Infections caused by hepatitis B virus (HBV) remain one of the most serious medical challenges worldwide, with approximately 350 million humans being chronically infected. There is currently no established effective therapy available. HBVs are small enveloped DNA viruses constituting the family Hepadnaviridae and propagating their genome by reverse transcription of an RNA intermediate (22). As well as in humans, orthohepadnaviruses have been identified so far in rodents such as woodchucks (woodchuck hepatitis virus), ground and arctic squirrels (ground squirrel hepatitis virus and arctic squirrel hepatitis virus), and recently in Old World as well as New World primates including woolly monkeys, orangutans, gorillas, and gibbons (17, 18). Naturally occurring infections by avian hepadnaviruses were first described in Pekin ducks (Anas domestica) (duck hepatitis B virus DHBV) from China and the United States (13, 33) and were subsequently found in various other duck strains (references 21 and 29 and references therein). DHBV-related viruses have also been isolated from four other avian species, namely, grey heron (Ardea cinerea) (heron hepatitis B virus (HHBV)) (24), Ross’ goose (Anser rossit) (Ross’ goose hepatitis B virus [RGHBV]), and snow goose (Anser caerulescens) (snow goose hepatitis B virus [SGHBV]) (2) as well as from white stork (Ciconia ciconia) (stork hepatitis B virus [STHBV]) (16). Comparative DNA analysis of the different viral strains revealed a relatively high variability of the DHBV isolates but not of HHBV and STHBV (14, 16).

Extracellularly, virions contain partially double-stranded DNA of exquisitely compact size (3.0 to 3.3 kbp). Following arrival in the nucleus, the incoming viral genome is converted into covalently closed circular DNA and serves as the template for viral transcription. The genes of hepadnaviruses are all extensively overlapping and encode the envelope (pre-S and S), the nucleocapsid (core or C), and nonstructural proteins such as the precore (pre-C) and its proteolytically processed and secreted form, designated e-antigen, as well as the multifunctional \( P \) protein, which has reverse transcriptase, DNA-dependent DNA polymerase, and RNase H activities. The \( P \) protein also serves as a primer for DNA minus-strand synthesis. A regulatory protein, designated \( X \), was thought until recently to be uniquely expressed only by mammalian hepadnaviruses. However, expression of an X-like protein from a DHBV open reading frame without a conventional start codon has recently been demonstrated (3). Notably, an X-like open reading frame is also present in other avian HBV genomes infecting herons, snow and Ross’ geese, and storks (14, 16); however, it is not known whether the corresponding proteins are indeed expressed during natural infection.

