis in ducks in which up to 80% of the liver was briefly infected lends credence to the idea that natural turnover of hepatocytes may be of major importance, perhaps accelerated in infected liver by cytokines produced by immune-effector cells. The present study does not provide enough information to allow us to conclude that any of the possible mechanisms listed above do not contribute to the clearance of an infection, and further studies are in progress to help define the mechanisms involved. Interestingly, prolonged (≥3-month) treatment of congenitally DHBV-infected ducks with an inhibitor of viral DNA synthesis also leads to a situation in which most of the hepatocytes (ca. 95%) are virus free (unpublished data), suggesting that the turnover of hepatocytes could be important in transient infections.

Whatever the mechanism of viral clearance, it should be emphasized that an understanding of how transient infections resolve is of practical importance. A better understanding of this process could lead to insights into how to modify antiviral protocols to completely terminate chronic productive infections, with their attendant liver damage and association with the eventual development of hepatocellular carcinoma.

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REFERENCES

1. **Arber, N., G. Zajicek, and I. Ariel.** 1988. The streaming liver II. The hepatocyte life history. Liver **8**:80–87.
2. **Barker, L. F., F. V. Chisari, P. P. Mc Grath, D. W. Dalgard, R. L. Kirschstein, J. D. Almeida, T. S. Edgington, D. G. Sharp, and M. R. Peterson.** 1973. Transmission of type B viral hepatitis to chimpanzees. J. Infect. Dis. **127**:648–652.
3. **Berquist, K. R., J. M. Peterson, B. L. Murphy, J. W. Ebert, J. E. Maynard, and R. H. Purcell.** 1975. Hepatitis B antigens in serum and liver of chimpanzees acutely infected with hepatitis B virus. Infect. Immun. **12**:602–605.
4. **Carp, N. Z., J. Saputelli, T. C. Halbherr, W. S. Mason, and A. R. Jilbert.** Lab. Anim. Sci., in press.
5. **Halpern, M. S., W. S. Mason, L. Coates, A. P. O'Connell, and J. M. England.** 1987. Humoral immune responsiveness in duck hepatitis B virus-infected ducks. J. Virol. **61**:916–920.
6. **Hoofnagle, J. H., T. Michalak, A. Nowoslawski, R. J. Gerety, and L. F. Barker.** 1978. Immunofluoresence microscopy in experimentally induced, type B hepatitis in the chimpanzee. Gastroenterology **74**:182–187.
7. **Jilbert, A. R., J. S. Freiman, C. J. Burrell, M. Holmes, E. J. Gowans, R. Rowland, P. Hall, and Y. E. Cossart.** 1988. Virus-liver cell interactions in duck hepatitis B virus infection: a study of virus dissemination within the liver. Gastroenterology **95**: 1372–1382.
8. **Jilbert, A. R., J. S. Freiman, E. J. Gowans, M. Holmes, Y. E. Cossart, and C. J. Burrel.** 1987. Duck hepatitis B vurus DNA in liver, spleen and pancreas: analysis by in situ hybridisation. Virology **158**:330–338.
9. **Labarca, C., and K. Paigen.** 1980. A simple, rapid, and sensitive DNA assay procedure. Ann. Biochem. **102**:344–352.
10. **Mandart, E., A. Kay, and F. Galibert.** 1984. Nucleotide sequence of a cloned duck hepatitis virus genome: comparison with the human and woodchuck hepatitis B virus sequences. J. Virol. **49**:782–792.
11. **Mason, W. S., J.-M. Lien, D. J. Petcu, L. Coates, W. T. London, A. P. O'Connell, C. Aldrich, and R. P. Custer.** 1987. *In vivo* and *in vitro* studies on duck hepatitis B virus replication, p. 3–16. *In* W. S. Robinson, K. Koike, and H. Will (ed.), Hepadna viruses. Alan R. Liss, Inc., New York.
12. **Ponzetto, A., P. J. Cote, E. C. Ford, R. H. Purcell, and J. L. Gerin.** 1984. Core antigen and antibody in woodchucks after infection with woodchuck hepatitis virus. J. Virol. **52**:70–76.

12a. **Pugh, J. C., and J. W. Summers.** 1989. Infection and uptake of duck hepatitis B virus by duck hepatocytes maintained in the presence of dimethyl sulfate. Virology **172**:564–572.

13. **Rogers, A. W.** 1979. Techniques in autoradiography, 3rd ed. Elsevier/North Holland, Amsterdam.
14. **Searle, J., B. V. Harmon, C. J. Bishop, and J. F. R. Kerr.** 1987. The significance of cell death by apoptosis in hepatobiliary disease. J. Gastroenterol. Hepatol. **2**:77–96.
15. **Sells, S.** 1990. Is there a liver stem cell? Cancer Res. **50**:3811–3815.

16. **Summers, J., P. M. Smith, and A. Horwich.** 1990. Hepadnavirus envelope proteins regulate covalently closed circular viral DNA amplification. J. Virol. **64**:3701–3709.
17. **Summers, J., P. M. Smith, M. Huang, and M. Yu.** 1991. Morphogenetic and regulatory effects of mutation in the envelope proteins of an avian hepadnavirus. J. Virol. **65**:1310–1317.
18. **Tagawa, M., W. S. Robinson, and P. L. Marion.** 1987. Duck hepatitis B virus replicates in the yolk sac of developing embryos. J. Virol. **61**:2273–2279.
19. **Tuttleman, J. S., C. Pourcel, and J. Summers.** 1986. Formation of a pool of covalently closed circular viral DNA in hepadnavirus infected cells. Cell **47**:451–460.
20. **Urban, M. K., A. P. O'Connell, and W. T. London.** 1985. Sequence of events in natural infection of Pekin duck embryos with duck hepatitis B virus. J. Virol. **55**:16–22.
21. **Vendrely, R.** 1958. La notion d'espece a travers quelques donnes biochimiques recentes et le cycle 1. Ann. Inst. Pasteur (Paris) **94**:142–166.
22. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzylmethyl-paper and rapid hybridisation by using dextran sulphate. Proc. Natl. Acad. Sci. USA **76**:3683–3687.

22a. **Wu, T.-T.** Unpublished data.

23. **Wu, T.-T., L. Coates, C. E. Aldrich, J. Summers, and W. S. Mason.** 1990. In hepatocytes infected with duck hepatitis B virus, the template for viral replication is amplified by an intracellular pathway. Virology **175**:255–261.