Identification and Analysis of a New Hepadnavirus in White Storks

Irmgard Pult,*[,1] Hans Jürgen Netter,*[,]†[,1] Michael Bruns,* Alex Prassolov,* Hüseyin Sirma,* Heinz Hohenberg,*
Shau-Feng Chang,*[,]‡ Kai Frölich,§ Oliver Krone,§ Erhard F. Kaleta,¶ and Hans Will*[,2]

*Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse 52, 20251 Hamburg, Germany;
†Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital, Brisbane, Queensland 4029, Australia; ‡Biomedical Engineering Center,
Industrial Technology Research Institute, Hsinchu, 310, Taiwan, Republic of China; §Institut für Zoo- und Wildtierforschung,
10252 Berlin, Germany; and ¶Institut für Geflügelkrankheiten der Justus-Liebig-Universität Giessen, 35392 Giessen, Germany

We identified, cloned, and functionally characterized a new avian hepadnavirus infecting storks (STHBV). STHBV has the largest DNA genome of all avian hepadnaviruses and, based on sequence and phylogenetic analysis, is most closely related to, but distinct from, heron hepatitis B virus (HHBV). Unique for STHBV among the other avian hepadnaviruses is a potential HNF1 binding site in the preS promoter. In common only with HHBV, STHBV has a myristylation signal on the S and not the preS protein, two C terminally located glycosylation sites on the precore/core proteins and lacks the phosphorylation site essential for the transcriptional transactivation activity of duck-HBV preS protein. The cloned STHBV genomes were competent in gene expression, replication, and viral particle secretion. STHBV infected primary duck hepatocytes very inefficiently suggesting a restricted host range, similar to other hepadnaviruses. This discovery of stork infections unravels novel evolutionary aspects of hepadnaviruses and provides new opportunities for hepadnavirus research. © 2001 Academic Press

Key Words: hepatitis; avian virus; liver tropism; chronic viral infection; HBV; hepadnaviridae; viral epidemiology; hepadnavirus; hepatopathogenesis; hepatocytes.

INTRODUCTION

Hepatitis B viruses (HBVs) are enveloped, mainly hepatotropic DNA viruses and belong to the Hepadnaviridae family, which is divided into two genera, *Orthohepadnaviruses* found in mammals and *Avihepadnaviruses* found in birds. The replication of all members of this family involves reverse transcription of an RNA intermediate. Therefore, they are pararetroviruses and classified together with members of the *Retroviridae* and the *Caulimoviridae* into the genome type of reverse-transcribing viruses (Mayo and Pringle, 1998).

In addition to humans (HBV), naturally occurring *orthohepadnaviruses* have been identified so far in rodents (woodchucks (WHV), ground and arctic squirrels (GSHV and AGSV)) and in old as well as in new world primates (woolly monkeys, orangutans, gorillas, gibbons) (see review, Schäfer *et al.,* 1998). Naturally occurring *avihepadnaviruses* have been described first to occur in Pekin ducks (*Anas domesticus*) (DHBV) from China and U.S. (Zhou, 1980; Mason *et al.,* 1980), and subsequently in various other duck strains (for review, see Schödel *et al.,*

1991; Triyatni *et al.,* 2001 and references therein). Viruses related to DHBV have also been found in three other avian species, namely in gray herons (*Ardea cinerea*) (HHBV), in a Ross' goose (*Anser rossii*) (RGHBV), and in snow geese (*Anser caerulescens caerulescens*) (Chang *et al.,* 1999 and references therein).

Hepadnavirus genomes are small (3.0–3.3 kb), partially double-stranded, open circular DNAs containing extensively overlapping genes. These encode both structural (envelope proteins, designated S and preS, as well as the nucleocapsid proteins) and nonstructural proteins (precore and its proteolytically processed and secreted form, designated e-antigen, as well as the viral polymerase P). A regulatory protein, designated X, is known to be expressed by mammalian viruses and from an open reading frame without a conventional start codon also from duck hepatitis B virus (Chang *et al.,* 2001). An X-like ORF has recently been detected also in genomes of heron and snow goose hepatitis B viruses (HHBV and SGHBV), and of a Ross' goose derived virus genome (Netter *et al.,* 1997), but it is not known whether the corresponding proteins are expressed. For unknown reasons, the X proteins of all avian hepadnaviruses, as deduced from the DNA sequence information, are shorter in length and differ drastically in primary sequence from those of mammalian viruses. The X proteins of mammalian hepadnaviruses are believed to play a role in hepatocarcinogenesis, whereas the X proteins of

---

[1] These authors contributed equally to the work.

[2] To whom correspondence and reprint requests should be addressed at Hans Will, Heinrich-Pette-Institut, Martinistrasse 52, 20251 Hamburg, Germany. Fax: ++49-40-48051222 or 1. E-mail: will@hpi.uni-hamburg.de.

AP

DHBV obviously does not because, unlike in mammals chronically infected with hepadnaviruses, there is no correlation between the development of hepatocellular carcinoma and chronic DHBV infection (Cova *et al.,* 1993). The failure to detect HCC in avian species may be linked to the timing and mode of transmission of these viruses since they are usually transmitted vertically by *in ovo* transmission, resulting in congenital infection with immune tolerance and an absence of liver disease. Most ducks that have been assessed for the development of HCC have been infected by *in ovo* transmission. In contrast, infection with the mammalian hepadnaviruses always occurs at the time of birth or later in development, leading to transient or chronic outcomes of infection with greater degrees of immune-mediated liver damage that are believed to play a role in the development of hepatocellular carcinoma (HCC). Interestingly, it has been reported that experimental infection of 4-month-old ducks with DHBV also leads to the development of transient and chronic infections with mild and marked liver disease (Jilbert *et al.,* 1998) and these animals have a greater chance of developing HCC. The ability of avian species to develop HCC may also be affected by their shorter life span.

Several promoters, one (DHBV) or two (mammalian hepadnaviruses) transcriptional enhancers, as well as many transcription-factor binding sites, are known to regulate hepadnavirus gene transcription (for review see Yen, 1993). The longer-than-genome sized mRNAs and the subgenomic mRNAs synthesized from the viral promoters have different 5′ ends but are all colinear at the 3′ end due to a unique processing/polyadenylation signal. While all mammalian HBVs are to various degrees pathogenic for their hosts, infection of ducks with DHBV is not or less harmful to the animal, except when mutated (Lenhoff *et al.,* 1999). Patients chronically infected with HBV are at high risk of developing cirrhosis and hepatocellular carcinoma. The lack of an association of HCC development with chronic DHBV infection is possibly due to structural and functional differences in the X proteins of DHBV and mammalian hepadnaviruses. Whether any of the other avian hepadnaviruses are pathogenic is not known.

All identified hepadnaviruses have a very restricted host range and tissue tropism. For instance, DHBV isolated from Pekin ducks infects some species of ducks and geese but can neither be transmitted to Muscovy ducks (*Cairina moschata*), a domesticated duck not descending from mallards (common ancestor of domestic ducks), nor to chickens (*Gallus gallus domesticus*) (Marion *et al.,* 1987). HHBV does not infect ducks (Sprengel *et al.,* 1988) and infects only very inefficiently primary duck hepatocytes (Ishikawa and Ganem, 1995). However, substitution of a small region of the HHBV-specific preS domain by the corresponding DHBV protein renders this hybrid virus infectious for ducks and primary duck

hepatocytes (Ishikawa and Ganem, 1995). This provided the first evidence that a region of the preS protein is a host range determinant of hepadnaviruses. Other studies on HHBV revealed new control elements involved in viral replication (Mueller-Hill and Loeb*,* 1996) and novel aspects in virus receptor recognition (Urban *et al.,* 1998). Taken together, these data demonstrate exemplarily the value of such comparative studies between different hepadnaviruses for the understanding of hepadnavirus biology. The elucidation of evolutionary relationships between different hepadnaviruses, of mechanisms of host adaptation, receptor recognition, and pathogenesis, are good reasons for the interest in new members of hepadnaviruses that may occur in additional species of birds. Moreover, the DHBV/duck system is well established and the most convenient animal model for hepadnavirus research. Furthermore, it allows both *in vitro* and *in vivo* infection experiments with novel members of avian hepadnaviruses and chimeric viruses. Last but not least, identification of viruses in endangered animals may provide information which may soon be inevitably lost forever.

In this study, we report the identification and structural, evolutionary, and functional characterization of a novel member of the avian hepadnavirus family which endemically occurs in white storks (*Ciconia ciconia*), a threatened species (Pastro *et al.,* 2001).

RESULTS AND DISCUSSION

Detection of HHBV-related large envelope proteins in stork sera

Based on the close evolutionary relationship between herons and storks (Slikas, 1997), we assumed that hepadnaviruses in both animal species are also closely related. Therefore, we used an antiserum raised against the entire HHBV preS-protein domain to search for hepatitis B viruses in sera of several members of the stork family (see Materials and Methods). With this antiserum a total of 17 sera derived from white storks were examined for the presence of HHBV-related envelope proteins by enzyme-linked immunosorbent assay (ELISA) and/or immunoblotting. Fourteen of 17 white stork derived sera scored positive for HHBV preS antigen in the ELISA (data not shown). On the immunoblot an immunoreactive band comigrating with the major HHBV preS protein of 36 kDa was observed with the ELISA-positive sera. No signal was seen with the ELISA-negative samples (for a representative blot, see Fig. 1). Interestingly, none of the 24 sera of 15 other members of the stork family analyzed (see Materials and Methods for details) was positive by immunoblotting. Notably, the heron sera derived HHBV preS-specific signals were much stronger on the immunoblot than those seen with an equivalent amount of stork sera (data not shown). This may be due to a low immunological cross reactivity of HHBV preS antiserum
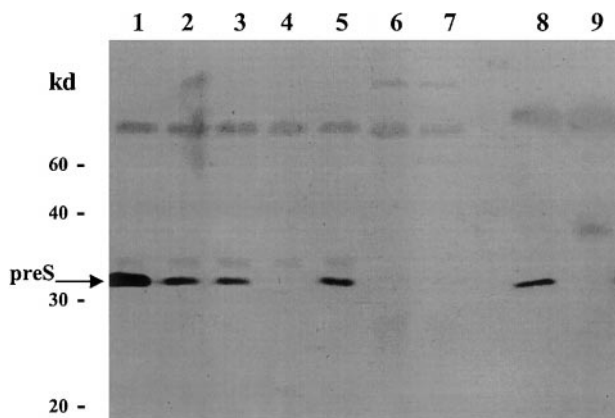
FIG. 1. A representative immunoblot for detection of HHBV-related large preS-envelope protein in sera of white storks (lanes 1 to 5) and black storks (lanes 6 and 7) with anti-HHBV preS antibodies. As a control, a HHBV-positive and HHBV-negative heron serum (lanes 8 and 9) were loaded.

with the corresponding protein in the stork sera and/or to a lower concentration of preS proteins expressed from a HHBV-related virus in storks (designated STHBV). The specificity of the signal on the immunoblot for an STHBV-derived virus protein is supported by the fact that a corresponding signal was neither detected in a DHBV-negative duck serum nor in HHBV-negative heron sera. When the stork serum proteins were analyzed by immunoblotting with a polyclonal antiserum raised against the DHBV preS domain, similar data were obtained as with the HHBV preS antiserum, except for even weaker but clearly positive signals at the position of a 36-kDa protein (data not shown). Taken together, the immunoblot and ELISA data suggest that 80% of the white storks tested are infected with an HHBV-related virus. Since all the STHBV-positive storks were located in two German zoos, it is currently questioned whether infection was acquired in the wild or in captivity. However, it is likely that the virus was originally present in wild stork populations that were taken into captivity, resulting in vertical transmission of the infection to their offspring. In any case, STHBV represents a new member of the avian hepadnavirus family.

