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Isolation and Characterization of a Hepatitis B Virus Endemic in Herons

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A new hepadnavirus (designated heron hepatitis B virus [HHBV]) has been isolated; this virus is endemic in grey herons (*Ardea cinerea*) in Germany and closely related to duck hepatitis B virus (DHBV) by morphology of viral particles and size of the genome and of the major viral envelope and core proteins. Despite its striking similarities to DHBV, HHBV cannot be transmitted to ducks by infection or by transfection with cloned viral DNA. After the viral genome was cloned and sequenced, a comparative sequence analysis revealed an identical genome organization of HHBV and DHBV (pre-C/C-, pre-S/S-, and pol-ORFs). An open reading frame, designated X in mammalian hepadnaviruses, is not present in DHBV. DHBV and HHBV differ by 21.6% base exchanges, and thus they are less closely related than the two known rodent hepatitis B viruses (16.4%). The nucleocapsid protein and the 17-kilodalton envelope protein sequences of DHBV and HHBV are well conserved. In contrast, the pre-S part of the 34-kilodalton envelope protein which is believed to mediate virus attachment to the cell is highly divergent (<50% homology). The availability of two closely related avian hepadnaviruses will now allow us to test recombinant viruses in vivo and in vitro for host specificity-determining sequences.

Human hepatitis B virus is the prototype member of the hepadnavirus family (10). Other members have been isolated from eastern woodchucks (woodchuck hepatitis virus [40]), ground squirrels (ground squirrel hepatitis virus [18]), and Pekin ducks (duck hepatitis B virus [DHBV] [20]). Related viruses seem to exist in tree squirrels and possibly in other animals but have not been characterized in detail (6, 12). The narrow host range and the difficulties in establishing viral infection in cultured cells have forced the use of animal systems in hepadnavirus research. DHBV-infected ducks represent the most convenient animal system which has been used successfully for elucidating many aspects of the molecular biology of hepadnaviruses (for reviews, see references 8 and 37 and F. Schödel, R. Sprengel, T. Weimer, D. Fernholz, R. Schneider, and H. Will, Adv. Viral Oncol., in press).

Hepadnavirus replication involves reverse transcription of an RNA pregenome which leads to a heterogeneous population of DNA and DNA-RNA replicative intermediates in the liver (19, 39). In the virion, the viral genome is a partially single-stranded circular DNA molecule with none of the DNA strands covalently closed (13). To the 5′ ends of the DNA plus and minus strands, an oligoribonucleotide and a protein, respectively, are covalently linked; these linkages serve as primers for DNA synthesis (9, 14, 15, 23, 31, 44). In vitro, the virion-encapsidated genome can be converted into a double-stranded DNA molecule by use of the virion-encapsidated polymerase (13).

DHBV is the smallest hepadnavirus (3.0 kilobase pairs [kb]), with a simple genome organization of three overlapping open reading frames (ORFs) designated pre-C/C-, pre-S/S-, and pol-ORF (16, 35, 37). The major nucleocapsid protein (duck hepatitis B core antigen [DHBcAg]) (34) and soluble derivatives thereof (duck hepatitis B e antigen [DHBeAg]) of unknown function are encoded by the pre-C/C-ORF (4, 30). This is similar for mammalian hepadnaviruses except for the nucleocapsid and e antigens, which are larger in DHBV. The pre-S/S-ORF encodes two major envelope proteins of 17 (duck hepatitis B surface antigen [DHBsAg]) and 36 kilodaltons (kDa) (pre-S) (17, 27, 29), whereas three envelope proteins (HBsAg, pre-S1, and pre-S2) are expressed from the corresponding ORF of mammalian hepadnaviruses (11). The pre-S proteins are believed to mediate specific binding of the virus to a cellular receptor (25, 27). The long pol-ORF overlaps with the pre-C/C- and pre-S/S-ORF and most likely encodes the viral reverse transcriptase (38, 41, 42, 45) and probably an RNase H activity (F. Schödel, T. Weimer, H. Will, and R. Sprengel, AIDS Res. Hum. Retroviruses, in press). Three major transcripts, 1.7, 2.1, and 3.4 kb in length, are used to produce these proteins (1), whereas only two major transcripts are consistently found for mammalian hepadnaviruses (2, 3, 5, 22, 44). A fourth ORF, designated X, is present in all mammalian viruses but not in DHBV (16, 35). Since DHBV is the only avian hepadnavirus identified so far, it is not known whether the lack of an X-ORF is characteristic for avian hepadnaviruses or whether this is correlated with the low or nonexisting pathogenicity of DHBV.