All known hepadnaviruses are highly cell type specific and have a very narrow host range, restricted to their natural host and a few closely related species. For instance, the prototype HBV infects only humans, chimpanzees, chacma baboons, and to a certain extent also *Tupaia belangeri* (8, 31), but it does not infect woolly monkeys, for which a naturally occurring related virus (woolly monkey hepatitis B virus) is known (4, 10). GSHV does not even infect all species of squirrels, just chipmunks, close relatives of ground squirrels (30). DHBV infects only certain duck and goose species (12) but does not
infect Muscovy ducks (*Cairina moschata*), a domesticated duck not descended from mallards (the common ancestor of domestic ducks), or chickens (*Gallus gallus domesticus*) or infects them only in very inefficiently (12, 15). This remarkably extreme host range restriction is, at least partially, determined at the level of viral entry. The pre-S domain, residing in part on the exterior of viral particles, mediates their attachment to hepatocytes. Pre-S and S proteins arise by differential translation initiation from the pre-S/S gene: the S protein, providing 80% of the surface protein content, and the pre-S protein, in which the S protein is N-terminally extended by the hydrophilic pre-S domain of 161 amino acids. These two envelope proteins are embedded in the lipid membrane of both the abundantly secreted, noninfectious subviral particles and virions (22). The L protein of DHBV is myristoylated at glycine 2, and its mutational prevention abrogates the infectivity of DHBV (11). Despite various reports of pre-S binding proteins, the actual cellular receptor(s) used by hepadnaviruses remains unknown. For DHBV, a cellular glycoprotein, designated gp180 or CPD, with features fulfilling several of the criteria of a bona fide receptor, has been identified (9, 28). Although gp180 can mediate virus attachment and internalization, this protein alone is not sufficient to mediate productive DHBV infection in non-permissive cells (1, 27). This and other findings suggest the requirement for additional cellular factors necessary for reconstitution of a de novo infection system. Although ducks and derived primary hepatocytes are not permissive for HHBV (7, 24), replacement of a small region of the HHBV-specific pre-S domain by the corresponding sequence from DHBV overcomes this species barrier (7). This appears to apply similarly to mammalian hepadnaviruses, as shown for woolly monkey hepatitis B virus pseudotyped with a small stretch of pre-S1 domain by the corresponding sequence from DHBV overexpression and cloning of CHBV DNA from virions. Crane sera were screened for hepadnavirus genome sequences by Hot-Start PCR without prior DNA extraction as described previously (14), with STHBV- or HHBV-specific full-length primers which anneal to the nick region of viral DNA flanked by the restriction site for *SalI* (underlined below). The sequences of the primers used were as follows: STHBV *salI* ap+, 5'-GAATCAGATGCTCTTCATTACCCCTCTCCAT-3'; STHBV *salI* ap-, 5'-GAATCAGATGCTCTTCATTACCCCTCTCCATACTCGAGACG-3'; HHBV *salI* ap+, 5'-GAATCAGATGCTCTTCATTACCCCTCTCCAT-3'; HHBV 2852-150F (positions 2183 to 2150), 5'-GGCGCAATATCCCATATCACCGGCGGG-3'; HHBV 2852-150R (positions 2851 to 2828), 5'-CATGCATGCCCTGTGTAGTCTGCC-3'. The PCR assay was used 2 μl of the isolated avian sera diluted 1:200 to 1:2,000 in water. All PCR amplifications were carried out with the Expand High-Fidelity PCR system (Roche, Penzberg, Germany), yielding a mixture of 3′ single-adenine overhang products and blunt-end products, respectively (5). The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). For cloning, the purified products were ligated into the pUC18-derived T/A cloning vector pXcmKn12 (GIBCO-BRL, Gaithersburg, Md.) using the Rapid DNA ligation kit (Roche). The cloning vector was digested with XcmI, leaving single 3′ thymidine overhangs. After ligation, the constructs were amplified in bacterial strain DH5α. Three full-length clones (CHBV 1, CHBV 2, and CHBV 3) were obtained. To exclude mutations which could have been artificially introduced into the nick region of the CHBV by using STHBV- and HHBV-specific primers, subgenomic fragments of the CHBV genome sequence were additionally amplified with primers annealing to CHBV-specific sequences located outside of the nick region: CHBV2183+ (positions 2183 to 2209), 5′-GGCGCAATATCCCATATCACCCGGCGG-3′; and CHBV2852+ (positions 2851 to 2828), 5′-CATGCATGCCCTGTGTAGTCTGCC-3′. Amplification products of about 668 bp were subsequently analyzed by direct sequencing (see below). All PCR products were purified and analyzed on 1% agarose gels stained with ethidium bromide.

**DNA sequencing of PCR-amplified products and cloned CHBV genomes.**
Purified products of PCR amplification were directly sequenced using infrared-dye (IRD)-labeled primers. The two strands of the full-length viral genome containing individual clones (CHBV 1, CHBV 2, and CHBV 3) were sequenced independently. Plasmid DNA was prepared by using a MaxiKit (Qiagen) and then sequenced with primer-specific sets of fluorescent-labeled primers annealing to the different nucleotide sequences on the viral genome. In addition, M13 forward and M13 rev primers were used for sequencing of pXcmKn12-derived clones (14).

