

A New Avian Hepadnavirus Infecting Snow Geese (*Anser caerulescens*) Produces a Significant Fraction of Virions Containing Single-Stranded DNA

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We describe the identification and functional analysis of an evolutionary distinct new avian hepadnavirus. Infection of snow geese (*Anser caerulescens*) with a duck hepatitis B virus (DHBV)-related virus, designated SGHBV, was demonstrated by detection of envelope proteins in sera with anti-DHBV preS and S antibodies. Comparative sequence analysis of the PCR-amplified SGHBV genomes revealed unique SGHBV sequence features compared with other avian hepadnaviruses. Unlike DHBV, SGHBV shows an open reading frame in an analogous position to orthohepadnavirus X genes. Four of five cloned genomes were competent in replication, gene expression, and virus particle secretion in chicken hepatoma cells. Primary duck hepatocytes were permissive for infection with SGHBV, suggesting a similar or identical host range. SGHBV was found to secrete a significant fraction of virion-like particles containing single-stranded viral DNA. This was observed both in cell culture medium of SGHBV DNA-transfected LMH cells and in viremic sera of several birds, suggesting that it is a stable trait of SGHBV. Taken together, SGHBV has several unique features that expand the knowledge of the functional and evolutionary diversity of hepadnaviruses and offers new experimental opportunities for studies on the life cycle of hepadnaviruses. © 1999 Academic Press

INTRODUCTION

The Hepadnaviridae family is divided into two genera, *Orthohepadnavirus* and *Avihepadnavirus*. Avihepadnaviruses are known to occur naturally in ducks [duck hepatitis B virus (DHBV); Schödel *et al.*, 1989], grey herons [heron hepatitis B virus (HHBV); Netter *et al.*, 1997; Sprengel *et al.*, 1988], and Ross geese [Ross goose hepatitis B virus (RGHV); J. Newbold, personal communication]. A DHBV-like virus has also been isolated from a white domestic goose, but it is not clear whether this virus was transmitted from ducks to geese inadvertently via vaccination with duck sera (Schödel *et al.*, 1989). Orthohepadnaviruses have been isolated so far from humans [hepatitis B virus (HBV)], a chimpanzee, woolly monkeys (woolly monkey HBV), woodchucks (woodchuck HBV), ground squirrels (ground squirrel HBV), and arctic ground squirrels (arctic ground squirrel HBV) (Lanford *et al.*, 1998; Schaefer *et al.*, 1998; Testut *et al.*, 1996).

All hepadnaviruses have a very narrow host range, are predominantly hepatotropic, and can cause acute and chronic infections in their hosts (Ganem, 1996). Characteristic for hepadnaviruses is a small (3.0–3.3 kb) relaxed circular (RC), partially double-stranded (ds)DNA genome

with neither DNA strand covalently linked. The viral P protein is covalently linked to the 5' end of the DNA minus strand. The 5' end of the DNA plus strand begins with a short oligoribonucleotide. Most of the HBV virion DNAs contain plus-strand DNAs approximately half the size of the full-length genome, whereas in DHBV, most DNA plus strands are close to full length. The unusual structure of the viral genomes reflects the replication strategy (Nassal and Schaller, 1996), which starts with the conversion of the virion DNA into covalently closed circular DNA, from which a terminally redundant RNA pregenome is synthesized. The RNA pregenome is then reverse transcribed into viral DNA minus strands primed by the virus-encoded P protein, a process during which the DNA remains covalently attached to this protein. Most of the RNA pregenome is degraded by a P protein intrinsic RNase H activity during DNA minus-strand synthesis. The DNA plus strand is copied from the DNA minus strand by the same enzymatic machinery but depends on a short oligoribonucleotide derived from the 5' end of the RNA pregenome. This RNA primer needs to be translocated to a direct repeat sequence located close to the 5' end of the DNA minus strand. Lack of RNA primer translocation can result in *in situ* priming of the DNA plus strand and formation of linear viral dsDNA. The viral DNA in virions is embedded in a nucleocapsid, formed by a single core protein. The core particle is surrounded by a

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lipid membrane into which one type of small (S protein) and one or several types of large virus-encoded envelope proteins, designated presurface S (preS) proteins, are inserted. The vast majority of the viral particles are subviral particles that consist of lipid and envelope proteins. They can block or enhance virus infection (Bruns *et al.*, 1998), depending on the ratio of virions to subviral particles and the multiplicity of infection.

Due to the small size of hepadnaviral genomes, these viruses have a very compact gene organisation with only three or four overlapping open reading frames (ORFs) and corresponding promoters, enhancers, and a single processing/polyadenylation signal. The preC/C gene codes for the nucleocapsid protein (C protein) and a precursor protein (preC protein) for a secretory protein, designated e-antigen. C protein expression is accomplished through initiation of translation at the second AUG of the C gene. Synthesis of the preC protein requires translation initiation at the first AUG. All envelope proteins are synthesized from the preS/S gene by translation initiation at different AUGs and termination at the same site. The gene coding for the P protein overlaps amino-terminally with the C gene, in the middle with the preS/S gene, and carboxyl-terminally with the X gene and is synthesized from the pregenomic RNA, presumably by ribosomal scanning. The X gene, shown to be expressed only in mammalian hepadnaviruses, codes for a protein with a variety of regulatory functions (Yen, 1996). An X-like ORF has recently been identified also in HHBV and RGHV genomes (Netter *et al.*, 1997) but is lacking in DHBV. It is currently not known whether HHBV and RGHV express X-like proteins.