Being interested in defining features that differentiate mammalian and avian hepadnaviruses and in searching for a convenient in vitro and in vivo system in which host range determinants and virus receptors can be studied, we started a systematic search for DHBV-related viruses. Here we describe the isolation and characterization of a new avian hepadnavirus closely related to DHBV which is endemic in grey herons (*Ardea cinerea*) and cannot infect Pekin ducks.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases were purchased from New England BioLabs and Boehringer Mannheim Biochemicals. Radiochemicals [α-^{35}S]dATP, [α-

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^{32}P]dXTPs, and ^{125}I-protein A were purchased from Amersham Buchler Braunschweig.

Liver and sera. Liver and serum samples of herons were kindly provided by colleagues from the Veterinärmedizinische Hochschule, Hannover, Federal Republic of Germany. One-day-old Pekin ducklings were purchased from commercial suppliers. Sera from Pekin ducks were obtained by cardiac puncture or bleeding of the jugular vein. Sera were assayed for the presence of DHBV-related DNA by dot blot hybridization with [α-^{32}P]dCTP-labeled DHBV DNA prepared from plasmid pDHBV16-t-27 by nick translation (34). Virus-negative ducks were infected with DHBV by injection of cloned virus stocks or by transfection of cloned viral DNA (34, 36).

Immunoblotting. Protein extracts were prepared by homogenization of liver tissue in PBS-NP buffer (10 mM phosphate [pH 7.5], 140 mM NaCl, 0.1% Nonidet P-40), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred onto nitrocellulose filters. The filters were saturated with bovine serum albumin (1.5% in phosphate-buffered saline [PBS]) and incubated with antiserum (dilution 1:1,500) in 1.5% bovine serum albumin-PBS overnight. The antiserum used was raised in rabbits against DHBcAg expressed in *Escherichia coli* (unpublished data). After being washed intensively with PBS-NP, the filter was incubated for 3 h with 2.5 μCi of ^{125}I-labeled protein A in 1.5% bovine serum albumin-PBS, washed several times in PBS-NP and H_2O, dried, and exposed to an X-ray film.

Isolation and cloning of HHBV DNA. For partial purification of heron hepatitis B virus (HHBV) particles, 2 ml of five viremic sera were pooled and pelleted for 30 min at 12,000 rpm in a Sorval SS34 rotor to remove aggregated proteins and other debris. The supernatant was passed through a sterile filter (Milex-GS; 0.22-μm pore size; Millipore Corp.), and virus particles of the flowthrough were pelleted in a SW40 Ti rotor for 2 h at 35,000 rpm. The sediment was suspended in 50 μl of 20 mM Tris hydrochloride (pH 7.5)–20 mM EDTA, and 5 μl of the virus pellet was subjected to SDS-PAGE for analysis of viral proteins or used for an endogenous polymerase reaction as described previously (34). For the isolation of viral DNA, 20 μl of protease K (5 mg of protease K per ml, 0.2% SDS, 100 mM NaCl) was added to 20 μl of the virus pellet and incubated for 4 h at 37°C. The sample was deproteinized by phenol-chloroform extraction, and viral DNA was precipitated by the addition of 2.5 volumes of ethanol. For molecular cloning, the viral DNA was digested with restriction enzyme *Kpn*I and inserted into bacteriophage vectors M13mp18 and M13mp19 linearized by *Kpn*I. For transfection studies, a plasmid carrying almost two HHBV genomes in a head-to-tail orientation (pUHHBV4-26T) was obtained after insertion of two subgenomic HHBV DNA fragments from phage mp18HHBV-4 (*Xba*I/*Kpn*I, 2,914 base pairs [bp]; *Kpn*I/*Pst*I, 2,945 bp) into the *Xba*I/*Pst*I-linearized plasmid pUC19. Alternatively, transfection was performed with a monomer of the HHBV genome in linear (released by *Kpn*I digestion) or recircularized (linear genome with *Kpn*I ends ligated at low DNA concentration) form.

DNA sequence analysis. The nucleotide sequencing reactions were carried out by using the dideoxy-chain termination method with [α-^{35}S]dATP (21, 28). Sequencing kits were obtained from New England BioLabs. The complete nucleotide sequences of both viral DNA strands were obtained by using DHBV-specific oligonucleotides and single-stranded phage M13mp18/19 DNAs containing subgenomic HHBV DNA inserts.