DNA- and protein-based phylogenetic analysis of CHBV. Comparative sequence analysis was performed using the MacVector 7.0 (Oxford Molecular Ltd.) and SplitsTree programs as described previously (16). All three individual CHBV sequences were aligned with 29 complete avian HBV genomes (14 HBV clones, 5 HHBV clones, 1 RGHBV clone, 5 SGHBV clones, and 4 STHBV clones) deposited in the EMBL nucleotide sequence database. The identification numbers, the name of the isolates (if indicated), and the GenBank accession numbers or, if not available, the respective references for the hepadnaviruses compared herein are as follows: DHBV: DHBV1/X58567; DHBV3 (25); HHBV: HHBV/AF110996; DHBVCG (DHBV/X74623; NCCOLR (DHBV16)/K013834; ALTA:16/AF047045; HB-DGA (DHBV18-S)/M21983; HBDSI3CC (DHBV-S)/M32291; DHBV350/AJ006350; HBDSSCG (DHBV5-S)/M32990; DHBVCg (DHBVCA34)/X62013; DHBV26/X58569; DHBV22/X58568; RGHBV: HBDGENM/M95589; SGHBV: SGHBV/A F110999; SGHBV9/A F110006; SGHBV13/A F110997); RGHBV: HGHBV/Cg (HHBV4)/M2056; HeronC-HeronD (14); STHBV (16). For phylogenetic analysis of complete avian HBV DNA sequences, the method of split decomposition, using a set of aligned sequences as input, was performed by using the SplitsTree program as described previously (16).

Circularization of full-length CHBV genomes for replication assays. CHBV and SGHBV full-length genome containing plasmids pCHBV1, pCHBV2, pCHBV3, and pSGHBV15 were digested with M13fwd and M13rev primers and ligated using T4 DNA ligase (New England Biolabs and Pharmacia Biotech, respectively). This resulted in release of the reporter plasmids pCHBV1, pCHBV2, and pCHBV3. The linearized viral genomes were then digested with I and Bgl II enzymes as described previously (16). All three individual CHBV genomes were cloned into plasmid M13K07 under transcription control of the lac promoter (Stratagene). For each 60-mm culture dish containing about 106 cells, 2.5 µg of plasmid DNA were transfected with FuGene 6 (Roche Diagnostics). For each 60-mm culture dish containing about 106 cells, 2.5 µg of plasmid DNA were transfected with FuGene 6 (Roche Diagnostics). For each 60-mm culture dish containing about 106 cells, 2.5 µg of plasmid DNA were transfected with FuGene 6 (Roche Diagnostics).

transfection of LMH cells. LMH cells were grown to 60 to 80% confluency at 37°C under 5% CO2 in Dulbecco's modified Eagle medium/nutrient mix F12 (GIBCO-BRL), supplemented with 2 mmol of l-glutamine per liter, 100 µg of penicillin per ml, and 100 µg of streptomycin per ml (all from GIBCO-BRL, Paisley, Scotland), and 10% fetal calf serum. The cells were transfected using FuGene 6 (Roche Diagnostics). For each 60-mm culture dish containing about 3 x 106 cells, 2.5 µg of digested CHBV DNA and 0.5 µg of pEGFP-C1 (Clontech, Germany) reporter plasmid were used. At 14 h after transfection, the cells were washed with PBS and further cultivated as indicated in the corresponding text and/or figure legends. Transfection efficiency was estimated 2 days after transfection by counting the number of green fluorescent protein-expressing cells under an epifluorescence microscope.

Analysis of replicative intermediates of CHBV. The replication competence of the cloned CHBV genomes was tested by Southern blot analysis of DNA replicative intermediates in both intra- and extracellular viral particles. Cell culture medium collected from LMH cells transfected with CHBV or SGHBV genomes was clarified by centrifugation at 1,462 g for 15 min. Viral particles were precipitated from culture medium by using 8% polyethylene glycol. DNA from viral particles of crane sera or precipitated from cell culture supernatants was extracted with phenol-chloroform as described previously (2). Extracted DNA was separated on a 1.5% agarose gel and blotted onto an Hybond-N nylon membrane (Amersham Life Science) by capillary transfer as described previously. The membrane was hybridized with 32P-labeled DHBV-16 DNA.

Infection of primary fetal duck hepatocytes. Primary duck hepatocytes were prepared from liver of 21-day-old duck fetuses by collagenase digestion as described previously (16). Liver cells were resuspended in William's medium E (GIBCO-BRL) supplemented with 1 mM insulin, 10 µM hydrocortisone, and 1.5% dimethyl sulfoxide (1.5%) (all from Sigma, Taufkirchen, Germany), 2 mM glutamine, 15 mM HEPES (pH 7.2), 100 µg of penicillin per ml, and 100 µg of streptomycin per ml (all from GIBCO-BRL) and seeded into 12-well plates. The cells were infected on day 2 or 3 after seeding by using clarified supernatants of transfected LMH cells as described previously (16). The cells were infected with PBS and incubated for 1 h at 37°C with DHBV core rabbit antiserum (diluted 1:400) or a mouse monoclonal or rabbit polyclonal DHBV pre-S antiserum (both diluted 1:400) (16). The cells were then washed three times with PBS and incubated at 37°C with secondary Alexa 488- or Alexa 594-conjugated secondary antibodies (diluted 1:1,000) for another 30 min. Nuclei were counterstained with Hoechst (4 µg/ml). Following mounting and embedding, stained cells were analyzed and photographed with an epifluorescence microscope (Axiovert; Zeiss).

