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Competition *in Vivo* between a Cytopathic Variant and a Wild-Type Duck Hepatitis B Virus

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Several examples of human hepatitis B virus strains with enhanced replication *in vitro* have been described. To understand whether this characteristic could be a cause of liver disease, we have studied a variant of the closely related duck hepatitis B virus (DHBV) that had enhanced levels of cccDNA accumulation, previously shown to be cytopathic *in vitro,* as a model for the pathogenesis of analogous viruses in humans. *In vivo* liver damage caused by this variant (G133E) occurred only during the first 2 weeks p.i., after which time cccDNA levels and liver histology returned to near normal despite continued virus replication. To determine whether recovery was due to the emergence of noncytopathic revertant, we tested whether wild-type virus would have a selective advantage in competition with the cytopathic mutant in a fully infected liver. In a mixed infection of ducklings with G133E and a small amount of wild-type virus, the wild-type virus was detected as the predominant genotype after recovery of normal liver histology. Two candidate revertant viral genomes were cloned directly from the serum virus of G133E-infected birds after recovery and tested for (i) control of cccDNA levels in primary hepatocyte cultures and (ii) their ability to compete with wild-type virus in a mixed infection. At least one noncytopathic revertant was identified by these two criteria. The results support the conclusion that the recovery from liver damage in G133E-infected ducklings was due to the emergence of spontaneous noncytopathic revertants rather than to host suppression of virus cytotoxicity. The results indicate that acute liver injury may result from infection with a cytopathic hepadnavirus but that such viruses may be rapidly replaced by noncytopathic variants during persistent infection.

INTRODUCTION

The hepadnaviruses make up a small family of enveloped DNA viruses that replicate through reverse transcription of an RNA intermediate (Mason *et al.,* 1982; Summers and Mason, 1982; Seeger *et al.,* 1986; Huang and Summers, 1991). These viruses have double stranded circular genomes of ~3 kb in size and a simple genetic structure. They have been described in only a few hosts, which include humans (Dane *et al.,* 1970; Robinson *et al.,* 1974; Summers *et al.,* 1975), marmots (Summers *et al.,* 1978), ground squirrels (Marion *et al.,* 1980; Testut *et al.,* 1996), and certain waterfowl (Mason *et al.,* 1980; Sprengel *et al.,* 1988). In all known hosts, hepadnaviruses infect primarily hepatocytes and establish a persistent productive infection of each cell. The mechanism for persistent virus production has been studied in the duck hepatitis B virus (DHBV) and has been shown to be due to control of the copy number of viral DNA molecules that are present in the nucleus of the infected cell (Tuttleman *et al.,* 1986; Summers *et al.,* 1990, 1991; Wu *et al.,* 1990; Huang and Summers, 1991; Lenhoff and Summers, 1994a). This form of viral DNA, covalently closed circular DNA (cccDNA) serves as the transcriptional template for all new protein synthesis and genome replication. Copy number control, and thereby control of replication rate, is exerted by the action of one of the two envelope proteins, preS, in inhibiting new cccDNA synthesis beyond a small number of molecules that are formed early in the establishment of infection. It is believed the preS protein acts by diverting the precursor of cccDNA, relaxed circular DNA (rcDNA), into a pathway for assembly of virus particles which are secreted from the infected cell (Lenhoff and Summers, 1994a).

The control of virus replication rate within the cell has been postulated to be necessary for persistence of hepadnavirus infections at the cellular level, i.e., the lack of cytopathicity. In recent years, however, HBV strains that replicate at high levels after transfection of cloned DNA into heterologous cultured cells have been described (Moriyama *et al.,* 1996; Pult *et al.,* 1997; Scalglione *et al.,* 1997; Baumert *et al.,* 1998). The existence of such variants has prompted speculation that enhanced replication might be responsible for direct or indirect cytopathic effects caused by such strains isolated from patients undergoing severe liver disease. We have examined the pathogenesis of a strain of DHBV that causes cytopathic damage in infected ducklings as a model for how analogous viruses in the human population might behave.

In DHBV, enhanced intracellular virus replication can be produced by mutations that decrease the ability of the preS protein to direct rcDNA into the virus assembly pathway, resulting in the accumulation of excess copies

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of cccDNA in the nucleus. The higher levels of cccDNA accumulated by cells infected with such variants can result in eventual cytotoxicity due to unknown causes associated with enhanced replication (Lenhoff and Summers, 1994b). One such mutant we have constructed (G133E) causes acute noninflammatory liver injury in infected ducklings, presumably due to direct viral cytopathic effects (Lenhoff *et al.,* 1998), but liver injury caused by G133E infection rapidly regresses even though infection persists. The mechanism for recovery from acute liver injury is not known.

In this paper we report the results of experiments designed to investigate the basis for recovery from acute liver injury in G133E-infected ducklings. Our evidence indicates that infection with the cytopathic variant creates an opportunity for the outgrowth of noncytopathic revertants and that such noncytopathic revertants arise during infection. The experiments illustrate the importance of cccDNA regulation in the mechanism of persistent infection and show how enhanced replication in vitro does not necessarily confer a selective advantage in a persistent infection.

RESULTS

In initial studies (Lenhoff *et al.,* 1998) we observed the occurrence of acute liver injury after infection of ducklings with a mutant of DHBV (G133E) shown to be cytopathic *in vitro* (Lenhoff and Summers, 1994b). The mutation consisted of a single amino-acid substitution in the preS envelope open reading frame that resulted in diminished regulation of cccDNA levels and enhanced replication in infected primary hepatocytes. Increased levels of cccDNA in livers of ducklings infected with this mutant and the occurrence of acute noninflammatory liver injury were consistent with direct cytotoxic effect of this virus *in vivo,* consistent with its phenotype *in vitro.* However, ducklings infected with this mutant quickly recovered from the acute injury, and histology of the liver returned to near normal despite persistent virus replication. This finding could be the result of two different effects: (i) suppression of cytotoxicity by a host response and (ii) selection and phenotypic dominance of noncytopathic revertants of the infecting virus. The phenotypic dominance of a revertant *in vivo* would be aided by the fact that the mutation that causes diminished cccDNA regulation in G133E can be complemented by wild-type preS envelope protein to restore normal cccDNA levels (Lenhoff and Summers, 1994a). Thus the presence of a noncytopathic revertant virus that expressed normally functional preS envelope protein would be expected to suppress cytopathic injury due to the G133E mutant in a cell expressing both genomes. For this reason, we tested whether infection with G133E would allow the outgrowth of wild-type revertants should they arise spontaneously and whether such outgrowth coincided with the recovery from liver injury.