Detection of viral particles in stork sera by electron microscopy

To obtain further independent evidence for the presence of HHBV-related stork virus, we searched by electron microscopy for viral particles in two stork sera. One serum was STHBV positive as assayed by ELISA and immunoblotting, the second serum was negative in both assays. This was achieved by selective attachment of the viral particles onto an HHBV preS-antibody-coated carrier, fixation, and ultrathin sectioning. Representative photomicrographs thus obtained (Figs. 2A–2D) showed viral particles very similar in morphology (mostly round,

bilayer lipid membrane, to some degree pleomorphic, and variable internal granular structure) and diameter (40–60 nm) to those known for HHBV and DHBV. These particles were observed only in the positive but not in the negative serum (data not shown). This provides further evidence that hepadnaviruses are present in sera of white storks.

PCR amplification and cloning of STHBV DNA genomes

Serum samples from the 17 white stork sera were analyzed for the presence of hepadnaviral DNA with a PCR-based full-length genome amplification procedure previously used successfully to screen heron sera for HHBV (Netter *et al.,* 1997). Using primers homologous to the so-called nick region of the HHBV genome (P1 and P2; Netter *et al.,* 1997) and diluted stork sera, amplification products about 3 kb in size were obtained from 14 samples, previously scored positive for STHBV in ELISA and/or immunoblotting. Additional PCR fragments smaller than 3 kb are likely to represent viral genomes derived from spliced viral RNA because they did not disappear when using higher stringency PCR conditions. Similar discrimination of positive and negative samples in the ELISA, immunoblot, and PCR assays suggest that these methods have similar levels of sensitivity, or that readily detectable levels of viral antigens and DNA are present in all samples that tested positive for HHBV-related viral genomes. After purification of the 3-kb amplification products derived from one of the positive sera, they were directly sequenced by using
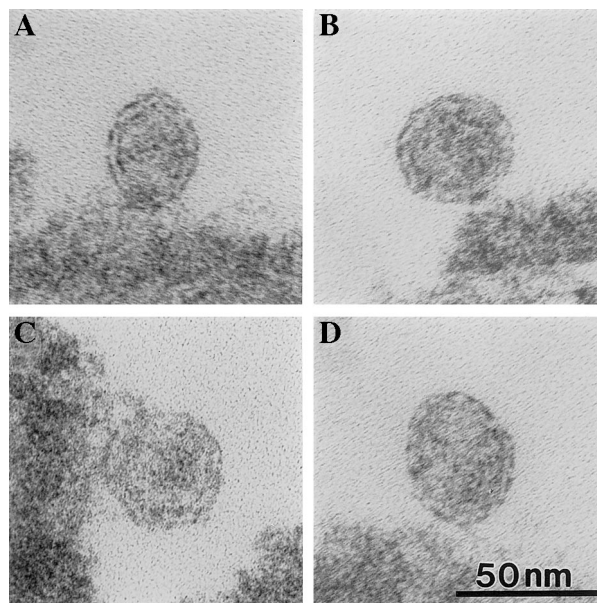

FIG. 2. Viral particles in stork sera as revealed by electron microscopy. Four representative photographs (A to D) demonstrate variable diameters of the particles ranging from 40 to 60 nm and different staining intensities, similarly as known for DHBV and observed for HHBV (data not shown).
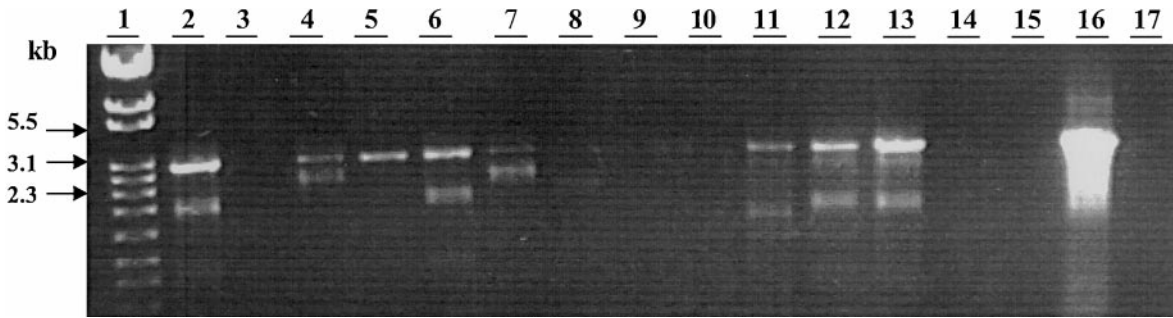
FIG. 3. Agarose gel analysis of PCR amplification products derived from stork sera obtained with STHBV primer pair PS1/PS2. The samples were from white storks (lanes 2–13) and black storks (lanes 14–15). The PCR product obtained with 1 ng of overlength STHBV genome DNA (lane 16, positive control) and with no DNA (lane 17, negative control).

various sequencing primers homologous to known avian hepadnavirus genomes. Full-length genome amplification was repeated with STHBV-specific primers and confirmed that 14 of 17 white storks but none of the sera from the other stork species were positive (representative examples shown in Fig. 3). The amplification products sequenced directly were also cloned, and four individual clones were sequenced (STHBV 1, 7, 16, and 21). To examine whether mutations were introduced artificially into the STHBV region to which the HHBV primers (P1 and P2) hybridized, we performed an additional PCR with primers hybridizing to known STHBV sequences up- and downstream of the nick region. The obtained PCR fragment (STON) was directly sequenced as well as after cloning (plasmid pSTON).

Sequence heterogeneity of the STHBV genomes

Direct sequencing of the full-length amplification product revealed heterogeneity only at very few positions (data not shown) and this heterogeneity was in close agreement with the variability at the same positions in the DNA sequences derived from the four cloned full-length genomes (see below). The sequence of one of the cloned genomes (STHBV 21) is representatively shown in Fig. 4, and those of all four cloned genomes are deposited in the EMBL GenBank (Accession Nos.: AJ251934, AJ251935, AJ251936, and AJ251937). Sequencing of fragment STON revealed two nucleotide substitutions when compared to the full-length amplification primer binding site P2 (T2532C and C2533T), and this was taken into account and corrected accordingly in the full-length genome sequence shown in Fig. 4 as well as in the genome sequences submitted to GenBank. DNA sequence alignment of the four cloned STHBV genomes revealed differences in only 19 positions (for details see Table 1), indicating little sequence heterogeneity. This is consistent with similar low-sequence heterogeneity between cloned DHBV and SGHBV genomes obtained from duck (Schödel et al., 1991) and snow goose sera (Chang et al., 1999) and from direct sequencing of HHBV genomes amplified from different heron sera (Netter et al., 1997). Ten of the 19 nucleotide changes are silent for the pre-

dicted proteins; four nucleotide changes result in amino acid changes in the preS domain of the large envelope protein, and three nucleotide changes result in the overlapping P protein domain (Table 2). One of the 19 nucleotide mutations was observed only in cloned genome STHBV 16 and changes the translation initiation codon of the small envelope protein to V (Table 1). Therefore, this genome is obviously defective in viral particle formation (for functional analysis of STHBV16, see below) and depends on complementation by an S-protein expression competent virus to be secreted in the form of viral particles. The defective virus is likely to represent a minor viral subpopulation in the serum from which it was cloned because no nucleotide variability was evident at this position by direct sequencing of the amplification products (data not shown). Alternatively, this mutation may have been introduced artificially by PCR. We consider this less likely because the PCR conditions used minimize PCR-derived mutations (Günther et al., 1998) and because similar naturally occurring mutants appear to exist also for other hepadnaviruses (Zöllner et al., 2001). Two of the 19 nucleotide substitutions predict conservative amino acid changes in the C-terminal end of the P protein. None of the nucleotide changes is located in regions that correspond to sequence elements known to be important or essential for replication and transcription of DHBV.

DNA sequence comparison and phylogenetic relationship of STHBV with known avian hepadnaviral genomes

The STHBV genome was compared to the most closely matching prototypes of the four known avian HBV genomes, namely DHBV, HHBV, RGHBV, and SGHBV. All cloned STHBV genomes are 3033 bp long and thus represent the longest avian HBV genomes known so far (Fig. 4). This is due to insertions in the preS region that is known to be one of the most variable region of HBV genomes possibly related to its functions in host and cell tropism as well as in receptor recognition. Consistent with the close relationship of storks and herons, the

STHBV CATACCCATCTTAGAGTATACACTAAATTAAATGAACAAGCATTGGATAA   GGCTAGGAGATTGCTTTGGTGGCATTATAATTGTTTGCTCTGGGGTGAAA   GTAACGTCACTAATTATATTTCTCGGCTTCGTACTTGGTTATCCACTCCA   150
HHBV   .....CT.AC.T..T..T.......G........T...C.....   A...C.C...........................C....CC.C.......A..G   CC.CT..T.A......................C.......C.T.T....C   150
RGHBV ...G.T..C..G.A..C...T0.A...A.....GG..T.T.A....G   ....................................C....AT.G....C.GC   C......T..C..C.......GA.AT.AA.A.........A....T   150
SGHBV ...G.A....A.A..CT..TG.A...A.T..C..GG..T.T.....TTCC   ......AC................C.C....A..G...A..G   C..CT..A..A..C.......C..C.C.......A....A...T   150
DHBV   ...G.A..C.A.A..C...TG.A...A.T..C..GG..T.......CG   .........................C....A..G...A..G   ....C.C...........C....G..A....T   150

STHBV GAAAAAATATCGTGGAAAAGGTGCCCCAACCATTGAAGCAATCACTAGACC   AATCCAAGTGGCTCAAGGCAGCAGAAATCAAACTAAGGGAGTTAGAAAAC   CTCGTGGACTTGAGCCTAGAAGAAGAAAAGTTAAAACCACAGTTGTCTAT   300
HHBV   .............A..C..G..   ......G.................................AC......... ..........C..A...........C....G..................... 300
RGHBV ...........CA.A........   ......G..G..AG........A.G...C..............T   .............C..A.........G..................A..... 300
SGHBV ...G...A..CA.A..TCG..........G   ......G..AG.....AAC.T...C..CTAC.....   ...........C..A..................A..............   300
DHBV   ......G...CA.A..TCG..   ......G..AG.....AAC.T..TC...AC...   ...........C..A..................T..............   300

                                                                                            preC/C *
STHBV GGGGAGAAGACGTTCAAAGTCCCGAGGGAGGAGATCCTCTCCATCCCAACG TGGCGGGCTCCCCTATCCCACGTAGTCGAGAAAACCAAAGTAGATCCTCCT CGCGTAGGGAATAGGGACCCCGCACGACATAAATTGGGAAAATTATCTGG 450

STHBV GTTATATCAAATGAAGGGATGTGAGTTTAATCCTGACTGGAAAATACCTG ATATTTCGAGAACTCATATCAATACTGAAATTGTAAATGAGTGCCCTTCA CGAAATTGGAAATATCTGACTCCAGCCAAATTTTGGCCCAAGAGCATTTC 600

                                HNF1   TATA box   •   ••
STHBV CTATTTTCCAGTCCATGCAGGAGTTAAACCCAAATACCCAGAATTCCAGA ATTGCCATGAGTCATTGGTTAATGATTACCTAAGCAAGCTCTTTGAAGCA GGAATCCTGTATAAGCGTGTGTCTAAACATTTGGTCACTTTTAAAGGGCC 750

                        preS
STHBV TAGTTTTTCTTGGGAACAACCATACCTTGTCCCGCAACATC--------- ATGGGGCATACACAAGCAAAATCCACAACAGACAGGAGAGTCGAAGGAGG AGAATTATTACTGCAACAATTAGCAGGAAGAAGAATGATCCCCAGAGAATTTC 891

                                HNF3
STHBV AGGGGGCTATTACAACGGCAGGGAAACTGCCTTCAATAGATCATGTTATG DATCATATAGACTCAGTGAAAGAACTGAGAACTATACAAAATCAGGGTCA TTGGCCCGAGGGCACAGGGAGGGAGGAGACTAGGATTAGACAAACCAACAACTA 1041