RESULTS AND DISCUSSION

Sera of demoiselle and grey crowned cranes contain HBV-related pre-S antigen and e-antigen. Based on the close evolutionary relationship between herons and cranes and on our previous work, we assumed that hepadnaviruses in the two animal species are antigenetically related. Therefore, crane sera were screened for HBV by immunoblotting using antisera raised against the entire HHBV pre-S protein domain or against DHBV core protein, the latter cross-reacting with the well-conserved e-antigen of all known avian hepadnaviruses. In total, 12 sera derived from Manchurian, paradise blue, sandhill, demoiselle, and grey crowned cranes were tested. Bands with a similar or identical electrophoretic mobility to the major HHBV pre-S protein at the position of a 36-kDa protein were revealed in three of the four demoiselle crane sera and all grey crowned crane sera tested, while all sera from Manchurian, paradise blue, or sandhill cranes were negative (Fig. 1A). Notably, the HHBV-positive serum showed a stronger signal than those seen with any of the crane sera. This may be due to a lower immunoreactivity of the HBV pre-S antiserum used with related proteins of the crane sera or to a lower viremia in demoiselle and grey crowned cranes. To test this, the membrane was re-incubated with DHBV pre-S-specific antiserum, which is known to react only weakly with the HHBV pre-S protein (16). The intensity of the putative p36 pre-S signals of the crane sera was much increased, while that of the HHBV pre-S antiserum became only marginally more intense (data not shown). This observation indicated that the putative pre-S proteins in crane sera may be antigenetically more similar to DHBV pre-S than to HHBV pre-S. All sera scoring positive for pre-S showed three major bands when analyzed for e-antigen (Fig. 1B), whereas those negative for pre-S were also negative for e-antigen. Two of the e-antigen bands comigrated with related proteins of the crane sera or to a lower viremia in demoiselle and grey crowned cranes. To test this, the membrane was re-incubated with DHBV pre-S-specific antiserum, which is known to react only weakly with the HHBV pre-S protein (16). The intensity of the putative p36 pre-S signals of the crane sera was much increased, while that of the HHBV pre-S antiserum became only marginally more intense (data not shown). This observation indicated that the putative pre-S proteins in crane sera may be antigenetically more similar to DHBV pre-S than to HHBV pre-S. All sera scoring positive for pre-S showed three major bands when analyzed for e-antigen (Fig. 1B), whereas those negative for pre-S were also negative for e-antigen. Two of the e-antigen bands comigrated with mono- and diglycosylated forms of HHBV e-antigen (19, 20); one showed a lower electrophoretic mobility and presumably represents another glycosylated or a nonglycosylated form of e-antigen.

PCR amplification and cloning of crane virus. To provide further independent evidence for infection of cranes with an HBV, the sera were screened for hepadnavirus DNA by PCR. Full-length viral genome amplification was performed using both HHBV- and STHBV-specific primers, which anneal to the sites positioned near the so-called nick region of the HBV genome. These allow full-length genome amplification of HHBV and STHBV as well as DHBV from diluted sera even

Downloaded from http://jvi.asm.org/ on October 15, 2017 by guest
without prior extraction of the viral DNA (14). With both primers, dominant amplification products of about 3 kbp were obtained with all sera of demoiselle and grey crowned cranes previously scored to be positive on pre-S immunoblots whereas all other crane sera were PCR negative (Fig. 1C [results obtained with STHBV primers only are shown]). Notably, there was a strong correlation between signal intensities in two independent assays, PCR and pre-S immunoblots, suggesting that both methods specifically detected an avian hepadnavirus and appear to reflect semiquantitatively the relative levels of viremia in the sera analyzed.