Mixed infection with G133E and wild-type virus

Three groups of ducklings were infected and analyzed in Experiment 1. In Group I, nine birds were infected with 10^9 vge G133E alone. This group was used to monitor the normal course of the G133E infection as it was previously reported. In Group II, nine ducklings were infected with a mixture of 10^9 vge G133E and 10^7 vge wild-type DHBV. This group was used to determine whether infection with G133E created an opportunity for selection of putative wild-type revertants, simulated by the wild-type virus added to the inoculum. In Group III, nine ducklings were infected with 10^9 vge of the mutant P130L mixed with 10^7 vge wild-type virus. This group served as a control that wild-type virus did not have a selective advantage over a phenotypically wild-type genome carrying the genetic marker, the absence of the *Sma*I site due to the C1178A mutation. Three birds from each group were sacrificed at 4, 9, and 21 days p.i. for analysis of tissues and for histological examination.

The viremias that developed in three birds of each group are shown in Fig. 1. Viremia achieved a maximum by Day 2 or 4 p.i. and thereafter fluctuated or declined by one or two orders of magnitude, as we have previously observed in experimental infections (Yang and Summers, 1998). Peak viremia coincided with full infection of the liver. The histological changes seen in the three groups of birds are summarized in Table 2. Changes in the appearance of the liver in Groups I and II at 4 days p.i. were similar to those previously described for G133E infection. In Group I extensive bile ductule proliferation was seen in the portal areas with small ductule-like cells infiltrating into the parenchyma (Figs. 2B and 2E). Similar but less extensive proliferative changes were seen in group II livers (Figs. 2C and 2F). Group III livers appeared normal (Figs. 2A and 2D). In all three groups, virtually all hepatocytes stained positive for viral preS envelope protein, while bile ductule and proliferating ductule-like cells remained negative (Figs 2D–2F). Livers at Days 4 and 9 showed evidence of hepatocellular injury in the form of apoptotic bodies and necrosis. At Day 21 p.i., livers from all groups were similar and appeared essentially normal (Table 2).

To detect enrichment for the added wild-type virus in the blood of infected ducks from Groups II and III, we carried out PCR amplification to produce a 664-bp fragment containing the region of the DHBV genome with the genetic tag; i.e., the presence or absence of the *Sma*I site at position 1178. The PCR products were then digested with *Sma*I to determine the proportion of wild-type (*Sma*I site present) to G133E or P130L virus (*Sma*I site absent). As seen in Fig. 3, the PCR product produced from the

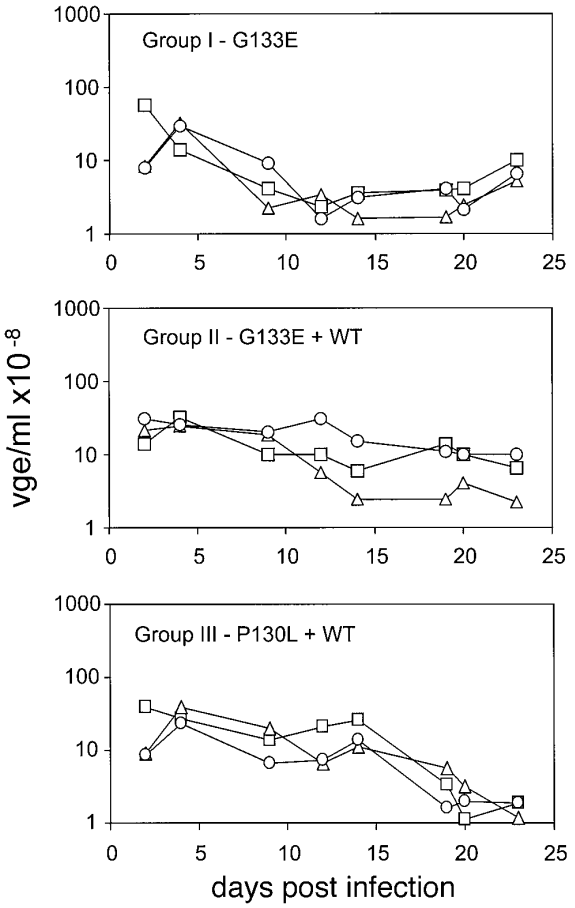

FIG. 1. Viremia in the three groups of infected ducklings of Experiment 1. The ducklings in each group were bled at the indicated times and the amount of viral DNA in the serum was determined by dot blot hybridization. The three birds shown for each group are representative examples.

serum of a duckling from Group I (Bird 1) lacked any detectable *Sma*I site, whether amplified from serum obtained at 4 or 21 days p.i., as expected. Serum from a duckling infected with wild-type virus in a parallel experiment (Bird 2) produced a PCR product that was uniformly digested with *Sma*I at both times p.i. In Group II birds, the PCR product contained little (Birds 3 and 4) or no (Bird 5) *Sma*I-digestible DNA at 4 days p.i., but the PCR product amplified from serum obtained at 21 days p.i. consisted predominantly of wild-type, *Sma*I-digestible DNA molecules. These results indicated that by 21 days p.i., coincident with recovery from acute liver injury, the major virus population found in the blood of birds from Group II had shifted from the G133E virus genotype to the wild-type genotype. This shift was dependent on the presence of the G133E mutation itself, since infection with the P130L noncytopathic strain mixed with wild type did not provide the opportunity for selection of the wild-type virus in birds from Group III (Birds 6–8). All digestions were monitored for completeness by means of an internal control plasmid DNA shown at the top of each lane.