Studies on DHBV have contributed substantially to the understanding of various aspects of the life cycle of hepadnaviridae, more than any other hepadnavirus. A major reason for this fact is the availability of ducks for experimental infection *in vivo* and the access to DHBV permissive primary hepatocytes that can be prepared from ducklings or embryos (Köck and Schlicht, 1993; Tuttleman *et al.*, 1986). In contrast to HBV and rodent hepadnaviruses, chronic infection with DHBV has little, if any, pathogenic consequence for ducks, except when preS protein of DHBV is mutated (Lenhoff and Summers, 1994), and is not associated with the development of hepatocellular carcinoma (Cova *et al.*, 1993). It has been speculated that the latter could be due to the fact that DHBV lacks an X gene (Yen, 1996).

The preS domain of the large envelope protein determines the host range of avian hepadnaviruses as shown with recombinants of HHBV and DHBV (Ishikawa and Ganem, 1995). Major progress has recently also been made in the elucidation of the early steps of host cell-receptor interaction using DHBV and primary duck hepatocytes (PDH). DHBV and HHBV preS proteins were shown to bind specifically to a carboxypeptidase D-like protein, gp180 (Eng *et al.*, 1998; Kuroki *et al.*, 1995; Tong *et al.*, 1995), and to mediate cell entry of DHBV (Breiner *et al.*, 1998; Urban *et al.*, 1998), suggesting that gp 180 is part of the DHBV receptor. In some studies, a rather long preS sequence and a specific conformation were found to be important for gp180 binding (Urban *et al.*, 1998), whereas other studies argue for short sequences and specific amino acids therein (Tong *et al.*, 1995). In any case, new avian hepadnaviruses with divergent preS sequences may be helpful for further elucidation of these early steps in infection.

Here we describe the identification and functional analysis of a new member of the avian hepadnavirus subfamily naturally infecting snow geese that provides new information and experimental opportunities for studies on hepadnaviruses.

RESULTS

Detection of HBV particles in sera of snow geese

The 15 sera from snow geese available were screened for the presence of DHBV-related envelope proteins by immunoblotting using rabbit polyclonal antisera raised against the DHBV preS domain and the DHBV S protein, respectively. Based on this assay, five sera (DW1, DW2, DW4, DW9, and DW13) scored positive with both antisera, and the corresponding virus was designated SGHBV. Using the anti-preS polyclonal antiserum, a dominant signal of two closely spaced bands at the position of 34/33 kDa as well as some minor bands were detected, whereas the dominant DHBV preS protein of viremic duck sera migrated slightly slower at a position of p36/35 (Fig. 1A). These data indicate that the signal at position p34/33 corresponds to the major phosphorylated (p34) and nonphosphorylated (p33) preS protein of snow goose virus. The less intense staining bands are most likely proteolytic degradation products and minor preS proteins, similar to those described for DHBV (Fernholz *et al.*, 1993). The polyclonal anti-S antiserum reacted predominantly with a protein of a molecular mass of 17 kDa when using viremic snow goose and duck sera, indicating that these signals correspond to the S protein of SGHBV and DHBV (Fig. 1B), respectively. As expected, the major SGHBV and DHBV preS proteins were also detected by the anti-S serum as weak bands at positions p34/33 and p36/35, respectively. Taken together, these data indicate that 5 of the 15 snow geese tested are infected with a DHBV-related virus with immunologically strongly cross-reactive envelope proteins. The slightly faster electrophoretic mobility of preS protein of SGHBV in all tested animals compared with that of DHBV suggests a difference in sequence, conformation, or size of the corresponding preS proteins.