STHBV CTCCTCCTCCAGCAATCACGTGGACCAGAGAAGAAGACGAGAGAGCAAAA CAATTCTTCAAACAATATCAGGAGAACAGACCCAAACAACCAAACACGGC TCCACCACCACTACCAGAACTACATGCAGCAGATCCTCCCCAGTGGAAAA 1191

                                                            s
STHBV TAAAGCCTGGGGACCCATTGCTGCAGGCCCAATCTCTAATCCCAAAGAAG GATCCAGACGTTCCCATTCTGAAGTTGCCGCAACTTCCAAAGTACAAGAA GAAGATGGGAGCTTCCTTCGGGGGGAATACTAGCTGGCCTAATCGGATTAC 1341

STHBV TGGTAGGATTTTTCTTGTTGACAAAAATTCTAGAAAATACTACGGAAGCTA GATTGGTGGTGGATTTCTCTCAGTTCTCCAAAGGAAAAAATGCTATGCGC TTTCCAAAGTACTGGTCACCAAACCTCACCGCATTACGTAGGATCGTGCC 1491

STHBV CTTGGGGATGCCCAGGATTTCCTCTGGACTTATCTCAGGCTTTCTATCATC FTTCTCCTTAATCCTGCTAGTAGCAGCAGGCTTGCTGTTTCTGACGGAAAA CAAGTCTATTATTTTCGGAAAAGCTCCAATGGGAGTCGGTCTCAGCCCTTT 1641

STHBV TCTCCTCCATCTATTTACTACTGCCATCGGAGCCGAAATCTCTCGTCGCT TTAACGTTGGACTTTTTCTTATATGGACGACTTCCTCCTCTGTCACCCA AGTGCTCGTCACCTTAACTCAATTAGCCACGCTGTCTGCACTTTTCTTCA 1791

       preS/S *
STHBV AGAATTCGGCATAAGAATAAACTTTGACAAAATGACTCCTTCACCTGTGA CTACGATTAGATTTCTGGGATATGAAATCTCCAATCAACATCTCAAGATT GAAGAAAGCAGATGGAATGAACTGAGACAAGTTATCAAGAAAATTAAAGT 1941

STHBV GGGACAATGGTATGACTGGAAATGTATTCAAAGATTAATTGGACATTTGA ATTTTGTTTTACCTTTTACTAAAGGAAATGTTGAAATGCTGAAACCTATG TATGATGCTTGTACTCACAGGGTTAACTTTGCTTTTTCTTCTAGGTACAA 2091

                                                           C/EBP   HNF3
STHBV AATACTCTTGTATAAATTAACTATGGGGGTTTGTAAATTAACTTTGAAAC CAAAGTCCAGTTTACCTTTGCCACGTGTAGCAACTGATGCTACCTTAACA CATGGCGCAATATCCCATATCACCGGCGGGAGCGCAGTGTTTACTTTTTC 2241

                        HNF1   SGHBV X
STHBV AAAGGTCAGGGACATTCATGTACAGGAATTGTTAATGGTTTGCCTAGCTA GATTAATGATTAAACCACGCTGTATCCTCACTGATTCTACCTATGTATGC CATAGGAAGTTCAACAAGCTACCATGGCATTTTGCAATGTATGCCAAGCA 2391

           X   RGHBV X   DR2   preC   P *
STHBV ATTATTAACCAGGCTGACATTATACTATGTGCCCAAGTAAATATAATCCTG CTGACGGCCCAACCAGGCATAAACCACCTGATTGGACAGCTGTTACACAAC ACCCCTCCTCTGAGAAACATATATATGTACCACATAGGCTATGTGGTCCTTA 2541

       DR1   E   ••
STHBV AG-AATAACACCCCTCTGCAATCGGAGCTGCGTGCCAAGGTATCTTTACGT TGCGTATCTCGCTGTTGTTGCTTGTGACTGTACCTTTGGTATGTACCATTGTT TATGAATACTTGCTTATATATATGGGATGTTAATGCTTCAAGAGCTCTTGCAAA 2690

                                                                                             PAS
STHBV TGTATACGATCTGCCAGATGATTTCTTTCCACAAATTGATGATTTAGTTC GCGGATGCAAAAGATGCTTTGGAACCTTATTGGAAAGCTGAAACAATAAAG AAAACATGTTTGATCGCAACTCACTTTGTGGGACTTGATTGAGGGATTTCTG 2840

STHBV GCAAAACTACACAGGGTATGAGCCAAATTGCAGATGCACTGAGAGCTGTAA TTTCCACCTACTACAACTCCAGTGCCTGACGGATATTTAATTTCTCACAAC GAAGCTCAAGAACTTCCACTAAATGATTTATTTGTAAATCAGGAAGAACG 2990

STHBV GATAGTAAATTTCCAACCTGACTATCCCATTACAGCTAGAATT   3033
HHBV   .....C..........................   3027
RGHBV AC..ACT..C..T......A..T.....AG...C..C..... 3018
SGHBV .....T.G..T.............A.......A...G..... 3024
DHBV   A.....C.G.................C..A.......A..... 3027

TABLE 1

Sequence Variation of Cloned STHBV Genomes

STHBV clone	Nucleotide position	Amino acid changes in different ORFs		
		preS	S	P
21	C48T			
1	A454G			
7	T472C			
1	A535T			
21	G977A	M62I		A270T
1	A1035G	T82A		N289S
1	A1037C	T82A		N290H
7	T1261C	L157P		
16	A1296G		M1V	D376G
7	T1596C		S101P	V476A
16	T2147C			S660P
21	C2186T			
21	G2293A			
7, 16	C2311A			
21	G2362A			
21	A2483G			I772V
7	A2763G			
1	T2847C			
1	C2937T			

Note. The amino acid change M to V in position 1 of the S-ORF should prevent translation initiation of the small S-protein.

STHBV genomes are most closely related in sequence and gene organization to the HHBV genomes (Netter *et al.,* 1997). The comparison of all avian hepadnavirus genomes available in GenBank (see Materials and Methods) showed that the DNA sequence divergence between STHBV and HHBV ranges from 14.2 to 14.5%. The differences between STHBV and DHBV range from 22.2 to 23.6% and between STHBV and SGHBV from 22.6 to 22.7%, and between STHBV and RGHBV the difference is 22.8%.

The likely evolutionary relationship between STHBV and the remaining avian hepadnaviruses was further examined by phylogenetic analysis of all DNA sequences deposited in GenBank by using the SplitsTree program. The tree-like network thus obtained (Fig. 5) indicates that all avian HBV genomes probably originate from a common ancestor. Moreover, the tree implies that STHBV is a distinct member of the family of avian HBVs and confirms the close relationship between STHBV and HHBV. DHBV subgroups correlate with the geographical

origin of virus-infected animals (Chinese–Australian origin vs Indo-European–U.S.–Canadian origin).

Due to the relatively high homology of HHBV with STHBV, the degree of sequence conservation and differences in regulatory sequences previously described for HHBV and other avian hepadnaviruses (Netter *et al.,* 1997) applies similarly to STHBV. Briefly, totally or similarly conserved are the direct repeated sequences DR1 and DR2, and the unique site for polyadenylation (PAS). The putative RNA encapsidation signal epsilon of STHBV is very similar to that of HHBV and, surprisingly, also to that of RGHBV, but quite different from that of DHBV (Hu and Seeger, 1997). A second region important for encapsidation of the DHBV pregenomic RNA (not present or essential in mammalian hepadnaviruses) mapped to nucleotides 551 to 719 (Calvert and Summers, 1994) is conserved between different STHBV isolates (Table 1) but varies considerably among different avian HBV genomes (data not shown). A splice donor and acceptor sequence, which give rise to a spliced transcript in DHBV and are reported to be essential for virus replication *in vivo* (Obert *et al.,* 1996), are also conserved in STHBV (Fig. 4).

Three transcription factors binding sites (C/EBP, HNF1, and HNF3) in the DHBV enhancer region (Lilienbaum *et al.,* 1993, Liu *et al.,* 1994) as well as viral promoter sequence motifs are all well conserved in STHBV (Fig. 4). However, the sequence of the binding site for HNF3, known to be important for the activity of the TATA-less S gene promoter of DHBV (Welsheimer and Newbold, 1996), is not conserved in STHBV and HHBV.

A perfect consensus sequence element for HNF1, located in appropriate distance to the TATA-box of the preS promoter, is unique for STHBV and less likely to be functional in HHBV due to two nucleotide substitutions. Interestingly, the preS1 promoter of human HBV has also a HNF1 site (Raney *et al.,* 1994). It remains to be shown whether the HNF1 in STHBV has a similar function for preS promoter activity as in HBV.

Comparative protein sequence analysis of all avian hepadnaviruses

At the protein level, the closest homology was observed for the STHBV- and HHBV-specific proteins. Considerable less homology was found for the remaining types of avian HBVs (Table 2). Also at the protein level, RGHBV is the next closest virus to STHBV, followed by

FIG. 4. Nucleotide sequence alignment of the cloned STHBV 21 genome with that of four known avian HBV genomes (HHBV (hbhcg.em_vi), RGHBV (hbdgenm.em_vi), SGHBV (sg15.gcg), and DHBV (hbds31cg.em_vi). Dots and dashes represent identical and deleted nucleotides, respectively. Deletions are artificially introduced into different genomes for optimal alignment. Translation initiation codons are indicated by arrows, and termination codons are indicated by asterisks. Additionally, both features are highlighted by gray rectangles. Transcription-factor binding sites, the TATA box of the preS promoter, and regulatory sequence elements are in bright boxes or in dark shaded ones. Double dots in bold above the STHBV sequence indicate the positions of a splice acceptor and splice donor sites, respectively. The corresponding branch-point sequence motif is indicated by a single dark dot.

TABLE 2

Sequence Differences between STHBV and Other Avian Hepadnavirus Genes

STHBV	% Homology				
	preC/C	PreS	S	Pol	X-like
HHBV	93.8–94.4	78.0–78.6	93.5–95.2	81.1–81.6	85.3–88.0
DHBV	80.6–81.9	47.1–50.6	80.8–83.8	68.5–70.1	69.7–74.3
RGHBV	80.7	52	84.4	69.5	83.1
SGHBV	80.6–80.9	49.7–50.9	82.0–83.2	70.7–70.8	69.9–71.2

SGHBV and DHBV. This is most obvious when the sequences of the most variable viral proteins (preS and X) are considered (Table 2). Amino acid substitutions in viral proteins that are unique for STHBV compared to all other known avian HBVs are predominantly found in the preS region and the overlapping spacer domain of the P protein (Figs. 6A–6D).

The preS/S gene encodes the large and small envelope proteins, S and preS. Sequence comparison revealed more amino acids unique for STHBV in the preS domain and in the overlapping spacer domain of the P protein than in any of the other viral proteins (Fig. 6A). Most of these amino acid changes are nonconservative. About half of them localize in the host range determining region and in the minimal binding domains for gp180/gp120, two cellular proteins proposed to represent components of the DHBV receptor (Urban et al., 1998, 2001; Tong et al., 1999; Li et al., 1999; and references therein). It is therefore conceivable that some or all of the amino acids in these positions contribute to host-specific receptor recognition and intracellular protein/protein interactions. Similar to HHBV, but unlike DHBV, SGHBV, and RGHBV, there is no PX[S/T]P motif in the preS domain in

STHBV which has been shown for DHBV to be phosphorylated and essential in its phosphorylated form for the transactivation activity of the preS protein (Borel et al., 1998, Rothmann et al., 1998). It is conceivable that the PXDP motif, present in the STHBV, HHBV, and also RGHBV preS proteins, mimics constitutive phosphorylation (Fig. 6A). This speculation is based on the observation that replacement of serine (S) by the negatively charged amino acid aspartate (D) in the PX[S/T]P motif of DHBV (S118D) does not interfere with the transactivation activity of the DHBV preS protein (Rothmann et al., 1998).