Characterization of two candidate wild-type revertants

We had previously cloned two candidate wild-type revertant genomes from the serum of ducklings originally infected with G133E virus and maintained to days 27 and 72 p.i., beyond the stage of recovery from liver injury (R. Lenhoff, unpublished). Nucleotide changes in the preS region of the envelope gene were characterized by sequencing and are depicted in Table 1. The single mutations detected in these two genomes were transitions that occurred within six residues of the original G133E mutation. In the mutant G133K, the upstream adjacent residue was changed from G to A, and in G133E/Q133R, an A at position 1204 was changed from A to G. Both mutations resulted in the indicated coding changes in preS and the overlapping P open reading frame. In both genomes, the original nucleotide substitutions present in the G133E genome were retained, indicating that these genomes were most likely revertants derived from the replicating virus.

The candidate revertants were tested for their replication phenotype in primary hepatocyte cultures compared with wild-type virus, G133E virus, and 1165A virus, a mutant that is totally defective in preS envelope protein production. LMH cells were transfected with appropriate head to tail dimers, and virus concentrated from the supernatants was used to infect primary duck hepatocytes. At 10 days p.i., replicative intermediates and cccDNA were extracted from the infected cell layers and assayed by Southern blot hybridization. The results, shown in Fig. 4, compare the levels of cccDNA in each infected culture with the amount of replicative intermediates in the same culture. The phenotype of two mutant viruses with defects in cccDNA regulation (1165A and G133E) is indicated by an increase over wild type in the ratio of cccDNA to rcDNA (60-fold and 4-fold, respectively). By this criterion, the ratio of cccDNA to rcDNA for the G133E/Q135R mutant was the same as wild type, while that of the G133K was only slightly higher than wild type. In this respect, both candidate revertants differed substantially from the G133E parent.

To test directly for the phenotype of these two candidate revertants we performed new series of infections (Experiment 2) in which each of these mutant viruses was competed with wild-type virus to determine whether it behaved like the cytopathic strain, G133E, or like the noncytopathic strain, P130L, in allowing wild-type virus outgrowth. Two groups of ducklings were infected, as previously, with 10^9 vge each of the test virus (G133K or G133E/Q135R in Groups I and II, respectively), mixed with 10^7 vge of wild-type virus, and viremia was followed by dot blot hybridization. Livers were removed at 4, 9, and 21 days p.i. for histological examination, and the out-

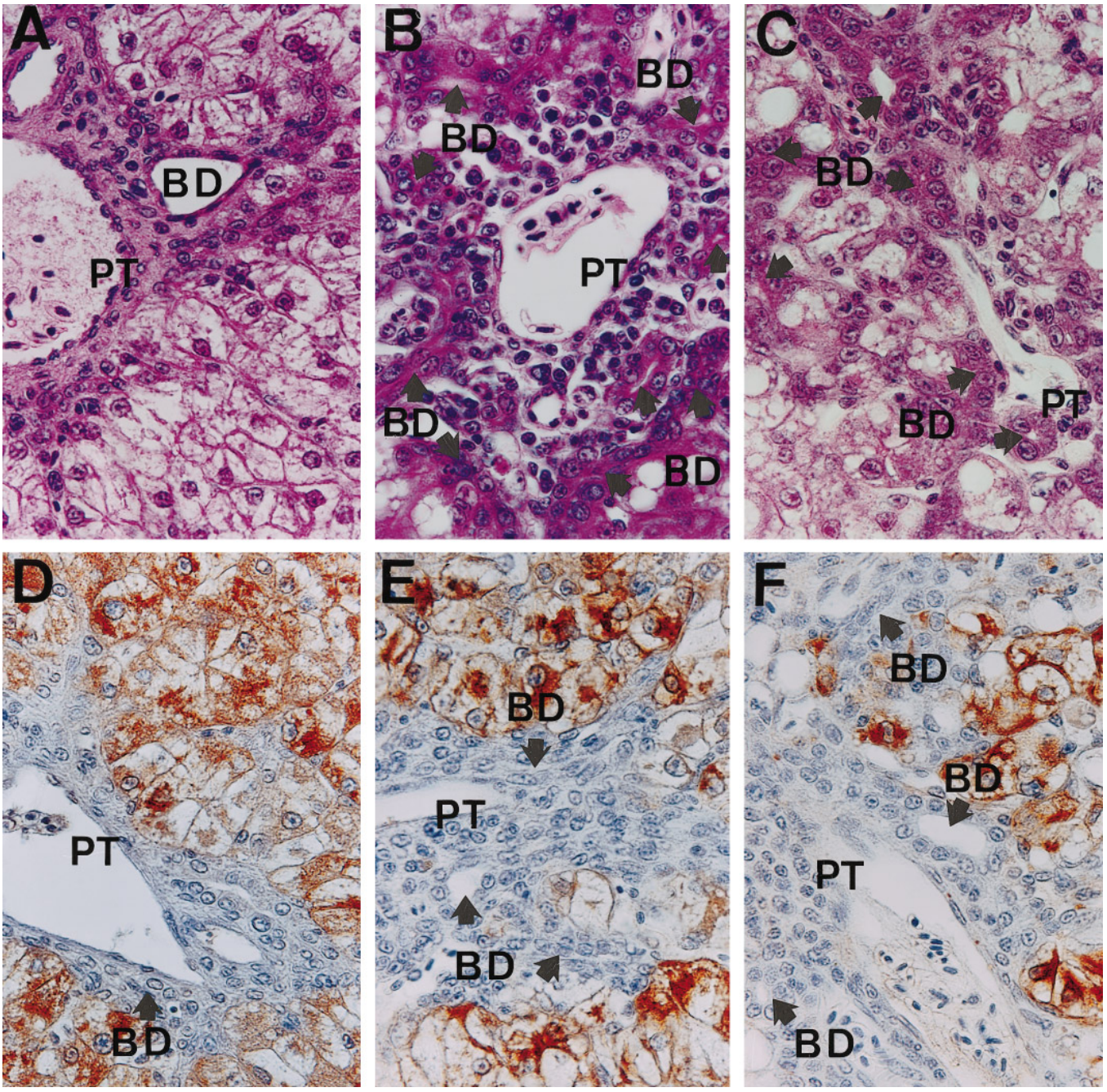

FIG. 2. Histology and immunohistochemistry of livers from Experiment 1. (A–C) Hematoxylin-eosin stain of livers from Day 4 p.i. of ducklings from Groups III (A), I (B), and II (C). (D-F) Immunohistochemical stain of preS protein in ducklings at 4 days p.i. from Groups III (D), I (E), and II (F). Arrows indicate specific features designated PT (portal tract), BD (bile ductule cells).