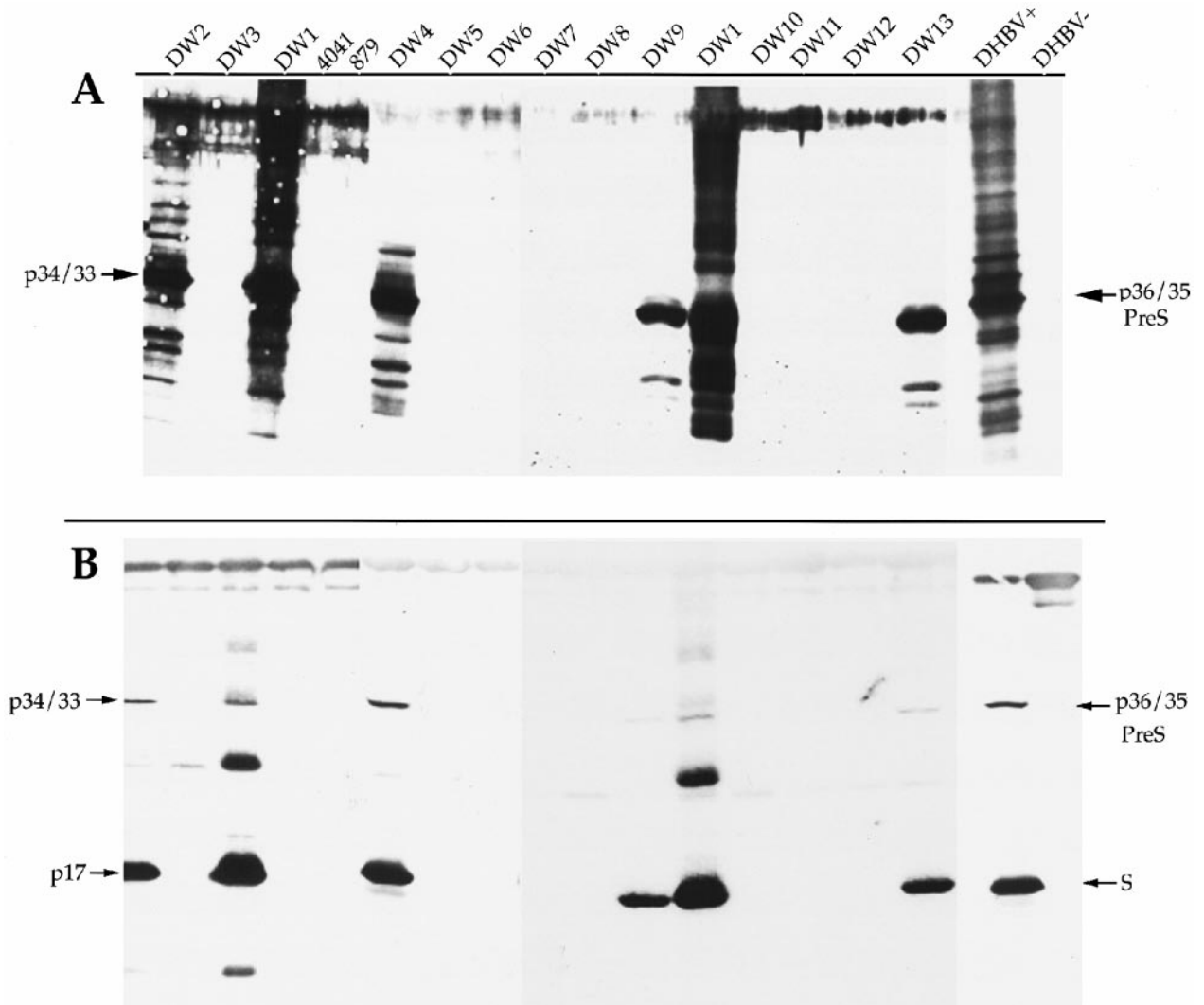


FIG. 1. Viral envelope proteins preS and S from snow goose sera (DW) and a Pekin duck serum (DHBV+) as revealed by immunoblotting with polyclonal anti-DHBV preS (A) or S polyclonal antibodies. The positions of the major phosphorylated and nonphosphorylated DHBV and SGHBV preS proteins (p36/35 and p34/33) as well as of the S protein (p17) are indicated by arrows.

Cloning and sequencing of SGHBV

All snow goose sera previously tested by immunoblotting were next screened by full-length genome PCR amplification with oligonucleotide primers P1 and P2 that are homologous to the well conserved sequence in the nick region of the DHBV genome (Günther *et al.*, 1997). With one serum (DW1), an amplification product of DHBV genome size was obtained, whereas all others were negative, presumably because of the low sensitivity of the assay used and the lower titers of the other sera. The amplification product obtained with serum DW1 was purified and cloned, and the amplified DNA was sequenced both directly and after cloning (five clones). To exclude potential P1 and P2 primer-induced mutations in the amplification product, we also amplified an approximately 1-kb-long SGHBV subgenomic fragment from the DW1 serum using primers P9 and P10, which anneal

upstream and downstream of the nick region of the SGHBV genome. The amplified fragment was also sequenced both directly and after cloning. The data obtained did not reveal any primer-induced mutations in the nick region (data not shown).

According to the sequence analysis, the genomes of SGHBV are 3024 bp in length, which is identical in size to most Chinese DHBV isolates (Fig. 2, and data not shown), and show all the genes (polymerase, precore/core, preS/S) known for DHBV (Fig. 2). However, in contrast to all DHBV genomes, SGHBV has an additional short ORF, which corresponds in location to the X gene of mammalian hepadnaviruses and to a similar putative gene in HHV and RGHV (Netter *et al.*, 1997). The DNA sequences derived from direct sequencing and sequencing of cloned SGHBV genomes were in close agreement and showed very little sequence variation.