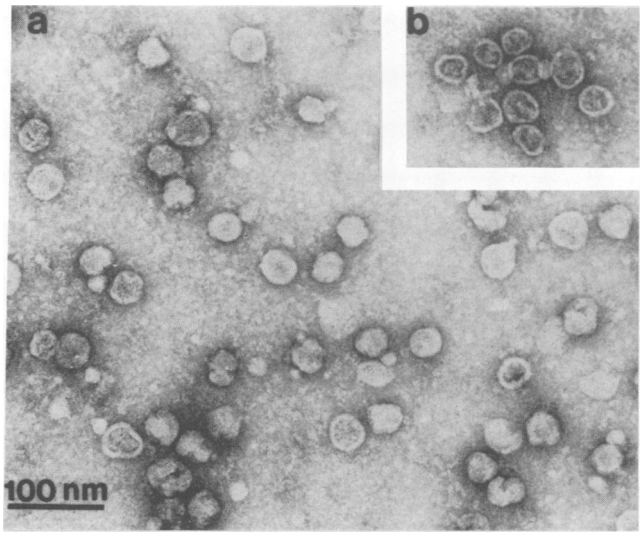

FIG. 1. Electron micrograph of negative-stained particles from ultracentrifuge deposits of HHBV- (a) and DHBV- (b) positive sera.

Comparative sequence analysis. For sequence analysis and alignments, the programs Seqed, Lineup, Gap, Bestfit, and Gapshow (version 5, 1987) of the University of Wisconsin Genetics Computer Group (UWGCG) computer software were used.

RESULTS

Identification of DHBV-related DNA and protein in sera and liver tissue of herons. To search for DHBV-related viruses in different avian species, 54 sera from herons were investigated by DNA spot test hybridization, using an α-^{32}P-labeled, cloned DHBV DNA (pDHBV16-t-27; 34) as a probe. Strong signals were observed with eight sera, and intermediate and weak signals were obtained with three sera (data not shown). With the cloned DHBV as a quantitative standard, the intensity of the signals corresponded to a viral titer of approximately 10^{10} to 10^{11} genome equivalents per ml. These data suggest that a DHBV-related virus is endemic in the herons of the area tested.

To get further information on this virus, viral particles present in sera of infected herons and DHBV-infected ducks were pelleted and analyzed morphologically by electron microscopy (Fig. 1). Essentially two types of particles were observed which closely resemble, in size (40 to 60 nm) and morphology, complete and empty viral particles of DHBV. As for DHBV, the putative empty HHBV viral particles (homogeneously staining) were in great excess compared to virions (densely staining core).

When the proteins of the virus pellets of HHBV- and DHBV-positive sera were analyzed by SDS-PAGE (Fig. 2A), virus-specific 17- and 36-kDa proteins (17, 27, 29) corresponding to the major envelope proteins were identified in both types of sera but not in virus-free samples. Thus, the numbers and sizes of the envelope proteins of both viruses seem to be very similar, if not identical. In the same virus pellets, neither a DHBV nor a putative HHBV nucleocapsid protein could be identified by Coomassie brillant blue staining, consistent with the electron microscopy data which showed a high prevalence of empty viral particles in sera.

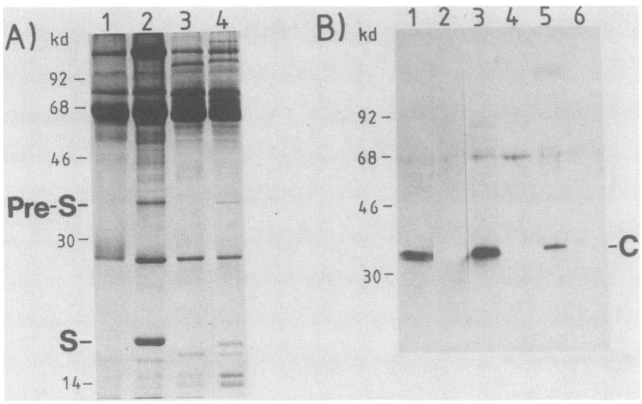

FIG. 2. Analysis by SDS-PAGE of envelope and core proteins of HHBV and DHBV. (A) Coomassie brilliant blue staining of proteins from ultracentrifuge deposits obtained from HHBV- (lane 2) and DHBV- (lane 4) containing and virus-free (lanes 1 and 3) heron and duck sera. The position of the pre-S and small S envelope proteins are indicated. (B) Immunoblot analysis of HHBV and DHBV core proteins performed with an anti-DHBV core antiserum (34) and ^{125}I-labeled protein A. Proteins of an HHBV-infected and uninfected heron liver were separated and probed in lanes 1 and 2. Core protein analysis was performed with ultracentrifuge deposits obtained from sera from ducks (lanes 3 and 4) and herons (lanes 5 and 6). Lanes 3 and 5 are from infected animals, and lanes 4 and 6 are from uninfected animals.