Analysis of viral particles in crane sera by immunoelectron microscopy. To obtain ultrastructural evidence for the presence of HBV-related viral particles in the crane sera containing pre-S antigen and viral DNA, we analyzed those sera by immunoelectron microscopy. DHBV-viremic duck serum was

FIG. 1. Screening of bird sera for pre-S (A) and e-antigens (B) and viral DNA (C) by immunoblotting and PCR, respectively. (A) Serum samples loaded were from manschurian cranes (lanes 1 and 2), a black stork (lane 3), demoiselle cranes (lanes 4 to 7), and crowned cranes (lanes 8 to 10). HHBV-positive and -negative heron sera used as controls were loaded in lanes 11 and 12, respectively. The pre-S antigens were detected with an HHBV pre-S antiserum. The full-length HHBV pre-S-specific band is indicated by an arrow. (B) e-protein in crowned (lanes 1 to 5 [lanes 4 and 5 correspond to different serum aliquots from the same cranes as in lanes 1 and 2, respectively]) and demoiselle (lanes 6 to 9) cranes as well as in HHBV-negative (lane 10) and -positive (lane 11) heron sera as detected by a DHBV-core- and e-antigen-specific antiserum. (C) Agarose gel analysis of PCR products amplified from crane sera. Serial dilutions (1/20 [lanes a], 1/200 [lanes b], and 1/2,000 [lanes c]) of four crane sera (lanes 2 to 13), one STHBV-positive stork serum (lanes 14 to 16), and one DHBV-negative serum (lanes 17 to 19) were subjected to PCR using STHBV full-length primers PS1 and PS2. A standard DNA size marker was loaded in lane 1.
tested in parallel for comparison, while nonviremic duck and crane sera served as negative controls. Viral particles from the sera were selectively immunoadsorbed onto microcarriers by using a mixture of HHBV and DHBV pre-S-specific antisera and analyzed by electron microscopy after ultrathin sectioning. Thus, in the pre-S immunoblot and PCR-positive but not -negative crane sera, viral particles with diameters ranging from about 40 to 60 nm (average, 40 nm) were observed (Fig. 2) which were indistinguishable from those in DHBV- and HHBV-positive sera (data not shown). Taken together, the data obtained by four independent assays (pre-S and e-antigen immunoblots, PCR, and electron microscopy) strongly indicated infection of two different species of cranes with avian hepadnaviruses.

**Direct sequencing and cloning of viral genomes from crane sera.** To determine the relationship of the viruses in the crane sera to known members of the *Avihepadnaviridae*, we first amplified and directly sequenced the pre-S region of the viral DNA from one crowned and one demoiselle crane, because this region is known to be most variable. Pre-S sequence analysis revealed that the HBV-related genomes in the crane sera differ from all other known avian hepadnaviruses by point
FIG. 3. Nucleotide sequence alignment of the cloned CHBV 1 genome with prototypic members of the avian hepadnavirus family (RGHBV, SGHBV 15, DHBV 16, HHBV 4, and STHBV 21). Dots and dashes represent identical and deleted nucleotides, respectively. Where necessary, deletions were introduced for optimal alignment. Translation initiation codons are indicated by arrows, and termination codons are indicated by asterisks. Transcription factor binding sites, the TATA box in the pre-S promoter, and other regulatory sequence elements are boxed.
mutations, inserts, and deletions and are very similar in sequence to each other (data not shown). The full-length viral genomes of two viremic demoiselle and crowned crane sera, amplified independently twice with primers homologous to the so-called nick region of avian hepadnaviruses, were then directly sequenced using combinations of different primers homologous to known avian hepadnavirus genomes or primers homologous to crane serum-derived pre-S sequences, as described above. An additional PCR performed with primers binding to sequences upstream and downstream of the nick region and subsequent direct sequencing of the amplification products excluded the notion that mutations were artificially introduced at the nick region by the primers used. Thus, the full-length sequences of the dominant virus population of at least one demoiselle and one crowned crane serum were obtained by PCR amplification and direct sequencing (data not shown).