growth of wild-type virus in the blood was monitored by PCR and *Sma*I digestion as before. As in Experiment 1, viremia in the two groups of infected birds, first detected by dot blot on Day 2 p.i., was observed to decrease or fluctuate throughout the experiment (Fig. 5). On histological examination it was noted that infection with the G133K mixture (group I) led to bile duct cell proliferation, abnormal, apoptotic, and lysed hepatocytes and mild mononuclear cell infiltrates, (Table 2, Fig. 6A). Livers from birds infected with the G133E/Q135R (group II) mixture appeared normal (Fig. 6B), except for the presence of moderate infiltrates of mononuclear cells. At 23 days p.i. ducklings from Group II showed normal hepatocellular architecture. In contrast, infected birds from Group I had evidence of sustained liver damage at Day 23 p.i. The histological changes in the livers of the two groups of ducklings in Experiment 2 are summarized in Table 2.

The genotype analysis (Fig. 7) showed that the virus

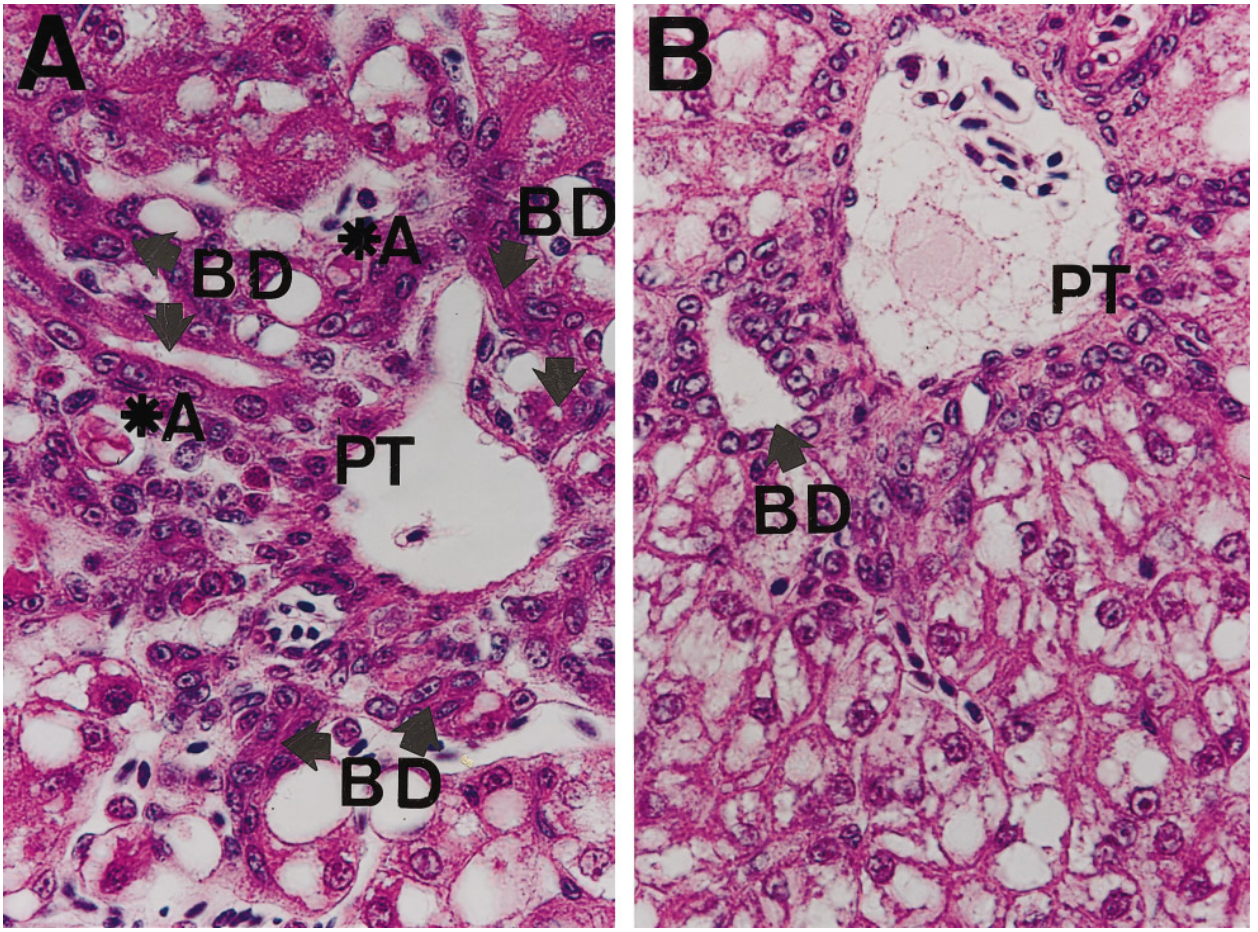

FIG. 6. Liver histology of infected ducklings in Experiment 2. Livers removed at Day 4 p.i. from a duckling infected with G133K + WT (A) or G133E/Q135R + WT (B) were stained with hematoxylin and eosin. Arrows indicate the location of the indicated features: BD, bile duct; PT, portal tract; *A, apoptotic or eosinophilic body.