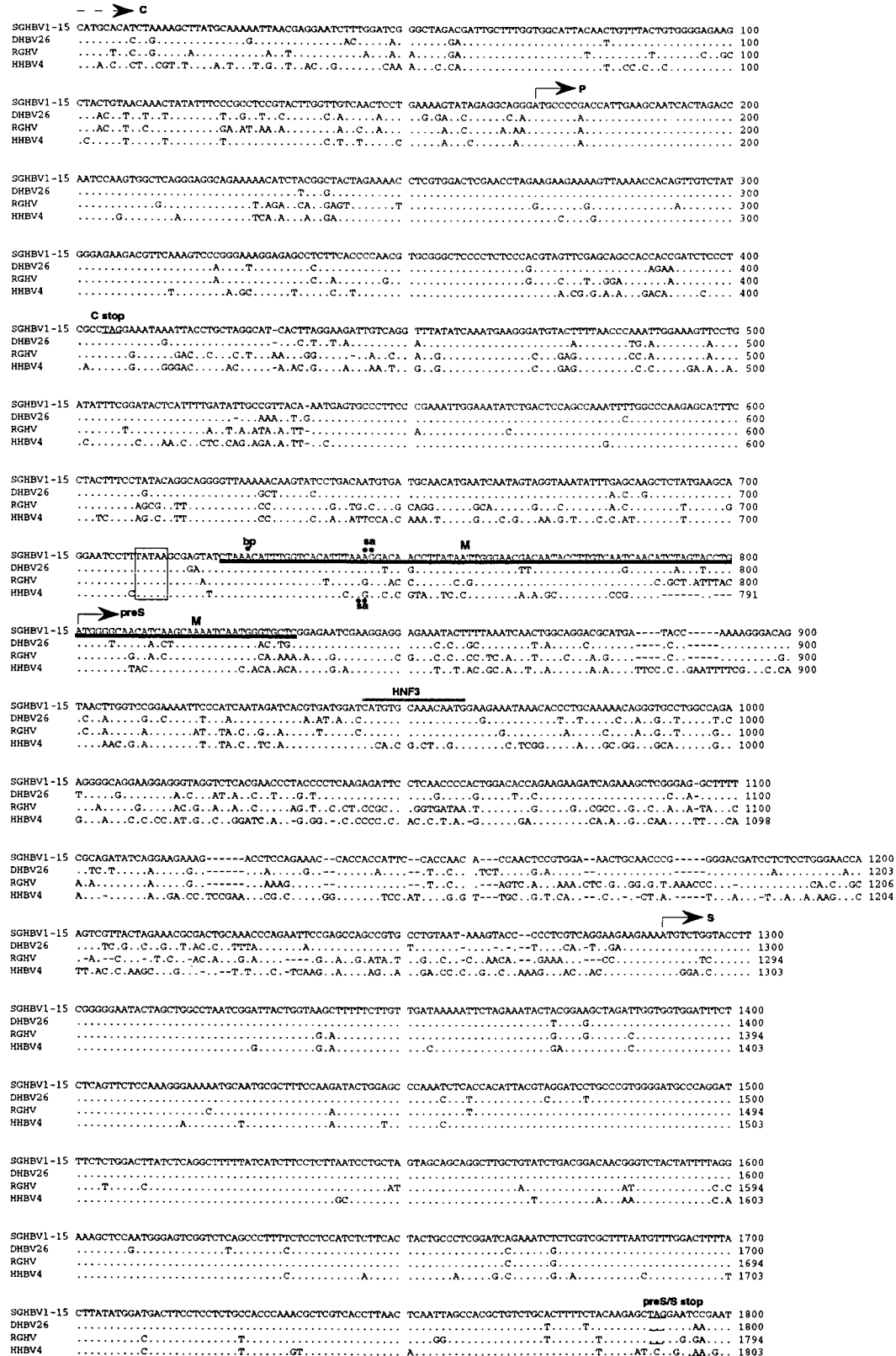


FIG. 2. DNA sequence alignment of the genomes SGHBV1-15, DHBV26, RGHV, and HHBV4. Start and termination codons for the viral genes and ORFs (P, polymerase; S and preS, envelope proteins; preC and C, precore and core) are indicated by arrows. Regulatory sequences (DR, direct repeats; pA, processing/polyadenylation signal; 3E, M, and 5E, regions important for DNA plus strand synthesis; enh, enhancer; sa, splice acceptor; sd, splice donor; bp, branch point for splicing) and transcription factor binding sites (HNF, hepatocyte nuclear factor; C/EBP, enhancer binding protein) are also given. TATA sequences regulating transcription initiation are boxed. E stands for encapsidation signal.