Unlike the preS domain, the coding region of the small envelope protein is highly conserved in all avian hepadnaviruses including STHBV that shows only a few conserved amino acid changes. However, it is interesting to note that both STHBV and HHBV S proteins differ from those of all other avian hepadnaviruses by a C-terminal two amino acid extension (Fig. 6A). A further common feature of HHBV and STHBV, different from all other hepadnaviruses (Macrae et al., 1991), is that the S protein instead of the preS protein has a putative myristylation site. Both features may play a role in host-specific virus/cell interaction only in species of the ciconiiformes order, to which both herons as well as storks belong.

The preC/C proteins of all avian HBVs including STHBV are all rather highly conserved (Fig. 6B). The highest sequence variability is found in the so-called large avian insertion domain, which is not present in mammalian hepadnaviral preC/C proteins (Bringas, 1997). The hydrophobic heptad repeat (hhr) domain, known to be important for assembly and dimerization of nucleocapsids (Yu et al., 1996), is highly conserved among different avian HBVs (Fig. 6B). In common with HHBV but unlike DHBVs, STHBV has two C-terminally located potential glycosylation sites; one of them is also present in RGHBV (Fig. 6B). Up to four putative phosphorylation sites (SPXX motifs), known to be important for modulation of different functions of the DHBV core protein (Yu and Summers, 1994), are located in the C-terminal domain of the C protein of all avian HBVs except STHBV, which lacks the most C-terminally located one (Fig. 6B). A recently identified threonine/serine kinase motif common to all hepadnaviral core proteins (Barrasa
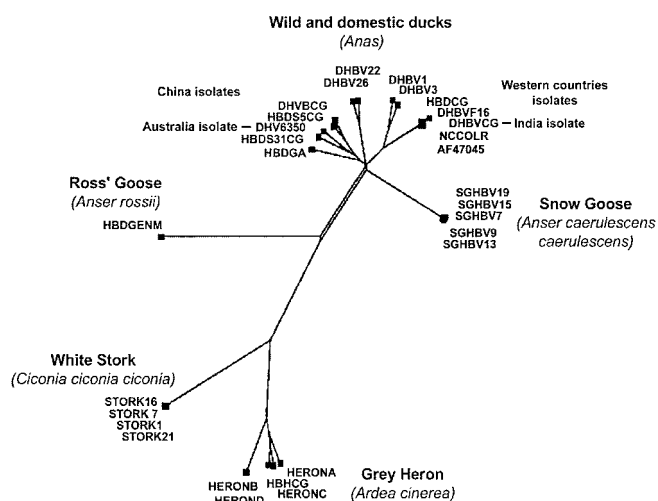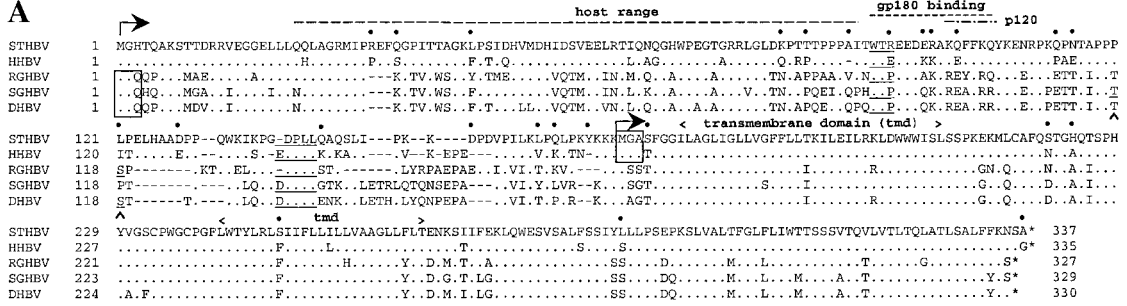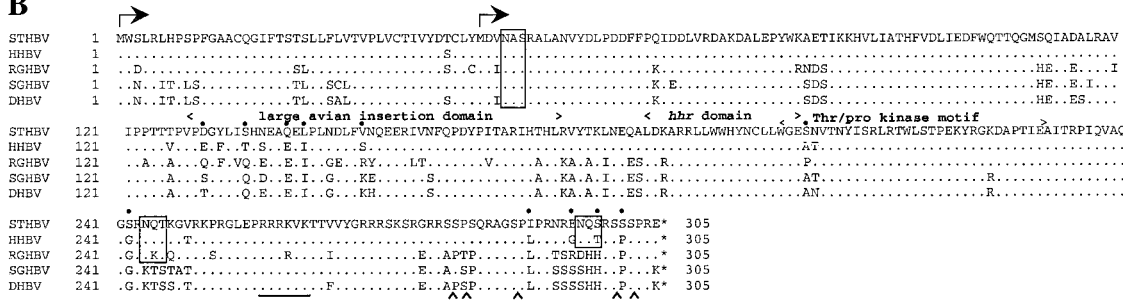

FIG. 5. Phylogenetic relationship of all known avian hepadnaviruses based on DNA sequences available from EMBL databank and established by using the SplitsTree software program (Huson, 1998).
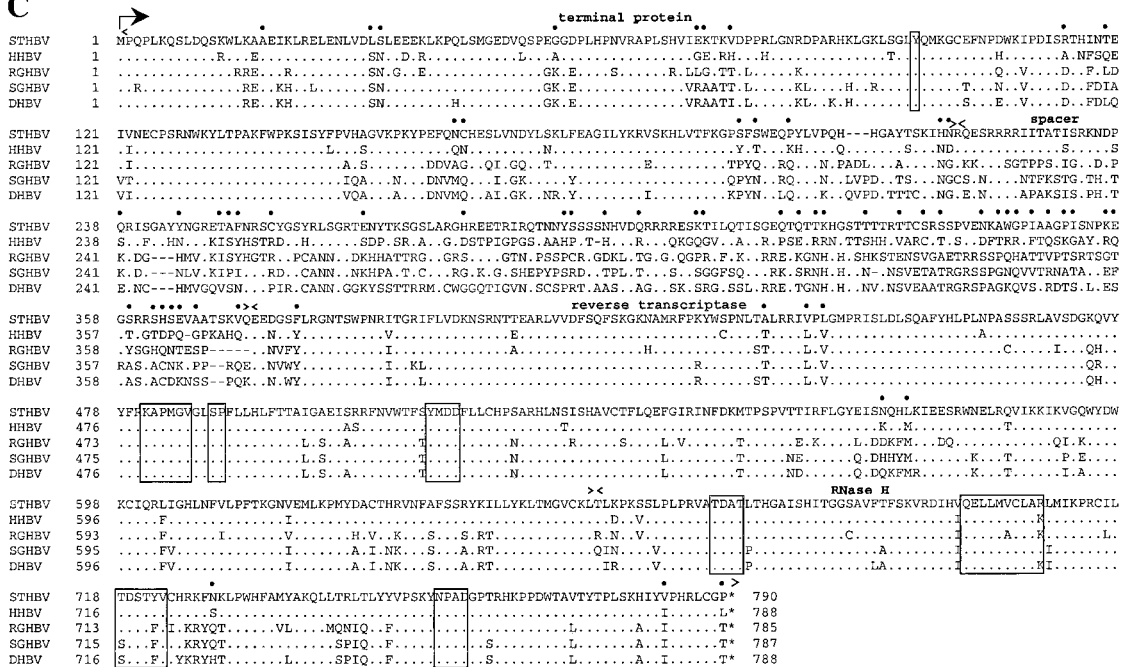
A

```
                                        host range                              gp180 binding
                             ------------------------------------------            ----------------  p120
STHBV   1  MGHTQAKSTTDRRVEGGELLLQQLAGRMIPREFQGPITTAGKLPSIDHVMDHIDSVEELRTIQNQGHWPEGTGRRLGLDKPTTTPPPAITWTREEDERAKQFFKQYKENRPKQPNTAPPP
HHBV    1  .......H.........P...S.......F.T.Q........L.AG.......A.......Q.RP....-...E....KK...E..........PAE.....
RGHBV   1  .QQP...MAE...A........---K.TV.WS..Y.TME.....VQTM..IN.M.Q..A......A.......TN.APPAA.V.N..P....AK.REY.RQ...E......ETT.I..T
SGHBV   1  .QHQ...MGA..I...I..N........---K.TV.WS..F.........LL..VQTM..VN.L.Q..A..A......V..TN..PQEI.QPH..P....QK.REA.RR....E..PETT.I..T
DHBV    1  .QQP...MDV..I........N........---K.TV.WS..F.T....LL..VQTM..VN.L.Q..A..A.A.....TN.APQE..QPQ..P....QK.REA.RR....E..PETT.I..T
                                                                                         <    transmembrane domain (tmd)   >
STHBV 121  LPELHAADPP--QWKIKPG-DPLLQAQSLI---PK--K-----DPDVPILKLPQLPKYKKKMGASFGGILAGLIGLLGLLVGFFLLTKILEILRKLDWWWISLSSSPKEKMLCAFQSTGHQTSPH
HHBV  120  IT.....E..--...S..-E...K.KA......V.--K-EPE---....V.K.TN-..J.T........................................N..A....
RGHBV 118  SP-------..KT..EL..-....ST.------LYRPAEPAE...I.VI.T.KV.---...SST...........I.......R.......GN.Q...N..A.I...
SGHBV 118  PT-------.---.LQ...D....GTK..LETRLQTQNSEPA---..VI.Y.LVR--K..SGT...........S...I.........G..Q...D..A.I...
DHBV  118  ST------.T.---.LQ...D....ENK..LETH.LYQNPEPA---...VI.T.P.R--K...AGT...........G..Q...D..A.I...
           ^                                                                              •
                <    tmd    >
STHBV 229  YVGSCPWGCPGFLWTYLRLSIIFLLILLVAAGLLFLTENKSIIFEKLQWESVSALFSSIYLLLPSEPKSLVALTFGLFLIWTTSSSVTQVLVTLTQLATLSALFFKNSA*   337
HHBV  227  ...................F......T............................S....S.......................................G*   335
RGHBV 221  ...............H....Y.D.M.T..A............SS....D.....M..L.......T......G........S*   327
SGHBV 223  ..................F......Y..D.G.T.LG......SS....DQ.....M...L...M...A..T........Y.S*   329
DHBV  224  .A.F...............F.......Y..D.M.I.LG.....SS....DQ.....M...L...M...A..T........Y..*   330
```

B

```
STHBV   1  MWSLRLHPSPFGAACQGIFTSTSLLFLVTVPLVCTIVYDTCLYMDVNASRALANVYDLPDDFFPQIDDLVRDAKDALEPYWKAETIKKHVLIATHFVDLIEDFWQTTQGMSQIADALRAV
HHBV    1  ..........................................S............................................................
RGHBV   1  ...D....................SL........S..C.I...........K...............RNDS..........HE..E....I
SGHBV   1  .N..IT.LS..........TL..SCL...........S..I...........K.E...............SDS..........HE..E.I.
DHBV    1  .N..IT.LS..........TL..SAL...........S..I...........K.E...............SDS..........HE..ES...
               <   large avian insertion domain       >  <   hhr domain    >  Thr/pro kinase motif
STHBV 121  IPPTTTPVPDGYLISHNEAQELPLNDLFVNQEERIVNFQPDYPITARIHTHLRVYTKLNEQALDKARRLLWWHYNCLLWGESNVTNYISRLRTWLSTPEKYRGKDAPTIEAITRPIQVAQ
HHBV  121  ......V...E.F..TS..E.I.....S..................................AT..................................
RGHBV 121  ...A..A...Q.F.VQ.E..E.I..GE..RY....LT.......V......A..KA.A.I..ES..R...........................
SGHBV 121  ...A....A...S...Q.D..E.I..G...KE......S...........A..KA.A.I..ES..R.........................AT............R..........
DHBV  121  ...A....A....T....Q.E..E.I..G...KH.......S...........A..KA.A.I..ES..R.........................AN............R..........
STHBV 241  GSHNQTKGVRKPRGLEPRRRKVKTTVVYGRRRSKSRGRRSSPSQRAGSPIPRNFNQSRSSSPRE*   305
HHBV  241  .G....]....T..................................L...G..T..P....*   305
RGHBV 241  .G..K.Q......S........R....I..........E..APTP.......L..TSRDHH..P....*   305
SGHBV 241  .G.KTSTAT....................E..A.SP.......L..SSSSHH..P...K*   305
DHBV  241  .G.KTSS.T...............F......E..APSP.......L..SSSSHH..P...K*   305
                NLS
```