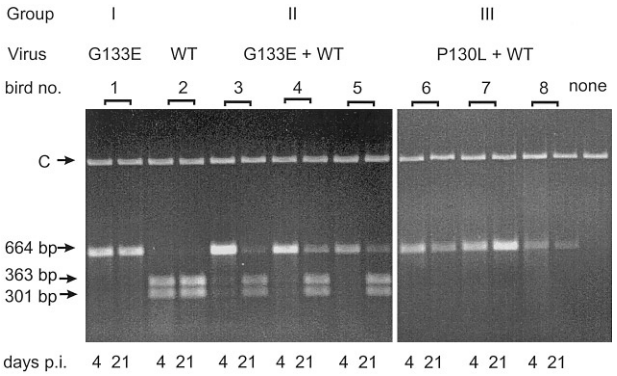

FIG. 3. PCR assay of viral genotypes in the serum from infected ducklings of Experiment 1 The region of DHBV DNA between nucleotides 835 and 1478 was amplified by PCR from 2 μl of serum obtained from ducklings at the indicated times. After PCR, each amplified DNA sample was mixed with 20 ng of a plasmid DNA containing a single *Sma*I site as an internal digestion control, and digested to completion with *Sma*I. Digestion was monitored by electrophoresis through a 15% agarose gel containing 1 μg/ml ethidium bromide and photographed under ultraviolet illumination. The positions of the uncleaved (664 bp) and cleaved (363 bp, 301 bp) PCR products, and the cleaved internal control (C) are indicated.

population in the G133K-infected ducklings shifted from the G133K genotype at 4 days p.i. to a mixture of G133K and wild-type virus at 23 days p.i., reflecting the outgrowth of the wild-type virus added in the inoculum. In contrast, the wild-type genotype virus was not detected in birds infected with the G133E/Q135R virus mixture at 23 days p.i. This result indicated that infection with the G133E/Q135R virus, like the noncytopathic P130L strain used in the previous experiment, did not allow outgrowth of wild-type virus, while infection with the G133K phenotype allowed wild-type outgrowth, probably due to cytotoxicity of the G133K infection.

DISCUSSION

The purpose of this study was to further elucidate the biological characteristics of cytopathic hepadnaviruses that might arise in a human population infected with HBV. As a model for the behavior of such viruses, we have used a cytopathic mutant of the avian hepadnavirus, DHBV, to produce chronic infections in ducklings. In this report, we have examined the basis for the transient

TABLE 1

DHBV Mutants Used in this Study

Genome designation	Nucleic acid sequence* 1188	1205	Amino acid sequence† 130	135	*Sma*I site at 1179
Wild type	CCA CTC CTG GGA AAT CAG		P L L G N Q		+
G133E	CCA CTC CTG G**A**A AAT CAG		P L L **E** N Q		−
P130L	C**T**A CTC CTG GGA AAT CAG		**L** L L G N Q		−
G133K	CCA CTC CTG **A**AA AAT CAG		P L L **K** N Q		−
G133E/Q135R	CCA CTC CTG G**A**A AAT C**G**G		P L L **E** N **R**		−

* Numbers indicate nucleotide positions according to Mandart *et al.,* 1984. Underlined letters indicate nucleotide differences with wild type.
† Numbers indicate the corresponding amino acid residues in the preS protein. Underlined letters indicate amino acid differences with wild type.

nature of liver injury induced by infection with the cytopathic strain of DHBV. Specifically, we tested the hypothesis that recovery from acute liver injury was due to the emergence of noncytopathic revertants during the chronic phase of infection.

We have used *in vivo* competition between genotypically marked viruses with differing cytopathicity to determine the relative fitness of each virus strain to predominate over the short term in a fully infected liver. Our experimental design was based on the assumption that the mixture of the competing genotypes found in the serum was representative of the virus replicating in the liver. In Experiment 1, a cytopathic variant of DHBV, G133E, was capable of spreading after inoculation to produce a fully infected liver but was thereafter rapidly replaced by wild-type noncytopathic virus that was added to the inoculum to simulate the presence of spontaneously occurring revertants. Presumably, noncytopathic revertants arising during growth of G133E would behave in the same way as the added wild-type virus, i.e., they would outgrow the mutant virus in a fully infected liver. Since the envelope defect in G133E that presumably gives rise to its cytopathic phenotype can be complemented *in trans* by a wild-type envelope protein, outgrowth of revertants would tend to suppress the cytopathic effects of the G133E strain in mixed infected cells and contribute to recovery of the G133E-infected duckling from virus-induced liver injury. A weak indica-

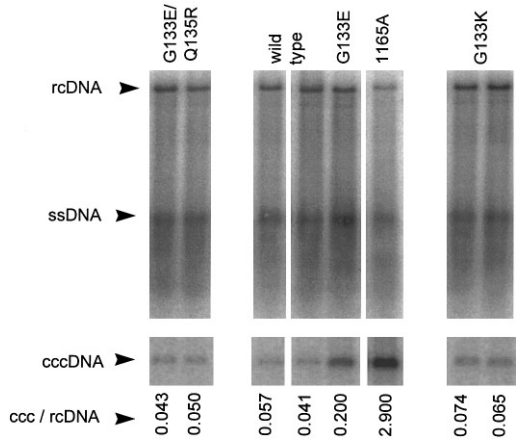

FIG. 4. Replicative intermediates and cccDNA in primary duck hepatocyte cultures. Primary duck hepatocytes were infected at Day 3 after plating with virus particles concentrated from LMH cell supernatants transfected with plasmids containing dimers of the indicated genomes. Virus from the equivalent of 20 ml of LMH cell supernatant was used to infect each plate, and the cultures were maintained for 10 days before extraction of the viral DNA. Viral DNA was analyzed by agarose gel electrophoresis and Southern blot hybridization. The positions of rcDNA, single-stranded DNA (ssDNA) and cccDNA are indicated. The radioactivity in the rcDNA band and the cccDNA band were quantitated by phophorimage analysis and the ratios of cccDNA to rcDNA are indicated.