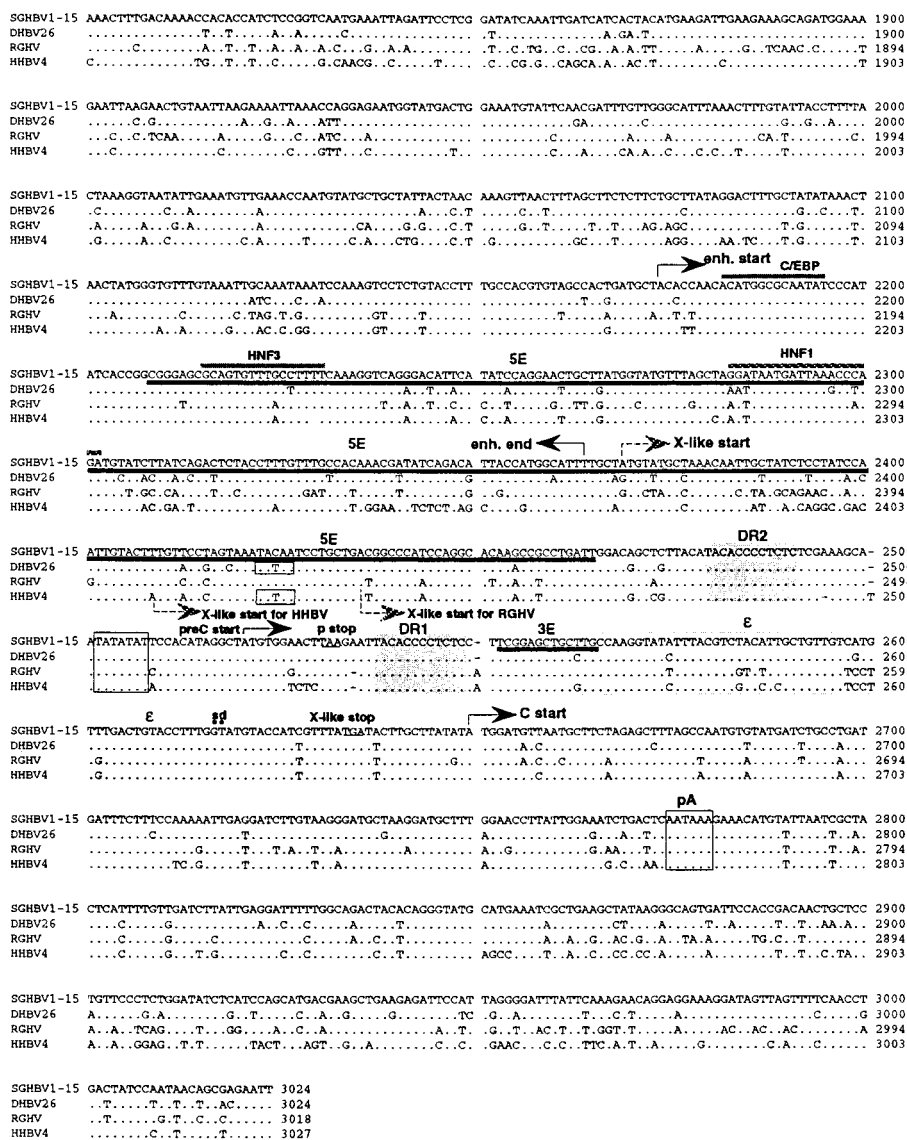


FIG. 2—Continued

For the five cloned genomes, DNA sequence variation was observed only at 34 positions (Table 1). These differences predict 28 amino acid changes in viral proteins, most of them located in the P protein and none located in the C protein (Table 1). Because of the calculated and experimentally determined very low PCR error frequency occurring during the full-length genome amplification procedure used (Günther *et al.*, 1998), most, if not all, of the sequence variation detected probably corresponds to the natural sequence variation of the SGHBV genomes present in the serum of the animal tested.

Phylogenetic relationship of SGHBV with other avian hepadnaviruses

All complete 23 avian hepadnavirus genomes, including the sequences of the 5 cloned SGHBV genomes, of 12 DHBV isolates (DHBV1, 3, 16, 22, 26, f1-6, i, p2-3,

qca34, s5, s18-B, and s31), of 5 HHBV isolates (HHBV-4, A, B, C, and D), and of 1 RGHV isolate, were aligned to elucidate the likely evolutionary relationship of SGHBV with all other avian hepadnaviruses. Based on this alignment, a split position analysis was performed using the SplitsTree program from Daniel Huson (Huson, 1998). According to this analysis all avian hepadnavirus genomes, including SGHBV, originate from a common ancestor (Fig. 3). The tree-like structure derived from this analysis indicates that SGHBV is most closely related to the DHBV isolates and places SGHBV in evolution between RGHV and DHBV. This is remarkable because SGHBV diverges in average by 11–13% in DNA sequence from DHBV isolates that differ in sequence among each other by less than 11%. The sequence divergence of SGHBV from RGHV and HHBV is about 22% and about 27%, respectively, which implies a less close evolution-

TABLE 1
Variation of SGHBV Sequences Amplified from
Snow Goose Serum DW1

NT Position	Clone	Amino acid changes in				X-like
		preC	P	preS	S	
C321A	7	—	—			
A441G	15		K91R			
A528C	15		D120A			
A574G	13		—			
T658C	9		—			
A829G	15		—	D10G		
A837G	9		E223G	R13G		
G914A	15		E249K	—		
A1130G	19		N321D	—		
A1151G	19		T328A	—		
A1276G	15		—	K150R		
G1303A	13		—		G6E	
A1358T	7		N397Y		K24N	
T1397C	7		F410L		—	
A1438C	9, 13		—		Q51P	
C1505T	19		—		—	
C1555T	19		—		A90V	
C1795T	19		—			
C1831T	13		—			
A1878G	13		K570R			
A1933T	19		—			
T2031C	7		M621T			
G2171C	19		A668R			
C2172G	19		A668R			
A2176G	19		—			
A2282G	13		R705G			
C2320T	15		—			
A2396G	7		I743V			—
A2398C	15		—			Y9S
T2405C	9		Y746H			—
C2459T	13		P764S			—
C2540G	19	T7R				N55stop
T2581C	9	S21P				V70A
C2964T	9	—				

ary relationship with these viruses compared with DHBV. Taken together, these data indicate that SGHBV is a distinct member of the avian hepadnavirus subfamily that has recently evolved from DHBV, or vice versa.

Comparison of regulatory SGHBV sequences with those of other avian hepadnaviruses

The alignment of the sequences shows that elements essential for replication, including the direct repeats DR1 (one mutation in isolate SGHBV clone 19 at position C2540G; Table 1) and DR2, the encapsidation signal sequence epsilon (one nucleotide mutation in a nonessential area at position 2581 in SGHBV clone 9), as well as known important sites involved in regulation of transcription in DHBV, are all completely or almost completely conserved in SGHBV (Fig. 2). The core promoter-specific and the preS promoter-specific TATA boxes (positions 2502–2508 and 710–714, respectively) are

conserved among SGHBV, DHBV, RGHV, and HHBV4 (Fig. 2). In contrast, the putative TATA box of the preC promoter in DHBV (nucleotide positions 2423–2427) is mutated in SGHBV (T2425C) and in RGHV (Fig. 2). The sequence of the binding site for HNF3 (positions 959–945), which was reported to be important for the activity of the TATA-less promoter of the DHBV S gene (Welsheimer and Newbold, 1996), is also conserved in SGHBV (Fig. 2) but not in HHBV. The SGHBV sequences between positions 2174–2365 correspond to the DHBV enhancer region, and all three important transcription factor sites (C/EBP, HNF3, and HNF1) within this region (Crescenzo-Chaigne *et al.*, 1995; Lilienbaum *et al.*, 1993; Liu *et al.*, 1994) are very well conserved. A splice donor and acceptor sequence that gives rise to a spliced preS transcript in DHBV *in vivo* (Obert *et al.*, 1996) is also present in SGHBV. The polyadenylation/processing signal sequence in SGHBV is identical to that of all known avihepadnaviruses (Fig. 2). Taken together, these data suggest that the strategies of replication and transcription of SGHBV are similar or identical to that of DHBV.