Although derived from different genera of cranes, the two viral genomes differed only in a few positions (data not shown) and therefore were considered to be variants of the same virus strain, designated CHBV. The low sequence heterogeneity is reminiscent of that of other avian hepadnaviruses from the same geographic area. The low sequence heterogeneity may be due to horizontal transmission in zoos, but this is unlikely because the viremic birds tested did not all originate from the same zoo. The amplification products obtained from two independent PCRs and two independent grey crowned crane sera originating from two different zoos were cloned into the pUC18 A/T cloning vector. Thus, in total, three individual clones were obtained from two different crowned crane sera (CHBV 1 to CHBV 3) and sequenced. Comparative sequence analysis of the DNA of these three genomes revealed nucleotide sequence variation in only 95 positions (data not shown), consistent with the rather low sequence divergence of crane viruses seen by direct sequencing. As expected, the two CHBV clones obtained from one serum sample were more similar to each other in sequence than to the clone obtained from the second serum sample from the crane originating from another zoo (data not shown), which argues against a cross-contamination of the PCR process. This conclusion is also supported.

FIG. 4. DNA-based phylogenetic relationship of CHBV to all known avian hepadnaviruses and evolutionary relatedness of the corresponding hosts based on comparative DNA analysis. (A) Evolutionary tree of all avian hepadnaviruses calculated by using the SplitsTree program. (B) DNA-based evolutionary tree of birds, including the natural hosts of avian hepadnaviruses (25).
by the fact that 58 of the 95 nucleotide changes were silent for the predicted proteins, 37 resulted in amino acid changes in the viral P protein (only 7 of them were conservative), 6 of them were in the pre-S domain (2 of the 6 were conservative), and only 1 (nonconservative) was in the S protein. Furthermore, almost none of the nucleotide changes are located in regions harboring sequence elements important or essential for replication and transcription of DHBV.

For comparative analysis with other avian hepadnaviruses, we present here only the sequence of one of the cloned crane
genomes (CHBV 1 [Fig. 3]), while the sequences of all three viral genomes, CHBV 1 to CHBV 3, have been deposited in the EMBL GenBank database.

DNA sequence comparison and phylogenetic relationship of CHBV to known avian hepadviruses. The CHBV 1 genome was aligned with the most closely matching (as shown by the BLAST search program) prototypes of the five known avianhepadnavirus genomes, namely, RGHBV, SGHBV 15, DHBV 16, HHBV 4, and STHBV 21 (Fig. 4). This analysis showed that the DNA sequence identity between CHBV and RGHBV clones is 84%, while it is estimated as 83% for DHBV 16, 83% for SGHBV 15, 79% for STHBV 21, and 77% for HHBV 4, indicating a close relationship of CHBV to duck and goose hepadnaviruses. This is also illustrated in a phylogenetic Splits-Tree diagram for which the DNA sequence data of all known avian hepadnaviruses were evaluated (Fig. 4A). The close relationship of CHBV to RGHBV and DHBV is a very surprising result, because cranes (members of the order Gruidae) belong, together with storks and herons (members of the order Ciconiiformes), to the superorder Passerimorphae, parvclass Passerae, and therefore are close relatives whereas ducks and geese (members of the order Anseriformes) belong to the superorder Anserimorphae, parvclass Galloanserae, and are therefore evolutionarily far remote from cranes, storks, and herons (Fig. 4B). As expected, the highest DNA sequence divergence of CHBV from other hepadnaviruses was evident in the pre-S region, known to be highly variable in sequence because of its multiple functions in host and cell tropism. All DNA sequences of CHBV with regulatory functions, such as promoters, enhancers, replication signals (DR1, DR2, ε-encapsulation signal), RNA processing/polyadenylation, and splicing signals, as well as transcription factor binding sites (Fig. 3), are rather highly conserved compared to other duck and goose hepadnaviruses.