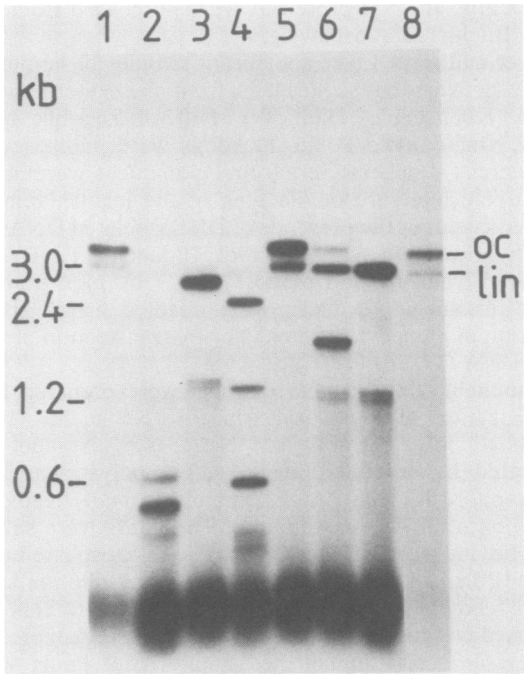

FIG. 3. Restriction enzyme analysis of HHBV genomes obtained from ultracentrifuge deposits of a pool of viremic heron sera. The viral genomes were labeled with [α-^{32}P]dCTP by using an endogenous polymerase assay (34). After proteinase K digestion, the viral DNA was extracted, digested with restriction enzymes (lanes 1 to 7, uncut, *Eco*RI, *Pst*I, *Bam*HI, *Bgl*II, *Hin*dIII, and *Kpn*I, respectively), and analyzed by agarose gel electrophoresis and autoradiography. Lane 8, DHBV DNA labeled and analyzed (uncut) as described above. Minor bands around 1.2 kb probably derive from a minor fraction of linearized viral genomes (34). OC, Open circular genomic DNA; lin, linear genomic DNA.

With an anti-DHBc antiserum, immunoblotting was performed with the same virus pellets and with liver protein extracts to visualize nucleocapsid proteins (Fig. 2B). This method revealed a specific immune reaction with a protein of approximately 32 kDa. This protein comigrates with DHBcAg of DHBV virions and core particles of DHBV-infected liver (34) and therefore might correspond to the HHBV nucleocapsid protein. Immunoblot analysis of proteins of a collection of heron livers revealed the 32-kDa viral core protein in 29 of 63 samples (data not shown), which is consistent with the high frequency of HHBV infection in herons as demonstrated by DNA dot spot hybridization.

The analysis of the viral DNA in liver tissue of infected herons by Southern blotting showed a pattern strongly reminescent of replicative intermediates of DHBV (data not shown). This suggests that HHBV replication, like that of all hepadnaviruses, involves reverse transcription of a pregenome.

To test whether the HHBV genome is partially single stranded and can be repaired by a virion-encapsidated polymerase, an endogeneous polymerase assay was performed. By using this assay, the HHBV genome could be radioactively labeled. Size fractionation of the DNA on agarose gels revealed two bands corresponding to the open circular and linear forms of the viral genome (Fig. 3). The labeled DNA comigrates with the corresponding genomic DNA of DHBV, which indicates that DHBV and HHBV have similar, if not identical, genome sizes. However, the restriction pattern obtained with HHBV (Fig. 3) was drastically different from that obtained with or predicted for other DHBV isolates (16, 35, 37) and thus predicted a major sequence divergence of HHBV and DHBV.

Infectivity of HHBV in Pekin ducks. To examine whether HHBV can infect Pekin ducks, 14 Pekin ducks, 1 to 3 days old, were inoculated intrahepatically with six different HHBV-positive sera and 4 animals were injected with a pool of five sera. At four weeks after injection, none of the animals became viremic as demonstrated by DNA dot blot analysis of serum samples (data not shown). In the same experiment, four control animals injected with DHBV-positive duck serum became DHBV positive (data not shown). When liver tissue was analyzed for the expression of viral nucleocapsid proteins by immunoblotting or for replicative intermediates by Southern blotting, sera from the four positive DHBV-injected ducks showed characteristic patterns of DHBcAg expression and viral replication, whereas all HHBV-infected animals were negative (data not shown). The immunoblot and the Southern blots were sensitive enough to detect even 10 to 50 times less-efficient replicative intermediate and core-protein synthesis in HHBV-infected ducks than in DHBV-infected ducks. It is therefore likely that HHBV cannot infect Pekin ducks. To confirm this interpretation further, 1-day-old ducks were transfected with a mixture of monomeric linear and covalently closed circular (16 ducks) or dimeric plasmid-integrated (12 ducks) cloned HHBV DNA (see below). As in the infection experiment, none of the animals transfected with HHBV developed viremia whereas cloned DHBV DNA tested in parallel was infectious in 6 of 6 animals tested (data not shown). Assuming that the cloned HHBV is infectious in herons, the results support our conclusion that HHBV and DHBV have a different host range.