Comparative protein sequence analyses

The amino acids of all SGHBV viral proteins were compared with those from all other avian hepadnavirus isolates to identify amino acid changes that are unique for SGHBV or are shared with non-DHBV avian hepadnaviruses only. In the core protein coding region were found four amino acids unique to SGHBV (E24, S87, E107, and A215) and three changes that SGHBV shares with HHBV only (V3, T160, and S248) but with none of the other avihepadnaviruses (marked in Fig. 4A in which only the sequences of one genome of 12 DHBV, 1 RGHV, and 5 HHBV isolates are shown). In the preS and S protein coding regions, amino acids unique for SGHBV were identified at seven positions (H4, A11, S42, H87, T143, Y155, and L157). At three positions, amino acids were identical to those in RGHBV and/or HHBV preS (E67, R140, and K193) but differed from all DHBV isolates (marked in Fig. 4B). The amino acids in the structural SGHBV proteins that are unique or shared only with RGHV/HHBV may have emerged as a consequence of host adaptation and C/preS protein interaction. In the P protein, there are more amino acids unique to SGHBV than in the structural proteins. Most of them are located in the amino-terminal domain (7 of 10). Four amino acids are shared with HHBV or RGHV but not with any of the DHBV isolates (marked in Fig. 4C). None of these amino acids have an obvious consequence for the various known functions of the P protein. The YMDD motif of the active site of the RTase and the tyrosine residue to which the DNA minus strand is covalently linked are conserved in SGHBV (Fig. 4C). The spacer domain of the P protein of SGHBV diverges most profoundly from DHBV and the other avian HBV species (Fig. 4C).

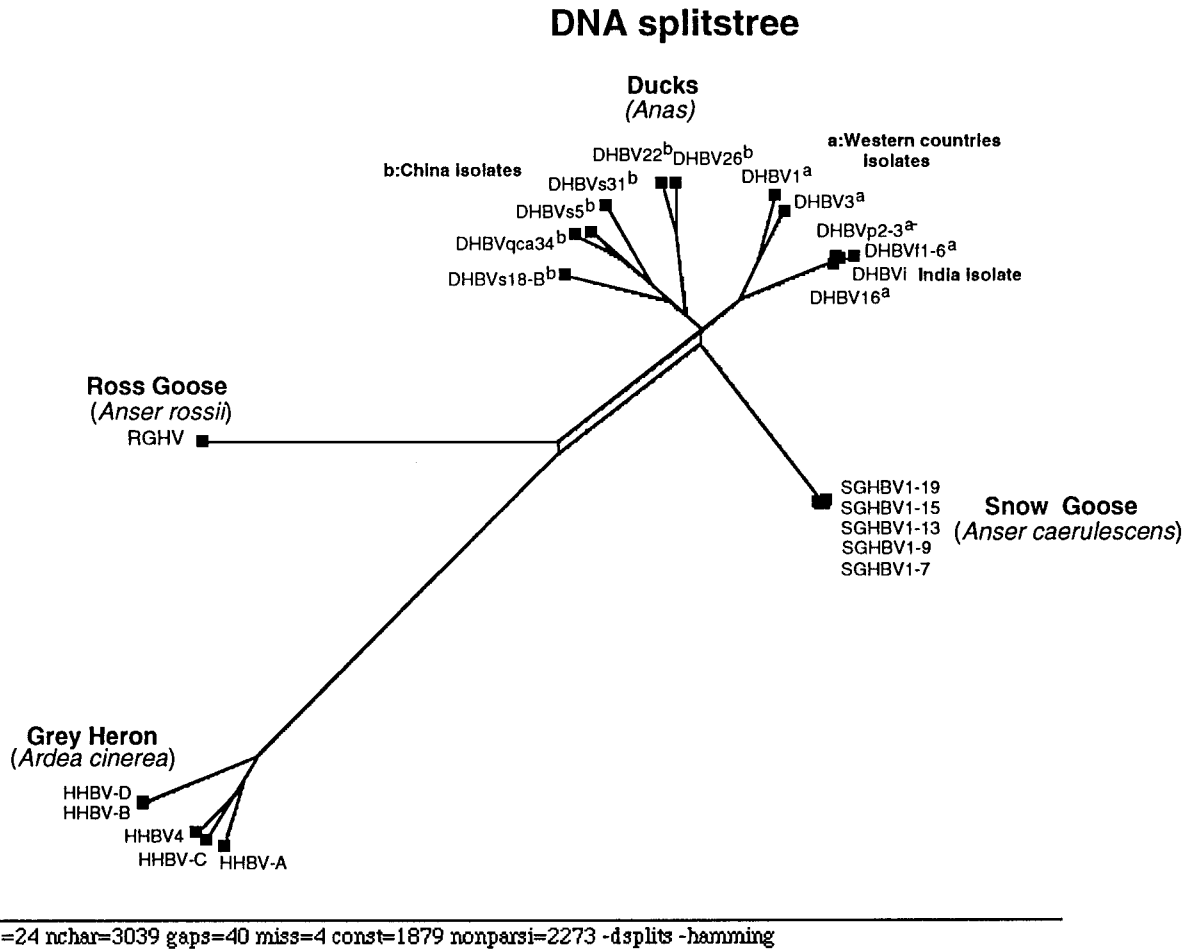


FIG. 3. Phylogenetic relationship of avian hepadnaviruses based on DNA sequences of all known viral genomes available from GenBank and established using the SplitsTree software program (Huson, 1998).