CHBV proteins and comparative protein sequence analysis of avian hepadviruses. Comparative protein sequence alignment confirmed the close homology of CHBV to duck and goose hepadnaviruses but also highlighted some interesting differences (Fig. 5). As expected, the pre-S protein sequence and the overlapping P-protein spacer region of CHBV exhibited more unique amino acid changes than did all other viral proteins of CHBV and proteins of all known avianhepadnaviruses. Most interestingly, the host-determining region of DHBV pre-S, located between amino acids 22 and 37, contains in CHBV a short insert of 3 amino acids (PMK), similar but not identical to the analogous region of HHBV (14) and STHBV (16), while all other known duck and goose hepadnaviruses have no such insert. However, unlike STHBV and HHBV, which lack a myristoylation consensus sequence at the amino terminus of pre-S (glycine at position 2) but instead have one at that of the small S protein, CHBV has such a signal for pre-S and not for S, identical to duck and goose hepadnaviruses. Myristoylation of DHBV pre-S is important for efficient infection of duck hepatocytes (11) but appears not to be essential for an HHBV recombinant virus containing the DHBV host-determining region that infects duck hepatocytes (7, 14) or for in vivo infection of herons and storks with HHBV and STHBV. The presence of six amino acids unique for an internal pre-S subdomain of CHBV (Fig. 6A, CPD binding region) and known to be essential for the high-affinity binding of DHBV pre-S (amino acids 30 to 115) to the putative DHBV receptor protein CPD may play a role in the host specificity of CHBV. The same may apply to an insertion in the pre-S protein of two amino acids at positions 126 and 127 required for optimal sequence alignment, which is unique for CHBV and RGHBV. The short sequence motif WTP, shown to be essential for infectivity in DHBV, is conserved in CHBV. The S protein of CHBV exhibits four unique amino acid changes not present in any other avian hepadnavirus. Taken together, CHBV has on the one hand unique pre-S and S sequence motifs and on the other hand sequence features in common with hepadnaviruses of ducks and geese but also with those of herons and storks. On the basis of these data on CHBV pre-S and S protein sequences, it was particularly interesting to determine the host range of this virus. The presence of an open reading frame in CHBV without a conventional translation start codon in an analogous position to that coding for the regulatory X-like protein of DHBV and its predicted protein sequence divergence from all other avian hepadnavirus X proteins may also play a role in host range specificity or reflect host adaptation mechanisms.

Cloned CHBV genomes are replication competent in chicken hepatoma cells. The competence of the three cloned CHBV genomes in viral protein expression, replication, particle formation, and secretion was tested by transfection of LMH chicken heptoma cells. The SGHBV genome was used as a positive control. Core proteins of the predicted sizes were observed on an immunoblot with anti-DHBV core antibodies and lysates from LMH cells transfected with each of the four genomes but not in mock-transfected cells (Fig. 6A). Expression of pre-S protein (the full-length protein and its major degradation product) from all four genomes was also revealed by immunoblotting of the same lysates with anti-HHBV pre-S antibodies (Fig. 6B). In the culture medium of LMH cells transfected with the four viral genomes, e-antigen of the expected size was also detected by immunoblotting with an antiserum against DHBV core protein and e-antigen (data not shown). These data indi-

FIG. 5. Amino acid sequence alignment of the CHBV 1 proteins with those of the five known avian hepadnaviruses. Only divergent amino acids are indicated. Dashes mark deletions introduced for optimal alignments. Translation initiation and termination codons are indicated by arrows and asterisks, respectively. Amino acids unique for CHBV are indicated by black dots above the sequence. (A) Pre-S and S proteins. Putative myristoylation sites are boxed. Known motifs involved in virus neutralization (WTP) (26) and core–pre-S interaction (GDPAL motif) (32), a phosphorylation site important for DHBV pre-S transactivation function, the host range-determining region, as well as the DHBV pre-S–CPD interaction domains, are indicated. (B) Pre-C and C proteins. The large avian insertion domain and the hydrophobic heptad repeat (Hhr) are shown. Putative glycosylation sites are boxed. (C) Polymerase protein. The terminal protein, the spacer region, the reverse transcriptase, and RNase H domains are indicated by < or >. Conserved functional motifs within the different domains and the amino acid to which the viral genome is covalently linked are boxed. (D) Protein sequence of the putative avian hepadnavirus X proteins, in comparison to that of DHBV as recently reported (3).
cate the competence of all three cloned CHBV genomes in viral protein expression.

The competence of these viral genomes in replication and viral particle secretion was analyzed by Southern blotting of DNA extracted from viral particles released from transfected LMH cells into the cell culture medium. The Southern blot revealed the relaxed circular and single-stranded DNA typical of both naked and enveloped core particle-encapsidated viral DNA produced and shed by all four genomes tested (Fig. 6C, lanes 6 to 9), indicating their competence in viral replication, virion assembly, and viral particle secretion. Viral DNA extracted from two viremic demoiselle and crowned cranes analyzed as an additional control on the same Southern blot was visualized as typical open-circular DNA of about 3.3 kbp (lanes 1 to 4) and in a similar concentration to that in a highly viremic DHBV serum (lane 5).