Cloning and sequencing of HHBV isolates. To search for an explanation on the sequence level for the biological differ-

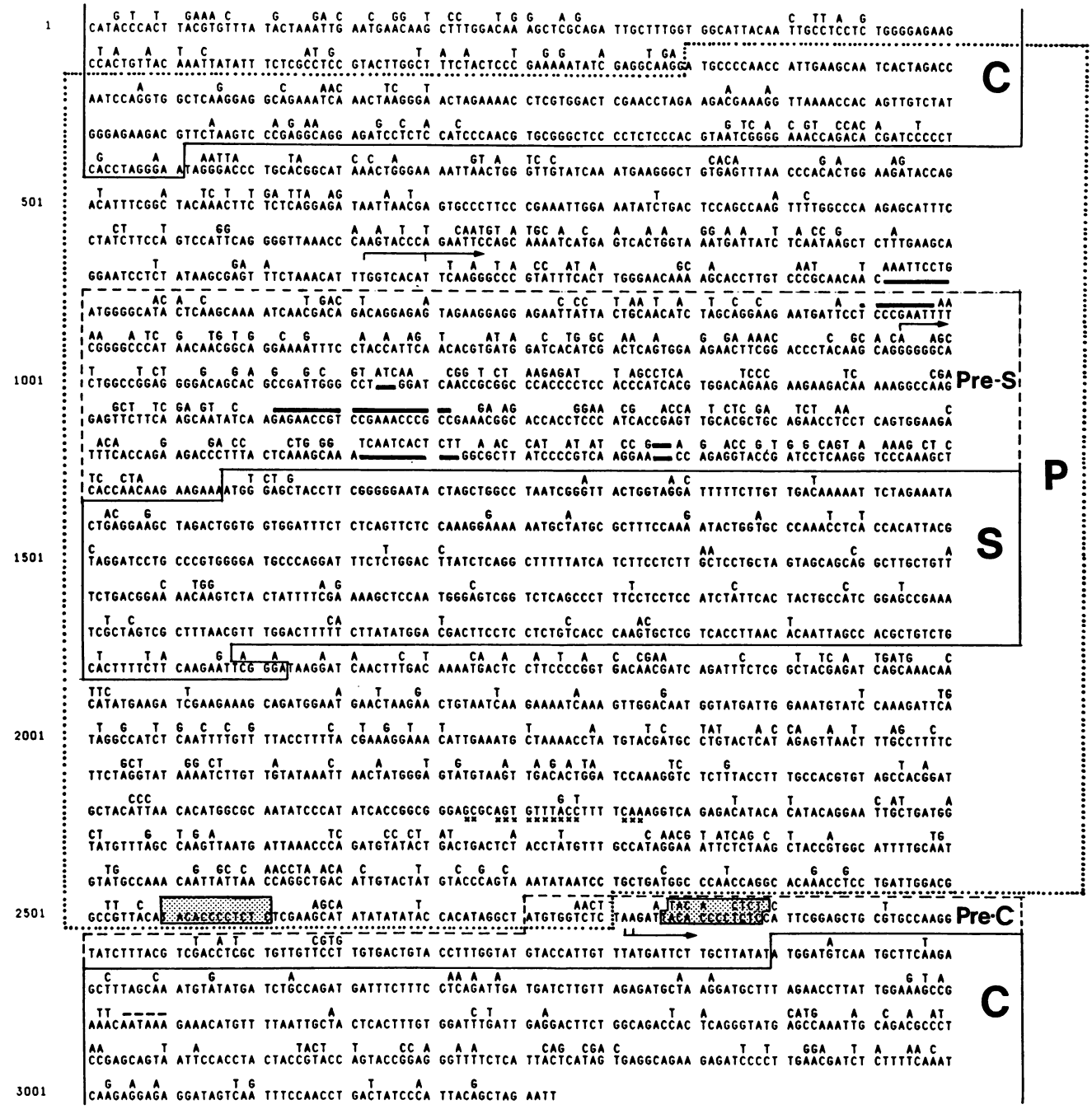

FIG. 4. Comparative DNA sequence analysis of the DHBV-3 genome (upper line; 35) and the HHBV-4 genome (lower line) linearized at the *Eco*RI site. Only nonconserved nucleotides are indicated for DHBV. For optimal alignment of both sequences, a few gaps (indicated by black bars) were introduced by using the GAP program of the UWGCG software (limit 1, 100; limit 2, 100). A gap of 3 bp introduced into both sequences at position 1266 has been made because of a 3-nucleotide insert at the corresponding position of two Chinese DHBV isolates which have been recently sequenced (Sprengel et al., unpublished). Known and predicted genes are boxed. The direct repeat sequences important for viral replication are shown in shaded boxes. A putative enhancer sequence is marked (×). Transcription start sites of DHBV (1) are indicated by arrows, and the consensus sequence for processing and polyadenylation is overlined.