In contrast to DHBV, an ORF located in a position similar to those of the mammalian hepadnavirus X genes is apparent in all SGHBV genomes. In HHBV and RGHV, similar X gene-related ORFs are present (Netter *et al.*, 1997). However, the SGHBV X gene-related ORF (87 amino acids) is longer than those of HHBV (75 amino acids) and RGHV (66 amino acids) and differs in sequence (Fig. 4D). Note, if one assumes use of a nonconventional translation initiation codon, an X-like protein similar in length and sequence to that of SGHBV could also be expressed by all known isolates of DHBV (Fig. 4D, and data not shown). In one of the SGHBV genomes (SGHBV1-19), there is a stop codon (N55stop) at the carboxyl-terminal end of the X-like gene that would allow the expression of only a amino-terminally truncated X-like protein. Preliminary evidence suggests that a full-length X-like protein in SGHBV has functional similarities with that of mammalian X proteins (unpublished data).

Viral protein expression of cloned SGHBV genomes

The cloned SGHBV genomes were transfected into LMH cells to analyse their competence for viral gene

expression. Four days after transfection, the viral proteins in the cells as well as in the culture medium were analysed. In extracts of cells transfected with the cloned SGHBV genomes (Fig. 5), the major SGHBV preS protein (p34/33), as well as smaller versions thereof, were detected by immunoblotting (Fig. 5A). The major DHBV preS protein, analysed in parallel as a control, migrated slightly slower than that of SGHBV. This finding is consistent with the analysis of the preS proteins in sera of birds naturally infected (Fig. 1A) and confirms the characteristic difference of the major DHBV and SGHBV preS proteins in electrophoretic mobility. It further demonstrates that the transfected cloned DNA is indeed derived from the snow goose serum and is not a DHBV contaminant. All SGHBV genomes expressed also a small surface protein S of 17 kDa (p17) identical in mobility to that of DHBV (Fig. 5B). Furthermore, all SGHBV genomes expressed a nucleocapsid protein (p35) with slower electrophoretic mobility than that of DHBV (p32) as revealed by immunoblotting (Fig. 5C). Taken together, these data imply that all five full-length SGHBV genomes isolated by

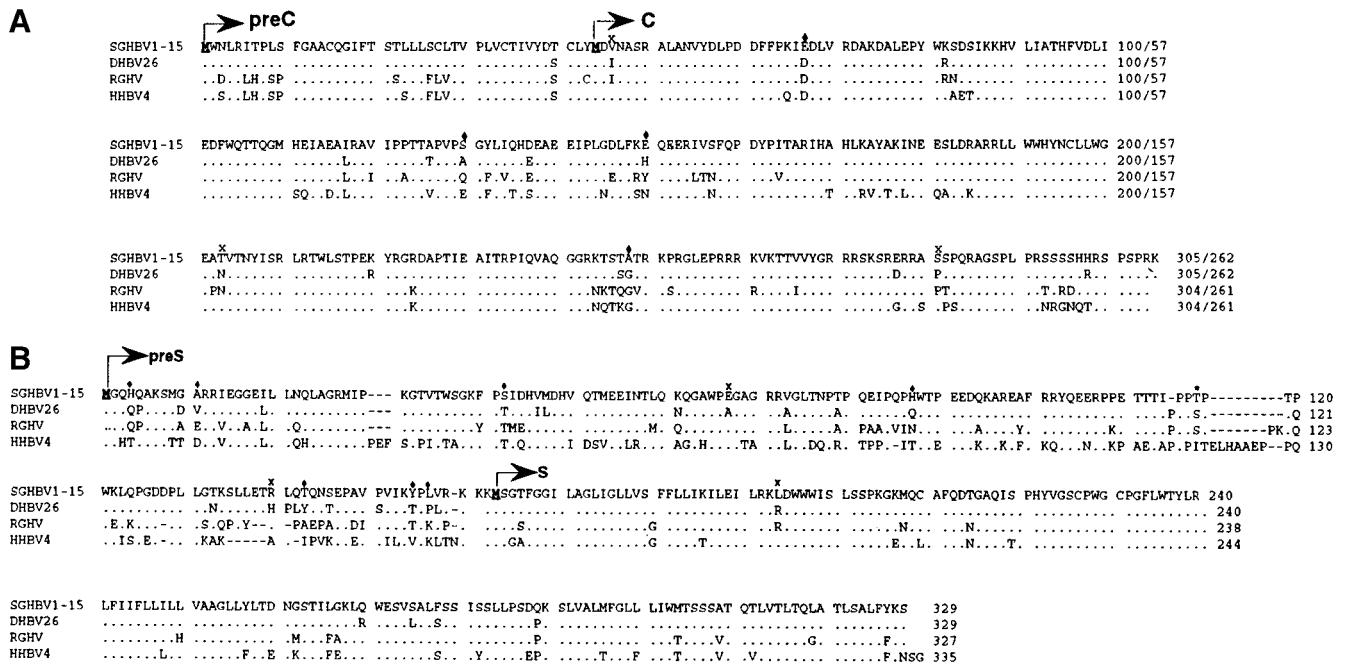


FIG. 4. Sequence comparison of the viral proteins from SGHBV1-15, DHBV26, RGHV, and HHBV4. (A) PreC/C proteins. (B) PreS/S proteins. (C) P proteins. (D) X-like ORF-derived proteins. ♦, Amino acids that are unique for SGHBV compared with all known avian HBV sequences. ×, The sequence of SGHBV is different from all known DHBVs but similar to RGHV or HHBV4. *Potential phosphorylation sites. Translation start codons are underlined.

cloning are competent in expression of the structural proteins with slightly different electrophoretic mobility (except S).