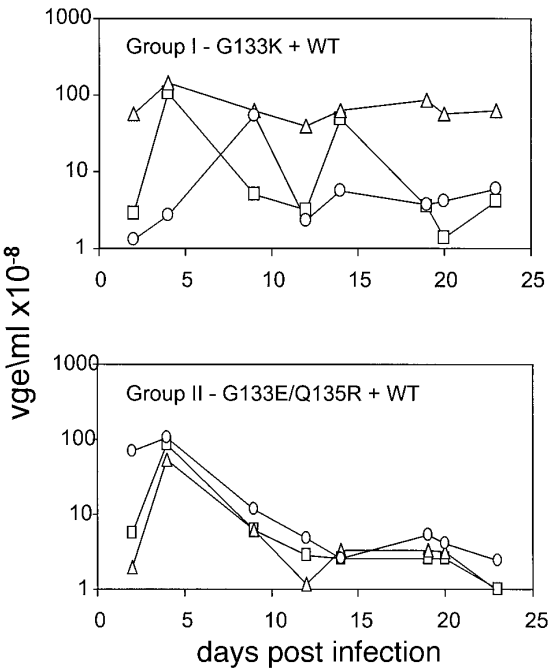

FIG. 5. Viremias in the two groups of infected ducklings in Experiment 2. The ducklings in each group were bled at the indicated times and the amount of viral DNA in the serum was determined by dot blot hybridization. The three birds shown for each group are representative examples.

TABLE 2

Summary of Liver Histology of Infected Ducks in Experiments 1 and 2

Group	Day 4*				Day 9				Day 23			
	BP†	EB	Ly	MNC	BP	EB	Ly	MNC	BP	EB	Ly	MNC
Experiment 1												
I (G133E)	+++	+	+	+	+++	+	+	+	+	−	−	+
II (G133E + WT)	++	+	+	+	++	+	+	+	±	−	−	++
III (P130L + WT)	−	−	−	−	−	−	−	±	−	−	−	±
Experiment 2												
I (G133K + WT)	++	+	+	+	++	+	++	+	++	+	+	+
II (G133E/Q135R + WT)	+	−	−	+	−	−	−	++	−	−	−	++

* Estimated severity of liver changes in three birds for each group at the times indicated. The severity noted was representative of that in each bird.

† Features, and grading in cells per mm^2 liver section: BP, bile duct epithelial cells and proliferating bile ductule cells; −, <150; +, 150–500; ++, 500–1000; +++, 1000–1500. EB, eosinophilic bodies; −, <1; +, 1–10. Ly, lysed hepatocytes; −, <1; +, 1–150; ++, 150–300. MNC, mononuclear cells; 0, <100; +, 100–250; ++, 250–500.

tion of such an effect was noted in the mixed infection with G133E and wild-type virus since liver injury appeared to be less severe in the mixed infection than in the single infection with G133E (Table 2). However, more birds would be needed to document the significance of the difference we noted in this experiment.

Consistent with the scenario that the outgrowth of wild-type revertants contributed to the recovery from liver injury in G133E-infected birds was the isolation of an apparent revertant, G133E/Q135R, from a recovered G133E-infected duck. This virus behaved in a manner that was not distinguished from wild type by (i) its phenotype in primary duck hepatocytes, (ii) evidence of liver injury in infected ducklings, or (iii) its ability to compete with wild-type virus in fully infected liver (Experiment 2). A second candidate revertant, G133K, did not seem significantly different from wild-type virus in its ability to control amplification of cccDNA in primary duck hepatocytes but was apparently cytopathic in infected animals. The basis for the outgrowth of this variant is therefore not apparent. It is possible that coinfection of hepatocytes with G133K and G133E was less cytopathic than infection with either virus alone, or that G133K was less cytopathic than G133E and was therefore capable of competing with G133E in a fully infected liver. Neither of these possibilities has been tested.

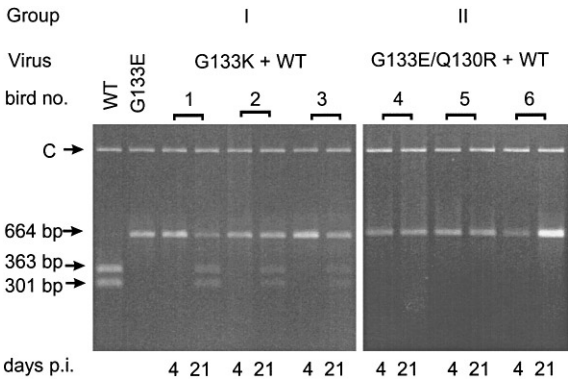

FIG. 7. PCR assay of viral genotypes in the serum from infected ducklings of Experiment 2. The region of DHBV DNA between nucleotides 835 and 1459 was amplified by PCR from 2 μl of serum obtained from ducklings at the indicated times. After PCR, each amplified DNA sample was mixed with 20 ng of a plasmid DNA containing a single *Sma*I site as an internal digestion control and digested to completion with *Sma*I. Digestion was monitored by electrophoresis through a 15% agarose gel containing 1μg/ml ethidium bromide and photographed under ultraviolet illumination. The positions of the uncleaved (664 bp) and cleaved (363 and 301 bp) PCR products and the cleaved internal control (C) are indicated.

It is curious that both candidate revertants retained the original nucleotide substitution of G133E, even when codon 133 was altered by a second site mutation in G133K (Table 1). Authentic wild-type revertants presumably could arise by a reversion of A to G at position 1198, the site of the original G133E mutation, but such revertants were not observed. In direct sequencing reactions of the PCR products of serum virus in several birds from preliminary experiments with G133E, the G133K second site mutation at nucleotide 1197 was observed as a predominant genotype occupying about 50% of the sequence ladder at that position (J. Summers, unpublished data). We do not know the phenotype of the complete genomes bearing these second site mutations at residue 1197, and therefore we do not understand the basis for the apparent repeated selection of this mutation in different birds. The wild-type nucleotide G at position 1198 was not seen in these mixed sequence ladders, indicating that "same site" reversion to wild type was not a frequent type of reversion.