ences between DHBV and HHBV, viral DNA was isolated from a pool of five viremic heron sera without previous repair of the gapped region. The virus pool contained at least two major sequence variants as revealed by restriction enzyme analysis of labeled virus DNA (data not shown). After digestion with restriction enzyme *Kpn*I, three DNA fragments, 1.2, 1.8, and 3.0 kb in size, were observed (data not shown), which suggests the existence of viral genomes with one and two *Kpn*I recognition sites. After insertion of the *Kpn*I fragments into phage vector M13mp18, three independent recombinants with a full-length genome (3.0 kb) and five subgenomic *Kpn*I inserts (1.8 and 1.2 kb) were obtained. As herons were not available for testing of the infectivity of the cloned viral DNA, one phage (mp18HHBV-

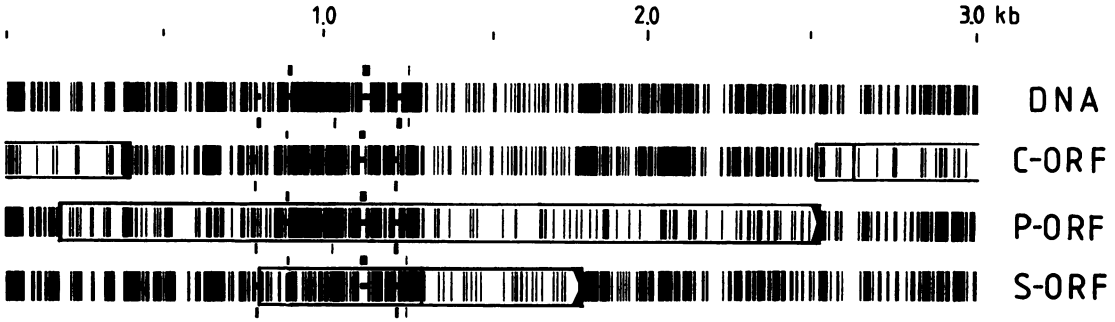

FIG. 5. Schematic presentation of a DNA (first row) and protein sequence alignment (rows 2, 3, and 4 represent reading frames A, B, and C) of the DHBV-3 and the HHBV-4 genome. Base exchanges and amino acid substitutions are indicated by vertical bars. Known and predicted genes are boxed. Small filled boxes above and below the lines indicate gaps introduced into the corresponding genomes for optimal alignments.

4) carrying a full-length genomic insert was selected arbitrarily for sequencing. Both strands of the HHBV DNA were completely sequenced (Fig. 4; for details, see Materials and Methods).

Comparative sequence analysis of HHBV and DHBV. The genome of HHBV is 3,027 bp in length, and compared with the DHBV-3 isolate (35), it shows a nucleotide sequence variability of 21.6%. To get both sequences aligned, gaps had to be introduced into the pre-S coding region (Fig. 4, 5, and 6) of both genomes. The computer-based alignment of both sequences reveals a conserved genome organization with three major ORFs, referred to as pre-C/C, pre-S/S, and pol (Fig. 4). The beginning and the endpoints of the pre-C/C- and pol-ORF were defined as described for DHBV (35). As initiation codon for the pre-S-ORF, the first ATG (position 801; Fig. 4) present on the analogous DHBV 2.1-kb pre-S mRNA transcript (1) was used. The S-ORF starts with an ATG at position 1317 (Fig. 4), which was shown to be used to initiate protein synthesis of the 17-kDa envelope protein of DHBV (29). No additional long ORFs were found in the HHBV genome (Fig. 5), and ORFs of the DNA minus strand were ignored. They are not conserved between the different DHBV isolates and HHBV, and for DHBV no transcripts derived from the DNA plus strand were identified. The comparison of DHBV and HHBV shows an asymmetric distribution of base exchanges which preferentially affect ORFs without coding capacity (Fig. 5). There is, however, one remarkable exception: the region coding for pre-S and overlapping with the middle part of the pol-ORF. The peptide sequence in the middle part of the pol-ORF is as variable as a peptide stretch of a noncoding frame, and the pre-S protein sequence is also strongly affected. Very long stretches without DNA sequence divergence are not present (Fig. 5).