FIG. 6. Cloned CHBV genomes are replication competent. Intracellular core and pre-S protein expression (A and B) in LMH cells transfected with cloned CHBV or SGHBV genomes and DNA in secreted viral particles (C) as well as in crane and duck sera (C). (A and B) Detection of core (A) and pre-S (B) proteins in the lysates of LMH cells transfected with the indicated cloned genomes and detected by immunoblotting with antisera against DHBV core and HHBV pre-S proteins. The positions of the corresponding viral proteins are indicated by arrows. (C) Southern blot analysis of CHBV DNA in crane sera and culture media of transfected LMH cells. The samples loaded were viral DNA from sera of two crowned cranes (lanes 1 and 2), two demoiselle cranes (lanes 3 and 4), and a DHBV-viremic duck (lane 5). In the remaining lanes, the DNA from viral particles in the culture medium of LMH cells transfected with genomes from three cloned crowned cranes (lanes 6 to 8) and a cloned snow goose (lane 9) or harvested from mock-transfected cells (lane 10) were loaded. The positions of the relaxed circular (rc) and single-stranded (ss) DNAs are indicated.
Both natural and recombinant CHBV are infectious for PDH. Based on the analysis of the CHBV protein sequences and those of the corresponding pre-S proteins in particular, we predicted that CHBV is potentially infectious for PDH. To test this, cultures were prepared from duck embryos and infected with recombinant CHBV, SGHBV, and DHBV 16 viral particles harvested from supernatants of LMH cells transfected with the corresponding constructs. In parallel, PDH were infected with viremic demoiselle and crowned crane sera and, as a control, with a viremic duck serum, all having similar viral genome equivalents. After infection, viral gene expression was tested by immunoblotting and by indirect-immunofluorescence staining of the fixed cells.

For immunofluorescence analysis, cells were fixed 3 days after infection and costained for core and pre-S proteins. Immunostaining revealed that CHBV from supernatants of transfected LMH cells infected PDH with almost similar efficiency to that of DHBV infection (Fig. 7A, data shown only for pre-S).
or SGHBV used as controls (data not shown). Immunoblot analysis of infected cells confirmed the immunofluorescence data and showed similar intracellular expression levels for core (Fig 7B). Extracellularly, we also detected similar levels of e-antigens (Fig 7C). Since we may have selected nonrepresentative CHBV genomes by cloning, we also analyzed the infectivity of CHBV viremic sera from demoiselle and crowned cranes for PDH. In both cases, efficient infection of PDH was observed by using the same techniques (data not shown).

Taken together, these data indicate that CHBV infects PDH, and presumably also ducks, with similar efficiency to that of DHBV. It remains to be shown which sequence features of the pre-S and other proteins of CHBV are responsible for this rather unexpectedly broad host range and whether the sequence of CPD, the putative receptor of DHBV, is conserved in cranes. It will also be interesting to study any potential pathogenic effects of CHBV in ducks (provided that they can be infected with CHBV and establish a chronic infection) and whether this virus also infects other bird species. It also remains to be determined whether CHBV and DHBV infect primary cranhepatocytes and whether DHBV infects cranes. Such experiments are restricted by the fact that cranes are highly endangered species.

In any case, the infectivity of CHBV for duck hepatocytes suggests similar entry pathways and viral receptors in crane and duck hepatocytes. An unusual host adaptation mechanism or divergent evolution rates of the two viruses and their respective hosts may be responsible for the unexpectedly broad host range. Ongoing analysis of the cellular receptor(s) and of the host-determining region of hepadnaviruses, as well as additional information on intracellular virus-host interaction partners, may provide answers to these and other important open questions in hepadnavirus research.

ACKNOWLEDGMENTS

We are grateful to H. Will from the Zoo Dresden, G. von Hegel from the Zoo Karlsruhe, K. Baumgartner and B. Neurohr from the Tiergarten Nurnberg, and A. Ochs from the Zoo Berlin for providing different avian sera. We are grateful to M. Bruns for his initial help in Tiergarten Nurnberg, and A. Ochs from the Zoo Berlin for providing the host-determining region of hepadnaviruses, as well as prospective hosts may be responsible for the unexpectedly broad host range.

REFERENCES