It is not entirely clear how wild-type revertant viruses replace the cytopathic virus in a fully infected liver. We expect that the cytopathic phenotype of G133E would be compensated for by its higher replication rate during the spread of infection, but that in a fully infected liver, where

further spread is limited by the scarcity of uninfected cells, the longevity of infected cells would become the factor most beneficial to a virus strain for its survival in the liver. The population of hepatocytes infected by a cytopathic strain of virus would surely be decreased by cell death, thus enriching the liver in cells infected by a noncytopathic strain; therefore, new hepatocytes formed in a regenerative response by division of preexisting hepatocytes would be preferentially derived from the population infected by a noncytopathic strain. Alternatively, if new hepatocytes were formed from uninfected stem cells, the two viruses would compete directly for infection of this cell population. Even in this case, noncytopathic revertants would hold an advantage over G133E because cells infected with G133E alone would fail to survive, while dually infected cells and cells singly infected with a noncytopathic revertant would tend to survive. Whether stable mixed infection of hepatocytes occurs *in vivo* is not known, however. Both mechanisms for replacement of G133E by wild-type virus imply a large amount of hepatocellular turnover during replacement. If G133E virus were completely unable to infect new cells, a minimum of 50% of the cells in the liver would still have to die and be replaced by wild-type virus-infected cells to account for the amount of replacement observed in Experiment 1. Any residual ability of G133E virus to compete with wild-type virus for infection of new hepatocytes or for outgrowth of regenerating hepatocytes would necessitate even higher amounts of cell death to account for the observed replacement. A kinetic model of this process indicates that the amount of cell death involved in 50% replacement of G133E by wild-type virus after full infection of the liver might be as high as two to three times the starting number of liver cells (J. Summers, unpublished data available on request).

The mechanism responsible for cytotoxicity of the G133E virus is not known. In a previous study (Lenhoff and Summers, 1994b), cytotoxicity among a series of mutants defective in cccDNA regulation was directly proportional to the severity of their regulation defect. Whether it is the accumulation of cccDNA itself, the accumulation of some viral gene product, or some other behavior of the mutant preS protein that also affects its ability to regulate cccDNA that mediates cell injury is not known. The apparent cytotoxic phenotype of G133K virus did not correlate with a seemingly significant increase in cccDNA levels, compared with other mutants, but absolute levels of cccDNA per cell were not directly measured in these experiments. For this reason, the exact phenotype of G133E and G133K mutants that is responsible for the cytopathic property of the two viruses is a matter of conjecture.

The experiments reported here support the hypothesis that outgrowth of wild-type revertants contributed to the recovery of G133E-infected birds from acute liver injury. These data do not directly refute an alternative second hypothesis, that a host response suppressed the cytotoxicity of G133E infection in individual hepatocytes. In our view, however, these two hypotheses are mutually exclusive if cytotoxicity of the G133E strain is the factor that provides the opportunity for outgrowth of wild-type virus. Thus suppression of cytotoxicity by a host response would tend to remove the selective pressure for outgrowth of wild-type virus, which clearly exists, according to our experiments, in G133E-infected liver. For this reason, our data favor the outgrowth of noncytopathic revertants as the major mechanism for recovery of G133E-infected birds from acute liver injury. It follows that cytopathic variants in which reversion was not so readily achieved or in which cytotoxicity could not be suppressed by complementation *in trans* might be capable of causing a more chronic liver injury by direct cytotoxicity.

The results of this study illustrate some limitations on the potential of cytopathic variants of HBV, even with an enhanced replication rate, to cause persistent infection and chronic liver disease in humans. While such variants may efficiently spread in the liver following transmission, it can be predicted that they would be at a disadvantage for persistence in competition with similar but noncytopathic strains of HBV such as, for example, spontaneously arising revertants.

MATERIALS AND METHODS

Plasmids and mutant genomes

The DHBV genome used in these studies was that sequenced by Mandart (Mandart *et al.,* 1984). Site-directed mutagenesis was used to produce a DHBV variant with altered regulation of cccDNA synthesis. This mutant, G133E, containing single nucleotide changes at two sites, has already been described (Lenhoff and Summers, 1994a,b). One mutation was silent in the overlapping P open reading frame but resulted in a single amino-acid substitution of glycine to glutamic acid at position 133 (G133E) in the large envelope protein. A second change at nucleotide 1178 from C to A (C1178A) was silent in both the preS and P open reading frames but destroyed the *Sma*I site at this position and was therefore useful as a convenient genetic tag. A second mutant, P130L, was used as a wild-type control in these studies (Lenhoff and Summers, 1994a). P130L was generated *in vitro* by substitution of the C residue at nucleotide position 1189 with T, causing a change of codon 130 from P to L (CCA→CTA). This mutant also retained the C1178A mutation as a genetic tag. Two spontaneously occurring variant genomes (G133K and G133E/Q135R) cloned directly from infected duck sera (R. Lenhoff, unpublished observations) were subcloned as head-to-tail dimers in plasmid pSP65. *Sma*I digestion and sequencing of these genomes from nucleotide 800 to 1285 confirmed that they were derived from G133E and

revealed the location of mutations in preS that were presumably responsible for their altered phenotypes. In one case, the original nucleotide substitution (G133K) resulting in the G133E mutation (GGA→GAA) was retained while codon 133 was altered to encode lysine (GAA→AAA) and the overlapping P open reading frame codon was changed from glycine to glutamic acid (GGA→GAA). In the variant G133E/Q135R, the original mutation was retained while the codon at position 135 was changed from Q to R (CAG→CGG). The mutation at this site did not result in a change in P protein coding.

Cloning of candidate revertant genomes

Viral relaxed circular DNA (ca. 1 ng) was purified (Lenhoff and Summers, 1994b) from serum taken from two G133E virus-infected birds at 24 and 72 days p.i., respectively. The viral DNA was linearized with *Eco*RI, ligated to a 50-fold excess of *Eco*RI-linearized pSP65 plasmid, and transformed into *Escherichia coli* strain DH5-α. Two DHBV genomes that were candidate revertants were identified by *Sma*I digestion, sequenced between nucleotides 800 and 1279 (preS region), and cloned as head to tail dimers in pSP65. Dimers were used for transfection of LMH cells.

Antibodies

The DHBV core protein specific rabbit antiserum used in the present study was previously described (Calvert and Summers, 1994). A DHBV preS reactive antibody was prepared as described (Luscombe *et al.,* 1994). Secondary antibodies reactive to the DHBV specific antibodies conjugated to horse-radish peroxidase were obtained a commercial source (Pierce, Rockford, IL).