Predicted proteins. As deduced from the nucleotide sequence, the nucleocapsid antigen and the 17-kDa envelope protein are the most conserved viral gene products, with the same sequence divergence of 16.5%. Similarily conserved (16.8%) is the carboxy-terminal part of the pol-ORF-derived protein (nucleotide position 1316 to 2561; Fig. 4) which carries amino acid motifs characteristic for reverse transcriptase (41, 42) and RNase H (Schödel et al., in press). The amino-terminal part of the *pol* gene product (nucleotide position 170 to 800; Fig. 4) is less conserved (divergence, 25.36%). The middle part of the *pol* sequences (nucleotide position 800 to 1316; Fig. 4) is highly variable, with only 33.2% amino acid identity, and appears not to encode enzymatic functions as speculated previously for DHBV (35, 37). A similar high sequence divergence (50.3%) is apparent in the pre-S part of the 36-kDa envelope protein (Fig. 6). This may be associated with the different host range of HHBV.

Transcription and replication signals. As described for DHBV (1), there is a TATA box sequence upstream of the C-mRNA/pregenome transcription initiation site (nucleotide position 2530 to 2540; Fig. 4 and 7) which probably represents part of the core gene promoter. Neither a TATA box upstream of the putative HHBV pre-S mRNA start site nor a putative S-promoter element with simian virus 40 late promoter sequence similarity, as suggested for human hepatitis B virus (2), was found upstream of the 5′ end of the DHBV S-mRNA of several DHBV isolates (Sprengel, unpublished data) or of the analogous region of the HHBV. An octamer sequence (TGTTTGCT; nucleotide position 2250 to 2258; Fig. 4) present in all hepadnaviruses sequenced so far and recently speculated to play a role in viral gene expression (7, 32, 33) is only partially conserved in the fully sequenced HHBV genome (Fig. 4). It is unlikely that this sequence divergence renders the HHBV defective since several HHBV isolates which were sequenced in this region exhibit the same mutation (data not shown). An AATAAA signal sequence for RNA processing and polyadenylation is strictly conserved for DHBV and HHBV, suggesting that the transcripts of both viruses are coterminal and are processed within the C gene.

Two direct repeat sequences (DR1 and DR2) which are important in initiating DHBV DNA plus- and minus-strand synthesis (14, 15, 31, 44) are strictly conserved between

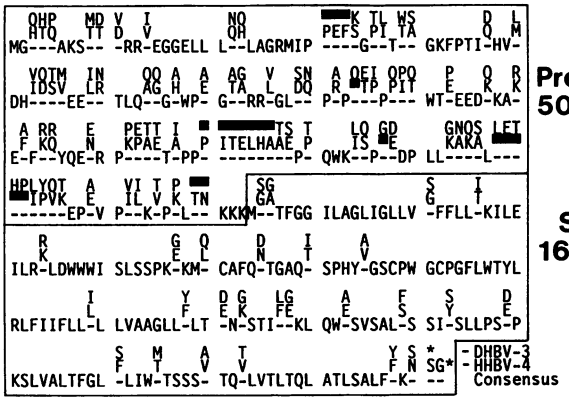

FIG. 6. Protein sequence alignment of the pre-S/S proteins of DHBV-3 and HHBV-4. Gaps introduced for optimal alignment (UWGCG program GAP; gap weight, 2.0; gap length weight, 0.3) are indicated by black bars.

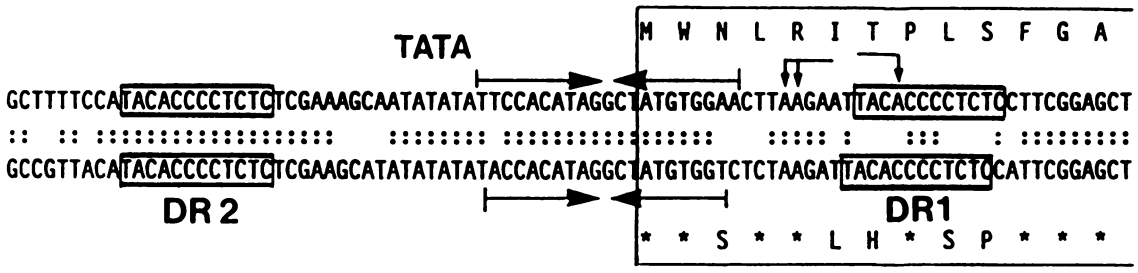

FIG. 7. Sequence comparison of the amino-terminal pre-C region of DHBV-3 (top) and HHBV-4 (bottom). The direct repeat sequences (DR1) of both viruses are boxed, and the inverted repeats are shown by arrows. Transcription initiation sites of the DHBV RNA pregenome upstream of DR1 and the site of DHBV DNA minus-strand initiation within DR1 are indicated by leftward and rightward arrows, respectively. The predicted amino-terminal protein sequence of the DHBV precore protein is shown at the upper line of the open box. Amino acid sequence substitutions found in HHBV versus DHBV are indicated at the bottom line of the open box.