C

```
STHBV   1  MPQPLKQSLDQSKWLKAAEIKLRELENLVDLSLEEEKLKPQLSMGEDVQSPEGGDPLHPNVRAPLSHVIEKTKVDPPRLGNRDPARHKLGKLSGIYQMKGCEFNPDWKIPDISRTHINTE
HHBV    1  ............R...E...........SN..D.R........L...A...........GE.RH...H...........T........H........A.NFSQE
RGHBV   1  .......RRE...R...........N.S.N.G.G.......GK.E...S.......R.LLG.TT.L....K...............Q..V....D..F.LD
SGHBV   1  .R...........RE..KH..L......SN...........GK.E.........VRAATT.L.....KL...H..R.......T...N..V....D..FDIA
DHBV    1  .......RE..KH..........SN.........H........GK.E.........VRAATI.L.....KL..K.H.......S...E..V....D..FDLQ
                                                                                   spacer
STHBV 121  IVNECPSRNWKYLTPAKFWPKSISYFPVHAGVKPKYPEFQNCHESLVNDYLSKLFEAGILYKRVSKHLVTFKGPSFSWEQPYLVPQH---HGAYTSKIHNRQESRRRRIITATISRKNDP
HHBV  121  ..I........L...S.........QN.............N.................Y.T...KH...Q....S...ND.........S....S
RGHBV 121  ..I.........A.S......DDVAG..QI.GQ..T.........E.........TPYQ..RQ...N.PADL...A....NG.KK....SGTPPS.IG..D.P
SGHBV 121  VT.............IQA...N...DNVMQ...I.GK....Y.............QPYN..RQ...N..LVPD..TS...NGCS.N....NTFKSTG.TH.T
DHBV  121  VI.............VQA...A...DNVMQ..AI.GK..NR.Y..........I.........KPYN..LQ...K..QVPD.TTTC...NG.E.N....APAKSIS.PH.T
STHBV 238  QRISGAYYNGRETAFNRSCYGSYRLSGRTENYTKSGSLARGHRREETRIRQTNNYSSSSNHVDQRRRRESKTILQTISGEQTQTTKHGSTTTTRTTCSRSSPVENKAWGPIAAGPISNPKE
HHBV  238  S..F..HN....KISYHSTRD...H.......SDP.SR.A..G.DSTPIGPGS.AAHP.T-H...R...QKGQGV..A..R.PSE.RRN..TTSHH.VARC.T.S...DFTRR.FTQSKGAY.RQ
RGHBV 241  K.DG---HMV.KISYHGTR..PCANN..DKHHATTRG..GRS....GTN.PSSPCR.GDKL.TG.G.QGPR.F.K..RRE.KGNH.H.SHKSTENSVGAETRRSSPQHATTVPTSRTSGT
SGHBV 241  K.D.---NLV.KIPI...RD..CANN..NKHPA.T..C..RG.K.G.SHEPYPSRD..TPL.T..S..SGGPSQ...RK.SRNH.H..N-.NSVETATRGRSSPGNQVVTRNATA..EF
DHBV  241  E.NC---HMVGQVSN...PIR.CANN..GGKYSSTTRRM.CWGGQTIGVN.SCSPRT.AAS..AG..SK.SRG.SSL.RRE.TGNH.H..NV.NSVEAATRGRSPAGKQVS.RDTS.L.ES
                                              reverse transcriptase
STHBV 358  GSRRSHSEVAATSKVQEEDGSFLRGNTSWPNRITGRIFLVDKNSRNTTEARLVVDFSQFSKGKNAMRFPKYWSPNLTALRRIVPLGMPRISLDLSQAFYHLPLNPASSSRLAVSDGKQVY
HHBV  357  .T.GTDPQ-GPKAHQ...N..Y..........V..............E..................C....T...L.V..............A.............
RGHBV 358  .YSGHQNTESP------...NVFY.......I.............A.................H........ST...L.V.............C....I...QH..
SGHBV 357  RAS.ACNK.PP--RQE..NVWY.........I..KL.............................R........ST...L.V..............QR..
DHBV  358  .AS.ACDKNSS---PQK..N.WY..........I..L............................R........ST...L.V..............QH..
STHBV 478  YFFKAPMGVGLSPFLLHLFTTAIGAEISRRFNVWTFSVMDEFLLCHPSARHLNSISHAVCTFLQEFGIRINFDKMTPSPVTTIRFLGYEISNQHLKIEESRWNELRQVIKKIKVGQWYDW
HHBV  476  ........................AS.............T...........................K..M.........T..............
RGHBV 473  .............L.S..A........T........N......R....S....L.V....T......E.K...L.DDKFM...DQ............QI.K....
SGHBV 475  .............L.S.........T..........N...............L....T...NE........Q.DHHYM....K...T.....P.E....
DHBV  476  .............L.S..A........T........N..............L....T....ND........Q.DQKFMR....K...T.....I.A....
                                                                   RNase H
STHBV 598  KCIQRLIGHLNFVLPFTKGNVEMLKPMYDACTHRVNFAFSSRYKILLYKLTMGVCKLTLKPKSSLPLPRVATDATLTHGAISHITGGSAVFTFSKVRDIHVQELLMVCLARLMIKPRCIL
HHBV  596  .......F..........I..........................D..V...........................................K.........
RGHBV 593  .......F......I.......V....H.V.K...S...S.RT.......R.N..V................C..............A..K........L..
SGHBV 595  .....FV........I.....A.I.NK...S...A.RT..........QIN...V...................P..........A..........I........
DHBV  596  .....FV........I.....A.I.NK...S...A.RT..........IR...V...................P..........LA..........I........
STHBV 718  TDSTYVCHRKFNKLPWHFAMYAKQLLTRLTLYYVPSKYNPAIGPTRHKPPDWTAVTYTPLSKHIYVPHRLCGP*   790
HHBV  716  ...................................................................L*   788
RGHBV 713  ....F.I.KRYQT......VL....MQNIQ..F...........L....A..I....T*   785
SGHBV 715  S...F..KRYQT...........SPIQ..F...........S........L....A..I....T*   787
DHBV  716  S...F..YKRYHT.......L.....SPIQ..F............S........L....A..I....T*   788
```

D

```
STHBV          MSQVNIILLTAQPGINHLIGQLLHTPLSRNIYMYHIGYVVLKITPLSIRSCVPRYLYVNLAVVPCDCTFGMYHCL*   75
HHBV           .YP......M....T.L...RP.......S..I........S.................D.............*   75
RGHBV          M....L....H.........KQ.IS....G......L....F..VF.............*   66
SGHBV   MLNNCYLLSNCTLFL..T.....H..TSR.......KQ.IF.....E..NYTPLL...L...I..YI...MF.......R.*   87
DHBV    W.N.CYLLYNC.LYR..I.....H..T.R...R........KQ.IF.....E..NYTPLL...L....DF...SF.......C.*   -
```
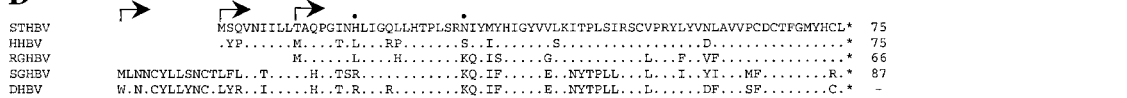
FIG. 6. Amino acid sequence alignment of the STHBV 21 encoded viral proteins with those of the four most closely matching prototypes of avian HBV genomes. Only divergent amino acids are indicated. Deletions introduced for optimal alignment are marked by dashes. Translation initiation and termination codons are indicated by arrows and asterisks, respectively. Amino acids unique for STHBV are indicated by dark black dots above the sequence. (A) PreS/S proteins: putative myristylation sites are boxed. Known motifs involved in virus neutralization (WTP) (Sunyach *et al.,* 1999) and core/preS protein interaction (XDPXL motif) (Yu *et al.,* 1998), phosphorylation sites, a host-range specificity-determining region, as well as the DHBV preS/gp180/p120 interaction domain, are indicated. (B) PreC/C proteins: The large avian insertion domain and the hydrophobic heptad repeat (Hhr) are shown. Putative glycosylation sites are boxed. Phosphorylation sites are also indicated. (C) Polymerase protein: the terminal protein, the spacer, the reverse transcriptase, and the RNase H domains are indicated by < or > and conserved motifs within the different domains are boxed. (D) X proteins: note, in DHBV, there is no conventional start codon and therefore the DHBV X-protein sequences beginning at the corresponding start codon of the X-like gene of SGHBV are given.

et al., 2001) and a nuclear localization signal described for DHBV (Mabit et al., 2001) is also conserved in STHBV (Fig. 6B).

The P protein of all avian hepadnaviruses including STHBV is well conserved, except for the nonfunctional spacer domain overlapping with the preS region (Fig. 6C). In this region many of the amino acid substitutions unique for STHBV are concentrated. Completely conserved are the tyrosine residue in the terminal protein domain (TP), to which the DNA genome is covalently linked, as well as the sequences known to be essential for the reverse transcriptase and the RNase H activities (Fig. 6C).

STHBV potentially encodes an X-like protein (Fig. 6D) identical in size (75 aa) and very similar in sequence to that of HHBV (Netter et al., 1997). This supports our assumption that these genes are expressed in vivo and are regulatory proteins similar as known for DHBV and mammalian X proteins. The coding region for the X proteins encoded by the other avian hepadnaviruses are slightly longer (SGHBV and possibly DHBV) or shorter (RGHBV). Except for DHBV which lacks a conventional start codon (Chang et al., 2001), the expression of X-like proteins from avian hepadnaviruses remains to be shown. The species-specific variation in length and sequence of the X-like proteins in avian hepadnaviruses may be necessary for interaction with cellular proteins that differ and/or are not conserved in the corresponding animal species.