Cell culture and transfection

Virus particles for infection of either primary duck hepatocytes or ducklings were produced by transfection of the chicken hepatoma cell line LMH (Kawaguchi *et al.,* 1987) as previously described (Summers *et al.,* 1991). Supernatants from the transfected LMH cells were collected daily from Days 3–10 posttransfection, clarified by centrifugation at 10,000 rpm for 10 min and stored prior to use at 4°C. Virus was concentrated by precipitation with polyethylene glycol (10% final concentration) followed by centrifugation and dissolved in a suitable volume. The titer of virus expressed in viral genome equivalents (vge) was determined by Southern blot hybridization following selective DNA extraction of enveloped virus particles as previously described (Lenhoff and Summers, 1994a). Viral DNA was quantitated by comparison of the hybridization signal from the equivalent of 1 ml medium from transfected LMH cells to a 300-pg hybridization standard using a scanning image analyzer (Molecular Diagnostics, Sunnyvale, CA). The number of pg of viral DNA per ml of LMH cell medium was then converted to vge per ml using a molecular weight for viral DNA of 2×10^6 Da.

Animals

Day-old ducklings were obtained from Metzer Farms (Redland, CA). Blood was collected at 1 day posthatch and the serum was assayed by dot blot hybridization for DHBV DNA. Primary hepatocyte cultures were prepared from DHBV negative ducklings 7–10 days of age as previously described (Tuttleman *et al.,* 1986). Infection for primary hepatocytes was carried out at 2 days postplating by addition of concentrted virus to the culture medium, which remained in contact with the cells for 20 h at 37°C. The culture medium, containing 1% dimethylsulfoxide (Pugh and Summers, 1989), was renewed daily.

For infection studies, DHBV-free ducklings were inoculated by intraperitoneal injection on Day 2 posthatch with either G133E at a dose of 10^9 vge or with a mixture of 10^9 vge of G133E, G133K, G133E/Q135R, or P130L, respectively, combined with 10^7 vge wild-type DHBV. Three birds from each group were sacrificed at Days 4, 9, and 21 p.i. Animals were killed by intravenous injection with phenobarbitol (68 g/ml) and their livers removed for virological and histological examination. All birds received humane care throughout the study in accordance with guidelines issued by the National Institutes of Health.

Analysis of viral DNA in the serum

Viremia was assayed daily for the first week and on alternate days for the next 2 weeks of each study. DHBV DNA in the serum was detected by dot blot hybridization. Serum (1 μl) was applied to a nylon filter (Hybond N, Amersham) and air dried. Viral DNA, was released by 1-min treatment with alkali (0.2 N NaOH) followed by neutralization in 0.2 N Trizma HCl. The filter was dried a second time and DHBV DNA was detected using a ^{32}P-labeled riboprobe specific for the detection of the viral minus strand (Pugh *et al.,* 1988). The amount of radioactive probe binding to serum-derived DHBV DNA was determined by comparison to a DHBV DNA standard on the same filter using phosphorimaging analysis (Molecular Diagnostics).

Purification and detection of viral DNA genomes in the serum using PCR

Amplification was performed on DNA isolated for the equivalent of 2 μl serum. Serum (10 μl) was mixed with 40 μl of TE 10:1 containing 2% SDS, 20 μg of Pronase, and 100 mM NaCl. The mixture was incubated for 60 min at 37 °C and then extracted with phenol. Nucleic acids were precipitated by the addition of 2 volumes of 100% ethanol and collected by microcentrifugation. The pellet was washed with ethanol, dried, and dissolved in 5 μl of TE 10:1, and 1 μl was used for polymerase chain reaction

(PCR). PCR was performed using as the plus strand primer an oligonucleotide nucleotide containing the DHBV sequence from position 816–834 and a minus strand primer with the DHBV sequence from nucleotide 1479–1459. A 664-bp fragment was produced from the viral DNA template using AmpliTaq Gold (Perkin-Elmer) and 30 amplification cycles in a Perkin-Elmer 9600. In products derived from wild-type DHBV, this 664-bp PCR product contained a single *Sma*I site; however, this site was absent in PCR products amplified from the P130L, G133E, G133K, or G133E/Q135R genomes. The presence or absence of the *Sma*I site was used to determine the genotype of the serum virus.

Assay of replicative intermediates and cccDNA in primary duck hepatocytes

DHBV DNA replicative intermediates were selectively extracted from primary duck hepatocyte cultures as previously described (Lenhoff and Summers, 1994b). Samples were analyzed by electrophoresis through a 1% agarose gel and transferred to a nylon filter. Viral DNA was detected by hybridization with a ^{32}P-labeled riboprobe specific for detection of the viral DNA minus strand.

Immunohistochemical detection of viral proteins in the liver

Fresh liver specimens were fixed for 30 min in ethanol: acetic acid (3:1) (EAA) and processed and embedded in paraffin using standard laboratory methods. Liver sections 4 μm in thickness were prepared, placed onto microscope slides, and hydrated through xylene and a graded alcohol series. Endogenous peroxidase was blocked by incubation in methanol containing 10% H_2O_2 for 30 min. To prevent nonspecific antibody binding, sections were blocked in 10% goat serum in 2 mM HEPES buffer, pH 7.4, containing 0.15 M NaCl (HBS) for a further 15 min. Primary antibodies specific for DHBV core or preS proteins were incubated on the liver sections for 60 min at room temperature. Unbound antibody was removed with three 5-min washes with HBS. The primary antibody was detected using the appropriate horse-radish peroxidase-conjugated goat secondary antibody (Pierce) for 60 min at room temperature.

Viral protein-antibody complexes was visualized by immunoperoxidase detection using diaminobenzidine (DAB) (Pierce) according to the manufacturer's instructions. All sections were counterstained with Mayer's hematoxylin. Sections were mounted with Cytoseal 60 (Cornwell Corp., Riverdale, NJ).

Grading of histological features was performed by examination of hemotoxylin-eosin stained sections of paraffin embedded EAA-fixed tissue. Slides were graded in comparison to reference slides that had been previously characterized by morphometric analysis for degree of bile ductule proliferation, eosinophilic bodies (presumably as a result of apoptosis), hepatocellular lysis, and mononuclear cell infiltration, as previously described (Lenhoff *et al.,* 1998).

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