DHBV and HHBV (Fig. 4). This suggests that HHBV replicates like DHBV and the primers for DNA plus- and minus-strand synthesis are similar for both viruses. However, the distance between both direct repeats is 1 nucleotide shorter (45 instead of 46 nucleotides) for HHBV, which affects predictably both the precore protein sequence and the structure of replicative intermediates. First, the mutations induce five amino acid exchanges at the amino terminus of the precore protein (Fig. 7). Second, if in analogy with DHBV the 5′ end of the HHBV pregenome is at position 2570 within DR1 (Fig. 7), and if initiation of DNA minus-strand synthesis takes place at an analogous position in DR1, the DNA minus strand of HHBV would have a 1-nucleotide-shorter terminal redundancy. Thus, the terminal redundancy which is probably essential for template switching in DNA plus-strand synthesis is predictably different in sequence between DHBV and HHBV. As in DHBV, there is an inverted repeat sequence between DR1 and DR2 (24) which may play a role in the viral life cycle. In HHBV, this repeat is conserved but 1 nucleotide shorter (9 instead of 10 nucleotides) and differs in sequence at the first nucleotide because of two complementary point mutations (Fig. 7). The same sequence motifs have been observed in five further HHBV isolates which were sequenced in this region (data not shown). Therefore, an altered precore protein sequence and sequence divergence in the origin of replication seem to be characteristic for the HHBV genome. Interestingly, the pre-C-ORF, which is dispensible for DHBV replication (4, 30), is present in all HHBV isolates sequenced, suggesting a selective advantage for viruses retaining pre-C-ORFs.

DISCUSSION

Screening of sera from several avian species for DHBV-related viruses revealed a new hepatitis B virus in 20 to 50% of grey herons tested in Germany. Several lines of evidence, including sequencing data, demonstrate a close relatedness of HHBV with DHBV. Since the sera were not from age-matched animals but were collected randomly in different areas of northern Germany, the high frequency of infection suggests that these animals are chronically infected. Conceivably, herons become infected congenitally, as do ducks (26, 43). In none of the livers of infected or uninfected herons were signs of primary liver carcinoma observed by visual inspection, nor was integrated viral DNA detected by Southern blot analysis (data not shown). Thus, as for DHBV, integration of HHBV DNA into the host chromosomes either is rare, if it occurs at all, or occurs only late in life. A gene, *X*, which is present in all mammalian hepadnaviruses and which may play a role in hepatocarcinogenesis is not present in DHBV or HHBV. The lack of an *X* gene may be a characteristic feature which distinguishes avian from mammalian hepadnaviruses, but further members of the avian hepadnaviruses have to be discovered and characterized to confirm this. On the basis of genome sequences, HHBV and DHBV are less closely related than the two rodent hepadnaviruses woodchuck hepatitis virus and ground squirrel hepatitis virus (78.5 versus 83.6% nucleotide identity). When the different hepadnavirus genomes were inspected, the most variable protein sequences were found in a short region of the pol frame and the pre-S region (F. Schödel, R. Sprengel, T. Weiner, D. Fernholz, R. Schneider, and H. Will, Adv. Viral Oncol., in press). The comparative sequence data presented here are in agreement with this finding and support the previous speculation that the corresponding pol region most likely is a tether between different enzymatic activities encoded in the pol-ORF (35). In contrast, the pre-S sequences are believed to play an important role in binding of the virus to the hepatocyte (25) and the highly restricted host range of hepadnaviruses could be determined by these sequences. Interestingly, despite a high pre-S sequence divergence of both rodent hepadnaviruses, GSHV is infectious in woodchucks (8), which renders the rodent viruses difficult to use for definition of sequences determining host-specific hepatocyte binding. When DHBV and HHBV pre-S sequences are compared, there is less sequence homology than observed with woodchuck and ground squirrel hepatitis viruses. Since HHBV appears not to be infectious for ducks, the pre-S sequence is the most obvious candidate that could determine host-specific hepatocyte binding. Strikingly, the longest continuous pre-S sequence identity of HHBV and DHBV does not exceed a 7-amino acid-long continuous peptide (Fig. 6). This suggests either an highly variable virus receptor protein or the involvement of only a very short peptide(s) or scattered amino acids of the pre-S protein in receptor binding. The availability of two closely related avian hepadnaviruses with different host ranges, and the possibility of testing recombinant viruses in vitro and in vivo in the most convenient hepadnavirus animal system (7, 34, 36), such as ducks, will allow us to identify sequences which determine host range specificity.

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