Functional analysis of STHBV genomes in chicken hepatoma cells

The competence of the cloned viral STHBV genomes in viral protein expression, replication, viral particle synthesis, and secretion was examined by transfection of LMH cells. Plasmids containing cloned overlength STHBV DNAs (STHBV 16 and 21) and as a control with head-to-tail tandem constructs of HHBV4 and DHBV3 were used for the transfection experiments. In cell extracts prepared from LMH cells transfected with DHBV3 DNA, a strong signal for the 32-kDa core protein was observed by immunoblotting with antiduck core antibodies. Much weaker signals at a slightly higher position (33 kDa) were also observed with extracts from STHBV and HHBV-transfected LMH cells but not in mock-transfected cells (Fig. 7A). The weaker signals are probably due to limited cross-reactivity of the anti-DHBV core antibody with the core proteins of HHBV and STHBV. The same samples were also investigated in separate blots for the presence of preS protein. Immunostaining of the lanes loaded with the samples derived from the HHBV DNA-transfected cells with anti-HHBV preS antibodies resulted in a strong signal for preS protein (35 kDa) and degradation bands or truncated versions thereof (Fig. 7B). A slightly weaker signal at a very similar position (34
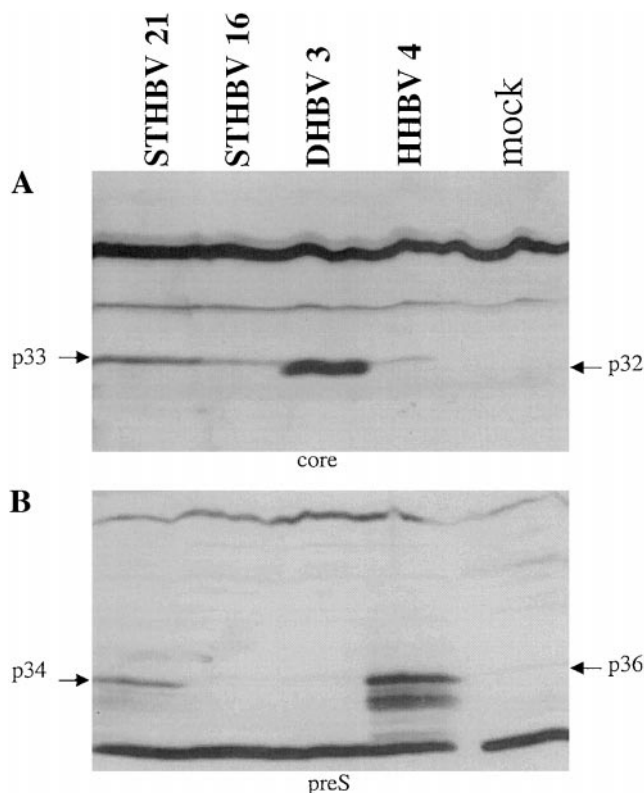

FIG. 7. Expression of core and large envelope proteins from STHBV, HHBV, and DHBV genomes in transfected LMH cells, as determined by immunoblotting. The core and preS proteins were detected with polyclonal anti-DHBV core (A) and anti-HHBV preS antibodies (B).

kDa) was observed with extracts from cells transfected with STHBV 21 DNA. At this and much longer exposures of the blot, no corresponding signals were detected with extracts derived from STHBV 16 DNA and mock-transfected cells (Fig. 7B). At longer exposures a weak signal was observed with the DHBV 3 sample. Reincubation of the same blot with anti-DHBV preS antibodies strongly increased the DHBV-specific preS signal. This demonstrates that the HHBV preS antibody reacts specifically with STHBV and only weakly with DHBV preS protein (data not shown). Moreover, these data imply that STHBV 21 is competent both in preS and core-protein expression, whereas STHBV 16 is competent only for core protein expression. The lack of the translation initiation codon for the small envelope protein in STHBV 16 appears to interfere with detectable preS-protein expression and/or lowers its stability, similarly as observed with a corresponding DHBV mutant (Fernholz, 1992).

To investigate whether STHBV 21 is also competent in replication, virion assembly, and secretion, DNA was extracted from cytoplasmic extracts and from viral particles from the culture medium of the transfected LMH cells. With the DNA extracted from all three cytoplasmic extracts, a typical pattern of replicative intermediates was revealed by Southern blotting (data not shown).
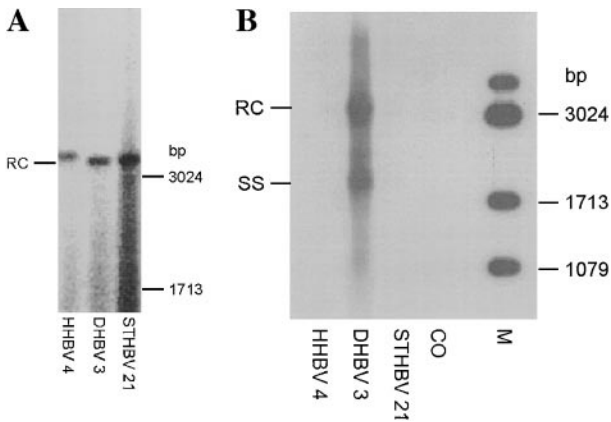
FIG. 8. Southern blot analysis with viral DNA from viral particles shed into the cell culture medium of LMH cells transfected with cloned genomic STHBV, HHBV, and DHBV DNA (left). Southern blot analysis of total DNA isolated from primary duck hepatocytes after incubation with the recombinant STHBV, HHBV, and DHBV particles (right).

Open circular DNA was detected in preparations derived from viral particles released from cells expressing and replicating STHBV-, DHBV-, and HHBV-specific genomes (Fig. 8A) but not in preparations derived from mock-transfected cells (data not shown).

Analysis of the infectivity of STHBV for duck hepatocytes

Primary duck hepatocytes were prepared from ducklings as described previously (Bruns et al., 1998) and infected with recombinant STHBV 21, HHBV4, and DHBV3 virions (m.o.i. 10) previously harvested from culture medium of LMH cells (see Fig. 8A) transfected with the corresponding plasmids. Total cellular DNA was extracted 7 days after infection and analyzed by Southern blotting. Typical and strong signals for replicative intermediates were observed with DNA from DHBV-infected cells but no signals with DNA from HHBV- and STHBV-infected cells (Fig. 8B). Since very inefficient infection of duck hepatocytes with the STHBV-related HHBV was reported previously when examined on the single-cell level by indirect immunofluorescence staining (Ishikawa and Ganem, 1995), we examined whether the same applies to STHBV. For this purpose about 10^6 primary duck hepatocytes were incubated for 16 h with DHBV- and STHBV-viremic serum containing approximately 10^6 genome equivalents, as determined by Southern blotting of viral DNA extracted from an aliquot of these sera (data not shown). Three days after infection the cells were fixed and stained for core protein with a polyclonal antiserum raised against DHBV core protein which cross-reacts with the STHBV core protein (see Fig. 7A). Bound antibodies were visualized with fluorescence-labeled anti-rabbit IgG under the microscope. Approximately 1% of all hepatocytes incubated with DHBV-viremic serum score positive in this assay, whereas after incubation

with STHBV only about 1 of 10^5 hepatocytes (0.0001%) were positive (Fig. 9). These data indicate that STHBV infects primary duck hepatocytes as inefficiently as or even less efficiently as reported for HHBV. Our data are also consistent with the absence of replicative intermediates in the Southern blot analysis of DNA from cells incubated with STHBV (Fig. 7B).

MATERIALS AND METHODS

Animals and sera

Serum samples from 17 white and 2 black storks (Ciconia ciconia and Ciconia nigra, respectively) were obtained from the Tiergarten Nürnberg and the zoo in Dresden, Germany. Additional stork sera (Ciconia abdimii (2), Ciconia boyciana (1), Ciconia episcopus (1), Ciconia maguari (2), Ciconia nigra (2), Ephippiorhynchus asiaticus (2), Ephippiorhynchus senegalensis (2), Jabiru mycteria (3), Leptoptilos crumeniferus (2), Leptoptilos javanicus (1), Mycteria americana (1), Mycteria cinerea (1), Mycteria ibis (2), Mycteria leucocephala (2)) were from various zoos and wildlife parks in the U.S.A. (San Diego, Miami, Audubon, Fort Wayne) and kindly provided by B. Slikas.

ELISA

Sera from storks were screened by enzyme-linked immunosorbent assay and/or by immunoblotting for the presence of STHBV proteins by using a rabbit polyclonal antiserum raised against the recombinant HHBV preS domain (Urban et al., 1998) as follows. Multiwell plates (MaxiSorp Immunoplates; Nunc, Roskilde, Denmark) were incubated for 2 h at room temperature with 10 μl stork sera, diluted 1:10 in EPBS (9.8 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 165 mM NaCl, 3.2 mM KCl, pH 7.4). After removal of fluid, the wells were blocked overnight at 4°C with 200 μl T-EPBS (EPBS supplemented with 5% of Tween 20). Then, the wells were further incubated for 2 h at room temperature with anti-HHBV preS antibody diluted 1:1000 in T-EPBS. After several washings, the wells were incubated for 1 h at room temperature with horseradish peroxidase coupled goat anti-rabbit IgG secondary antibodies (Dianova) diluted 1:1500 in T-EPBS. Immunoreactive proteins were then visualized by adding 0.5 mg/ml ortho-phenylenediamine and 0.5 μl/ml H_2O_2. Extinction was measured at a wavelength of 490 nm by using an automatic multiscan device (Titertek Multiscan; Flow Laboratories, Bonn, Germany).

Western blotting

An aliquot of each stork serum (0.2–1.0 μl) was diluted in 10 μl PBS (phosphate-buffered saline, 8.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), 2 μl 1 M DTT, and 8 μl twofold-concentrated Laemmli buffer. Then the samples were boiled for 5 min, centrifuged at 13 krpm for 10 min to remove the cellular debris, and separated by
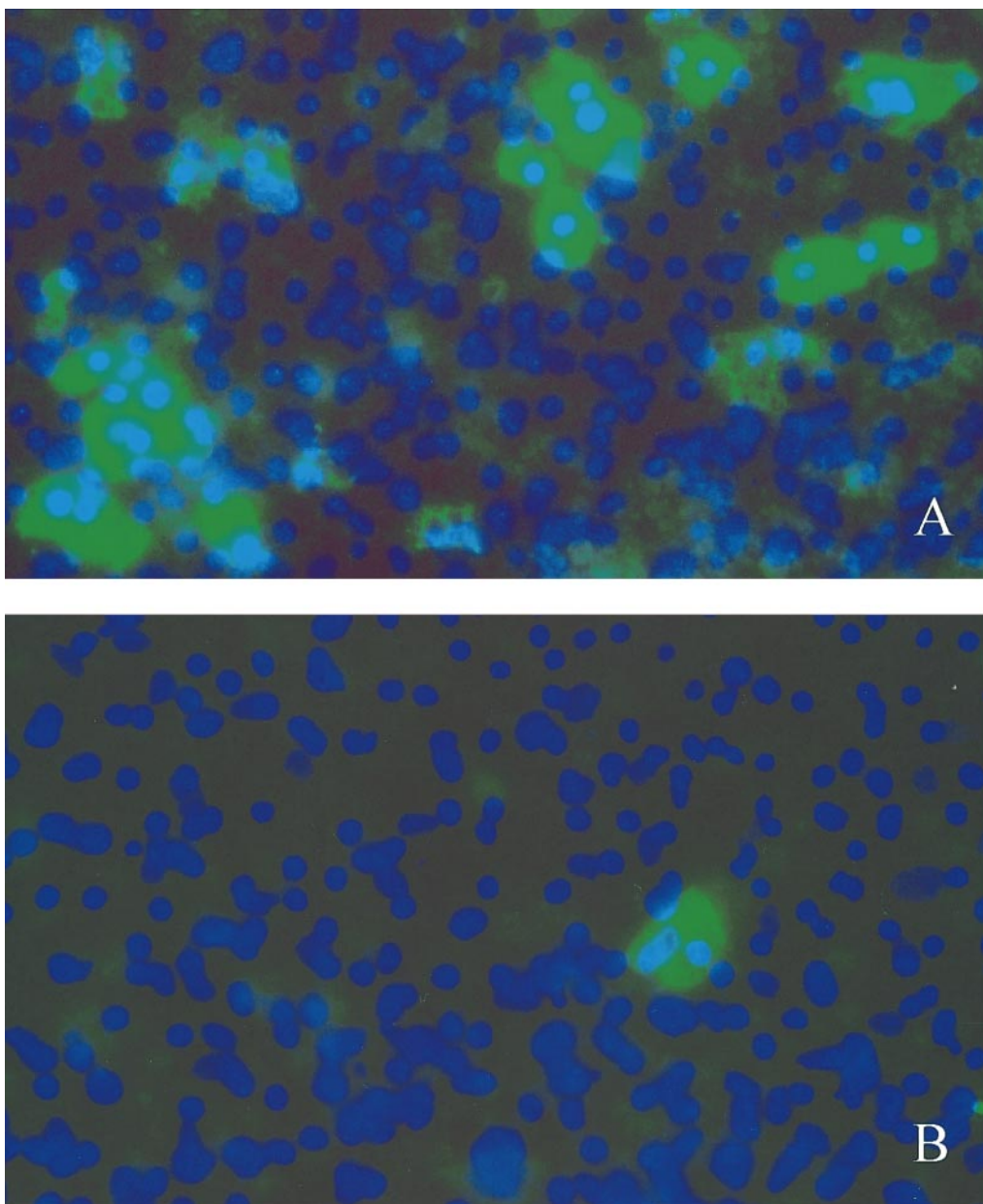
FIG. 9. Testing of the permissiveness of primary duck hepatocytes for DHBV (A) and STHBV (B) by indirect immunofluorescence staining for core (green). Nuclei are counterstained in blue with Hoechst dye. The photograph in B is not representative and has been selected to show one of the very few STHBV-infected cells.

20% PAGE. The proteins were transferred to PVDF or Protran nitrocellulose membranes after electrophoresis. Then the membranes were blocked for 2 h at room temperature in 5% dried milk diluted in TBS (Tris-buffered saline, 50 mM Tris/HCl, 150 mM NaCl), and incubated overnight at 4°C with anti-HHBV preS at a 1:1000 dilution in 1% dried milk dissolved in TBS. After several washings, the membranes were then incubated with horseradish peroxidase coupled goat anti-rabbit IgG antibodies at a dilution of 1:10,000 or 1:50,000. Proteins were then visualized by chemiluminescence (ECL Kit; Amersham Buchler, Braunschweig, Germany).

For the analysis of intracellular viral proteins, trans-

fected cells were lysed in an equal volume of Laemmli buffer, and proteins were analyzed as described above. For detection of core proteins, a rabbit polyclonal anti-DHBV core antigen serum (anti-C2989, Schneider *et al.,* 1991) was used at a dilution of 1:10,000.

Electron microscopic analysis of viral particles in stork sera

Stork sera were incubated with microcarriers decorated with anti-HHBV preS immunoglobulins. After washing, the carriers were collected by low-speed centrifugation. The pellet was washed, resuspended in PBS, and

then transferred into capillary tubes as described (Hohenberg et al., 1994).

For morphological investigations the sample-containing tubes were fixed for 1 h in 2.5% glutaraldehyde in PBS and washed, and cells were postfixed within the tubes for 30 min with 1% OsO_4 in PBS followed by washing in PBS and 30 min of staining in 1% uranyl acetate in water. For ultrathin sectioning the capillary tubes were dehydrated in a graded series of ethanol and embedded in ERL resin. Ultrathin sections were counterstained with 2% uranyl acetate and lead citrate. All electron micrographs were taken with a Philips CM 120 transmission electron microscope at 60 kV.

PCR amplification and cloning of STHBV DNA from virions

For cloning of the full-length STHBV genomes, the viral DNA was PCR-amplified without prior DNA extraction by boiling serum samples for 10 min. The PCR conditions were as described recently (Netter et al., 1997) and included the use of primers (P1 and P2) which anneal to the nick region of HHBV flanked by heterologous sequences at their 5′ ends containing restriction sites for BglII and SapI (underlined below). Sequences of the primers were as follows: P1 (positions: 2538–2561): 5′-GAAGATCTGCTCTTCATTACACCCCTCTCCATTCGGAGC-3′; P2 (positions: 2542–2519): 5′-GAAGATCTGCTCTTCGTAATCTTAGAGACCACATAGCCT-3′.

Alternatively, the following primers perfectly homologous to the nick region of STHBV and containing additional heterologous 5′ sequences (underlined) were used: PS1 (positions: 2544–2567): 5′-GAATCGATGCTCTTCATTACACCCCTCTCCATTCGGAGC-3′; PS2 (positions: 2548–2525): 5′-GAATCGATGCTCTTCGTAATCTTAAGGACCACATAGCCT-3.

For the PCR assay with the PS1 and PS2 primers, we used 2 μl of the sera diluted 1:200 in water. Nomenclature of avian HBV sequences was according to Sprengel et al. (1988). All PCR reactions were carried out with the Expand High Fidelity PCR System (Roche, Penzberg, Germany), yielding a mixture of 3′ single-adenine overhang products and blunt-ended products, respectively (Günther et al., 1995). PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). For cloning, the purified products were ligated into pUC 18 (GIBCO/BRL, Gaithersburg, MD) -derived T/A cloning vector (pXcmKn12) by using a rapid DNA ligation kit (Roche). The vector was digested with XcmI, leaving single 3′ thymidine overhangs. After ligations the sample was transformed into DH5-alpha bacterial cells (GIBCO/BRL). Four full-length clones (pSTHBV1, pSTHBV7, pSTHBV16, and pSTHBV21) were obtained.

To exclude that mutations were artificially introduced into the nick region of the STHBV clones during the full-length genome amplification with the HHBV-specific

primers P1 and P2 and to be able to construct overlength STHBV genomes required for hepatoma cell transfection experiments (see below), STHBV-sequences were amplified with primers (P3 and P4) annealing to STHBV-specific sequences located outside of the nick region: P3 (positions 2151–2172): 5′-GTTTACCTTTGCCACGTGTAGC-3′; P4 (positions 3029–3007): 5′-CTAGCTGTAATGGGATAGTCAGG-3.

The P3/P4-specific amplification product is about 880 bp long. It was purified, ligated into a T/A cloning vector, and cloned as described above. The pSTON construct was obtained which contains the STHBV-authentic nick region. All PCR products were analyzed on agarose gels.

DNA sequencing of PCR-amplified products and cloned STHBV DNA

Purified PCR-amplified products were directly sequenced (see below). For the four constructs containing the full-length STHBV sequences (pSTHBV1, pSTHBV7, pSTHBV16, and pSTHBV21) and also for pSTON, both strands were sequenced independently. Plasmid DNA was prepared by the Qiagen plasmid maxi kit (Qiagen) and sequenced with avian HBV-specific synthetic IRD fluorescence-labeled primers according to the manufacturer's instructions (SequiTherm long read cycle sequencing kit; Epicentre Technologies, Madison, WI). In addition, for pUC18-derived clones, M13fwd and M13rev primers were used. Primer sequences can be obtained from the authors upon request. The nucleotide sequence data of the four full-length genomes STHBV1, STHBV7, STHBV16, and STHBV21 have been deposited in the EMBL nucleotide sequence database under Accession Nos. AJ251934, AJ251935, AJ251936, and AJ251937, respectively.

Comparative DNA and protein sequence and phylogenetic analyses

Comparative sequence analyses were performed by using the PileUp and FastA programs from the Wisconsin sequence analysis package software version 9.1 for UNIX (Genetics Computer Group, Madison, WI) facilitated by the Rechenzentrum at the Universtiy of Zürich, Switzerland. The four different STHBV sequences were aligned to 25 complete avian HBV viral genomes (14 DHBV, 5 HHBV, 1 RGHBV, and 5 SGHBV isolates) found in the EMBL nucleotide sequence database (EMBL release 60.0). The identification numbers, the name of the isolates (if given), and the GenBank accession numbers or, if not available, the respective references for the hepadnaviruses given herein are as follows: DHBV: DHBV1/X58567; DHBV3, (Sprengel et al., 1991; HBDCG, (DHBVP2-3)/M60677; DHBVF16/X12798; DHBVCG (IDHBV)/X74623; NCCOLR (DHBV16)/K01834; ALTA-16/AF047045; HBDGA (DHBVS18-B)/M21953; HBDS31CG (DHBVS-31)/M32991; DHV6350/AJ006350; HBDS5CG (DHBVS-5)/M32990; DH-

VBCG (DHBVQCA34)/X60213; DHBV26/X58569; DHBV22/X58568. RGHBV: HBDGENM/M95589. SGHBV: SGHBV7/AF110999; SGHBV9/AF111000; SGHBV13/AF110996; SGHBV15/AF110997); SGHBV19/AF110998. HHBV: HBHCG (HHBV4)/M22056; HeronA–HeronD (Netter *et al.,* 1997). For phylogenetic analysis of complete avian HBV DNA sequences, the method of split decomposition (Bandelt and Dress, 1992), using a set of aligned sequences as input, was applied by Udo Toenges, University Bielefeld, Bielefeld, Germany, with the SplitsTree program (Bandelt and Dress, 1992; Dress *et al.,* 1996; Huson, 1998).

Construction of replication-competent overlength STHBV genomes for transfection experiments

A PCR-based cloning procedure (see above) was used to generate two overlength STHBV genomes, pSTOL16 and pSTOL21, used in transfection experiments (see below). pSTON was digested either with *Nco*I and *Hin*dIII or with *Hin*dIII and *Dra*III, respectively. The two full-length STHBV plasmids, pSTHBV16 and pSTHBV21, respectively, were each digested with *Dra*III and *Nco*I. The 3325-bp *Nco*I-to-*Hin*dIII fragment, the 696-bp *Hin*dIII-to-*Dra*III fragment, and the respective 2584-bp *Dra*III-to-*Nco*I fragments were used, ligated in a triple ligation assay in low-melting agarose (GIBCO/BRL), and cloned into pUC18, yielding the two STHBV overlength genomes pSTOL16 and pSTOL21, respectively.

Transfection of LMH cells with viral DNA and analysis by Southern blotting

LMH chicken hepatocarcinoma cells (Kawaguchi *et al.,* 1987) were grown to near confluency at 37°C with 5% CO_2 in Dulbecco's MEM/Nutrient Mix F12 (GIBCO/BRL), supplemented with 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. Cells were transfected by calcium phosphate coprecipitation of 20 μg pSTOL 16 or pSTOL 21 plasmids together with 2 μg reporter plasmid pCMV/SEAP (TROPIX, Bedford, MA) per 10-cm culture dish. The cells were incubated for 14 h with the precipitate, washed with PBS, and incubated for another 2–5 days. Transfection efficiency was determined 3 days after transfection by a standard colorimetric assay for placental alkaline phosphatase (SEAP) secreted into the medium (Berger *et al.,* 1988). Viral DNA was purified from intracellular core particles and from extracellular viral particles, subjected to 1.5% agarose gel electrophoresis, and transferred onto Hybond N+ nylon membranes (Amersham Life Science, Buckinghamshire, U.K.) by vacuum blotting as previously described (Pult *et al.,* 1997). Viral DNA was detected by hybridization of membranes with PCR-generated full-length DHBV, HHBV, and STHBV DNA probes, ^{32}P-labeled by *redi*prime DNA labeling system (Amersham Life Science).

Infection of primary fetal duck hepatocytes

Thirty milliliters of cell culture medium collected 4 days after transfection of LMH cells with avian HBV DNAs (see above) was clarified by low-speed centrifugation at 3000 rpm for 15 min. The viral particles from the supernatant were subsequently centrifuged at 25,000 rpm for 6 h in a SW28 rotor (Beckman, München, Germany) through a cushion of 20% sucrose. The pelleted material was then resuspended in PBS, and viral DNA was isolated and analyzed by Southern blotting (see above) from an aliquot corresponding to one-tenth of the original material. The remaining part was further diluted in modified William's medium E (WME; GIBCO/BRL) supplemented with 1.5% DMSO, 1 nM insulin, 2 mM glutamine, 10 μM hydrocortisone, 15 mM HEPES pH 7.2, 100 U/ml penicillin, 100 μg/ml streptomycin, and used for infection experiments of primary fetal duck hepatocytes. Primary hepatocytes were isolated from 21-day-old duck embryos by digestion liver tissue with 0.5% collagenase (Sigma, Deisenhofen, Germany) and subsequently infected as described (Bruns *et al.,* 1998). Cells were harvested at day 7 after infection for Southern blot analysis of intracellular viral replicative intermediates and of DNA from extracellular viral particles. Cells inspected by indirect immune fluorescence staining with a polyclonal antiserum raised against DHBV core protein were harvested 3 days after infection to visualize primary infections only.

REFERENCES

Bandelt, H. J., and Dress, A. (1992). A new and useful approach to phylogenetic analysis of distance data. *Mol. Phylogenet. Evol.* **1,** 242–252.

Barrasa, M. I., Guo, J. T., Saputelli, J., Mason, W. S., and Seeger C. (2001). Does a cdc2 kinase-like recognition motif on the core protein of hepadnaviruses regulate assembly and disintegration of capsids? *J. Virol.* **75,** 2024–2028.

Berger, J., Hauber, J., Hauber, R., Geiger, R., and Cullen, B. R. (1988). Secreted placental alkaline phosphatase: A powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene* **66,** 1–10.

Borel, C., Sunyach, C., Hantz, O., Trépo, C., and Kay, A. (1998). Phosphorylation of DHBV Pre-S: Identification of the major site of phosphorylation and effects of mutations on the virus life cycle. *Virology* **242,** 90–98.

Bringas, R. (1997). Folding and assembly of hepatitis B virus core protein: A new model proposal. *J. Struct. Biol.* **118**, 189–196.

Bruns, M., Miska, S., Chassot, S., and Will, H. (1998). Enhancement of hepatitis B virus infection by non-infectious subviral particles. *J. Virol.* **72**, 1462–1468.

Calvert, J., and Summers, J. (1994). Two regions of an avian hepadnavirus RNA pregenome are required in *cis* for encapsidation. *J. Virol.* **68**, 2084–2090.

Chang, S.-F., Netter, H. J., Bruns, M., Schneider, R., Frölich, K., and Will, H. (1999). A new avian hepadnavirus infecting snow geese (*Anser caerulescens*) produces a significant fraction of virions containing single-stranded DNA. *Virology* **262**, 39–54.

Chang, S.-F., Netter, H. J., Hildt, E., Schuster, R., Schäfer, S., Hsu, Y. C., Rang, A., and Will, H. (2001). Duck hepatitis B virus expresses a regulatory HBx-like protein from a hidden open reading frame. *J. Virol.* **75**, 161–170.

Cova, L., Duflot, A., Prave, M., and Trepo, C. (1993). Duck hepatitis B virus infection, aflatoxin B1 and liver cancer in ducks. *Arch. Virol. Suppl.* **8**, 81–87.

Dress, A. W. M., Huson, D. H., and Moulton, V. (1996). Analyzing and visualizing sequence and distance data using SplitsTree. *Discrete Appl. Math.* **71**, 95–109.

Fernholz, D. (1992). Studien zur Synthese und Funktion der Hüllproteine bei Hepatitis B Viren. Ph.D. thesis, Universität München, Germany.

Günther, S., Li, B. C., Miska, S., Krüger, D. H., Meisel, H., and Will, H. (1995). A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J. Virol.* **69**, 5437–5444.

Günther, S., Sommer, G., Von Breunig, F., Iwanska, A., Kalinina, T., Sterneck, M., and Will, H. (1998). Amplification of full-length hepatitis B virus genomes from samples from patients with low levels of viremia: Frequency and functional consequences of PCR-introduced mutations. *J. Clin. Microbiol.* **36**, 531–538.

Hohenberg, H., Mannweiler, K., and Müller, M. (1994). High-pressure freezing of cell suspensions in cellulose capillary tubes. *J. Microsc.* **175**, 34–43.

Hu, J., and Seeger, C. (1997). RNA signals that control DNA replication in hepadnaviruses. *Semin. Virol.* **8**, 205–211.

Huson, D. H. (1998). SplitsTree: Analyzing and visualizing evolutionary data. *Bioinformatics* **14**, 68–73.

Ishikawa, T., and Ganem, D. (1995). The pre-S domain of the large viral envelope protein determines host range in avian hepatitis B viruses. *Proc. Natl. Acad. Sci. USA* **92**, 6259–6263.

Jilbert, A. R., Botten J. A., Miller, D. S., Bertram, E. M., Hall, P. M., Kotlarski, J., and Burrell, C. J. (1998). Characterization of age- and dose-related outcomes of duck hepatitis B virus infection. *Virology* **244**, 273–282.

Kawaguchi, T., Nomura, K., Hirayama, Y., and Kitagawa, T. (1987). Establishment and characterisation of a chicken hepatocellular carcinoma cell line, LMH. *Cancer Res.* **47**, 4460–4464.

Lenhoff, R., Luscombe, C. A., and Summers, J. (1999). Acute liver injury following infection with a cytopathic strain of duck hepatitis B virus. *Hepatology* **29**, 563–571.

Li, J., Tong, S., and Wands, J. R. (1999). Identification and expression of glycine decarboxylase (p120) as a duck hepatitis B virus pre-S envelope-binding protein. *J. Biol. Chem.* **274**, 27658–27665.

Lilienbaum, A., Crescenzo-Chaigne, B., Sall, A. A., Pillot, J., and Elfassi, E. (1993). Binding of nuclear factors to functional domains of the duck hepatitis B virus enhancer. *J. Virol.* **67**, 6192–6200.

Liu, C., Mason, W. S., and Burch, J. B. (1994). Identification of factor-binding sites in the duck hepatitis B virus enhancer and in vivo effects of enhancer mutations. *J. Virol.* **68**, 2286–2296.

Mabit, H., Breiner, K. M., Knaust, A., Zachmann-Brand, B., and Schaller, H. (2001). Signals for bidirectional nucleocytoplasmic transport in the duck hepatitis B virus capsid protein. *J. Virol.* **75**, 1968–1977.

Macrae, D. R., Bruss, V., and Ganem, D. (1991). Myristylation of a duck hepatitis B envelope protein is essential for infectivity but not for virus assembly. *Virology* **181**, 359–363.

Marion, P. L., Cullen, J. M., Azcarraga, R. R., Van Davelaar, M. J., and Robinson, W. S. (1987). Experimental transmission of duck hepatitis B virus to Pekin ducks and to domestic geese. *Hepatology* **7**, 724–731.

Mason, W. S., Seal, G., and Summers, J. (1980). A virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. *J. Virol.* **36**, 829–836.

Mayo, M. A., and Pringle, C. R. (1998). Virus taxonomy—1997. *J. Gen. Virol.* **79**, 649–657.

Mueller-Hill, K., and Loeb, D. D. (1996). Previously unsuspected cis-acting sequences for DNA replication revealed by characterization of a chimeric heron/duck hepatitis B virus. *J. Virol.* **70**, 8310–8317.

Netter, H. J., Chassot, S., Chang, S.-F., Cova, L., and Will, H. (1997). Sequence heterogeneity of heron hepatitis B virus genomes determined by full-length DNA amplification and direct sequencing reveals novel and unique features. *J. Gen. Virol.* **78**, 1707–1718.

Obert, S., Zachmann-Brand, B., Deindl, E., Tucker, W., Bartenschlager, R., and Schaller, H. (1996). A splice hepadnavirus RNA that is essential for virus replication. *EMBO J.* **15**, 2565–2574.

Pastro, N., Lopez-Lazaro, M., Tella, J., Baos, R., Hiraldo, F., and Cortes, F. (2001). Assessment of genotoxic damage by the comet assay in white storks (*Ciconia ciconia*) after the Donana ecological disaster. *Mutagenesis* **16**, 219–223.

Pult, I., Chouard, T., Wieland, S., Klemenz, R., Yaniv, M., and Blum, H. E. (1997). A hepatitis B virus mutant with a new hepatocyte nuclear factor 1 binding site emerging in transplant-transmitted fulminant hepatitis B. *Hepatology* **25**, 1507–1515.

Raney, A. K., Easton, A. J., and McLachlan, A. (1994). Characterization of the minimal elements of the hepatitis B virus large surface antigen promoter. *J. Gen. Virol.* **75**, 2671–2679.

Rothmann, K., Schnolzer, M., Radziwill, G., Hildt, E., Moelling, K., and Schaller, H. (1998). Host cell-virus cross talk: Phosphorylation of a hepatitis B virus envelope protein mediates intracellular signaling. *J. Virol.* **72**, 10138–10147.

Schäfer, S., Tolle, T., Lottmann, S., and Gerlich, W. H. (1998). Animal models and experimental systems in hepatitis B virus research. *In* "Hepatitis B Virus: Molecular Mechanisms in Disease and Novel Strategies for Therapy" (R. C. Koshy and W. H. Caselmann, Eds.), pp. 51–74. Imperial College Press, London.

Schneider, R., Fernholz, D., Wildner, G., and Will, H. (1991). Mechanism, kinetics and role of duck hepatitis B virus e-antigen expression in vivo. *Virology* **182**, 503–512.

Schödel, F., Weimer, T., Fernholz, D., Schneider, R., Sprengel, R., Wildner, G., and Will, H. (1991). The biology of avian hepatitis B viruses. *In* "Molecular Biology of Hepatitis B Viruses" (A. McLachlan, Ed.), pp. 53–80. CRC Press, Boca Raton, FL.

Slikas, B. (1997). Phylogeny of the avian family Ciconiidae (storks) based on cytochrome *b* sequences and DNA-DNA hybridization distances. *Mol. Phylogenet. Evol.* **8**, 275–300.

Sprengel, R., Kaleta, E. F., and Will, H. (1988). Isolation and characterization of a hepatitis B virus endemic in herons. *J. Virol.* **62**, 3832–3839.

Sprengel, R., Schneider, R., Marion, P. L., Fernholz, D., Wildner, G., and Will, H. (1991). Comparative sequence analysis of defective and infectious avian hepadnaviruses. *Nucleic Acids Res.* **19**, 4289.

Sunyach, C., Rollier, C., Robaczewska, M., Borel, C., Barraud, L., Kay, A., Trepo, C., Will, H., and Cova, L. (1999). Residues critical for duck hepatitis B virus neutralization are involved in host cell interaction. *J Virol.* **73**, 2569—2575.

Tong, S., Li, J., and Wands, J. R. (1999). Carboxypeptidase D is an avian hepatitis B virus receptor. *J. Virol.* **73**, 8696–8702.

Triyatni, M., Ey, P., Tran, T., Le Mire, M., Qiao, M., Burrell, C., and Jilbert, A. (2000). Sequence comparison of an Australian duck hepatitis B virus strain with other avian hepadnaviruses. *J. Gen. Virol.* **82**, 373–378.

Urban, S., Breiner, K. M., Fehler, F., Klingmüller, U., and Schaller, H.

(1998). Avian hepatitis B virus infection is initiated by the interaction of a distinct pre-S subdomain with the cellular receptor gp180. *J. Virol.* **72,** 8089–8097.

Urban, S., Schwarz, C., Marx, U. C., Zentgraf, H., Schaller, H., and Multhaup, G. (2001). Receptor recognition by a hepatitis B virus reveals a novel mode of high affinity virus-receptor interaction. *EMBO J.* **19,** 1217–1227.

Welsheimer, T., and Newbold, J. E. (1996). A functional hepatocyte nuclear factor 3 binding site is a critical component of the duck hepatitis B virus major surface antigen promoter. *J. Virol.* **70,** 8813–8820.

Yen, T. S. B. (1993). Regulation of hepatitis B virus gene expression. *Semin. Virol.* **4,** 33–42.

Yu, M., and Summers, J. (1994). Multiple functions of capsid protein phosphorylation in duck hepatitis B virus replication. *J. Virol.* **68,** 4341–4348.

Yu, M., Miller, R. H., Emerson, S., and Purcell R. H. (1996). A hydrophobic heptad repeat of the core protein of woodchuck hepatitis virus is required for capsid assembly. *J. Virol.* **70,** 7085–7091.

Yu, M., Emerson, S. U., Cote, P., Shapiro, M., and Purcell, R. H. (1998). The GDPAL region of the Pre-S1 envelope protein is important for morphogenesis of woodchuck hepatitis virus. *Hepatology* **27,** 1408–1414.

Zhou, Y. Z. (1980). A virus possibly associated with hepatitis and hepatoma in ducks. *Shanghai Med. J.* **3,** 641–644.

Zöllner, B., Petersen, J., Schroter, M., Laufs, R., Schoder, V., and Feucht, H. H. (2001). 20-fold increase in risk of lamivudine resistance in hepatitis B virus subtype adw. *Lancet* **357,** 934–935.