Replication competence of the cloned SGHBV genomes

To examine whether the cloned SGHBV genomes are replication competent, we also analysed the replicative intermediates isolated from cytoplasmic core particles of the LMH cells transfected with the cloned DNA (see above) by Southern blotting. As in DHBV DNA-transfected LMH cells, open circular (RC) and single-stranded (ss) minus SGHBV DNAs were observed as major forms of intracellular replicative intermediates in core particles (Fig. 6A). The amount of replicative intermediates observed with the various SGHBV genomes was variable. This was not due to differences in transfection efficiencies because both the amounts of β-galactosidase, expressed from a cotransfected reporter plasmid (data not shown), and that of the expressed SGHBV proteins, as determined from the same transfected cells (Fig. 5), were very similar. No replicative intermediates were detected in cells transfected with the viral genome of clone SGHBV1-7 (Fig. 6A, lane SGHBV1-7), indicating that it is replication defective. These data imply that four of the five cloned SGHBV genomes are replication competent and use a similar or an identical replication strategy as DHBV. The single-point mutation in DR1 of the

SGHBV1-19 genome (Table 1) resulted in no obvious defect in viral replication (Fig. 6A).

Type of SGHBV viral particles secreted from transfected LMH cells

To analyse whether the cloned SGHBV genomes are competent in viral particle assembly and secretion, viral particles from the supernatant of the transfected cells were partially purified by ultracentrifugation through a sucrose cushion. The Southern blot analysis of the DNA of these samples showed RC DNA in the medium of the cells transfected with all replication-competent SGHBV clones and the DHBV genome but not with replication-defective genome SGHBV1-7 (Fig. 6B). Unlike DHBV, all viral particles of the replication-competent SGHBV genomes produced in addition strong signals corresponding to ssDNA minus strands. These molecules were even more prevalent than RC DNA (Fig. 6B), suggesting that they were produced at the expense of RC DNA. Taken together, these data indicate that four of the five SGHBV genomes are competent in virus particle assembly and secretion. However, secretion of a fraction of viral particles containing ssDNA is a unique feature of SGHBV genomes.

The unique characteristic of some secreted SGHBV particles containing DNA of the size of ssDNA minus strands prompted us to investigate whether this DNA

C

SGHBV1-15	MPRPLKQSLD QSKWLREAEK HLRLLLENLVD SNLEEEKLKP QLSMGEDVQS PGKGEPLHPN VRAPLSHVVR AATTDLPRLG NKLPARHHLG RLSGLYQMKG 100
DHBV26	..Q..... ..V..... ..I..... ..R..... ..R..... ..E..... ..K..... ..K..... 100
RGHV	..Q..... ..R..... ..I..... ..E..... ..G..... ..E..... ..S..... ..R..... ..LL..... ..GT..... ..D..... ..K..... ..K..... 100
HNBV4	..Q..... ..R..... ..K..... ..I..... ..K..... ..E..... ..D..... ..R..... ..L..... ..EA..... ..D..... ..IG..... ..ETRH..... ..P..... ..H..... ..RD..... ..K..... ..K..... 100
SGHBV1-15	CTFNPWKVP DISDTHFDIA VINECPSPRW KYLTFARFVP KSISYFPQA GVKNKYPDNV MQHESIVGKY LSKLYEAGIL YKRVSXHLVT FKGQPYNWER 200
DHBV26	..E..... ..MQ..... ..IV..... ..V..... ..A..... ..A..... ..TR..... ..I..... ..I..... ..I..... 200
RGHV	..E..... ..Q..... ..NLD..... ..II..... ..AHS..... ..P..... ..D..... ..AG..... ..Q..... ..Q..... ..T..... ..P..... ..E..... ..T..... ..Q..... 200
HNBV4	..E..... ..H..... ..I..... ..A..... ..N..... ..SQE..... ..II..... ..L..... ..VHS..... ..P..... ..EFQ..... ..QN..... ..L..... ..ND..... ..N..... ..F..... ..PYFT..... ..Q..... 200
SGHBV1-15	QYLVNQHLVP DGATSSKING CSENRRRRNT FKSTGRTHDT KR---DSNLVRKI PINRSRDGSC ANNGRNKHPA KTGCLARRGR KEGRSHEPYP SRDSSPLDPT 300
DHBV26	..K..... ..Q..... ..S..... ..TC..... ..RA..... ..TP..... ..A..... ..IS..... ..P..... ..P..... ..H..... ..M..... ..GO..... ..SN..... ..HIR..... ..P..... ..G..... ..SS..... ..PRG..... ..CW..... ..G..... ..S..... ..INQSCS..... ..A..... ..V..... ..S..... 300
RGHV	..PADL..... ..H..... ..AT..... ..RKKS..... ..SGTP..... ..PSTI..... ..KD..... ..P..... ..G..... ..GHM..... ..SYHGT..... ..Y..... ..P..... ..D..... ..H..... ..T..... ..RG..... ..G..... ..S..... ..E..... ..TGNTQ..... ..SS..... ..PCR..... ..GDK..... 300
HNBV4	KH..... ..P..... ..Q..... ..H..... ..Y..... ..D..... ..RQ..... ..S..... ..II..... ..TATSS..... ..KN..... ..S..... ..S..... ..IGFAH..... ..NG..... ..SYHST..... ..H..... ..RLS..... ..TSD..... ..T..... ..SR..... ..A..... ..G..... ..D..... ..STPIGPGSTA..... ..AHP..... ..THHV..... ..R..... 299
SGHBV1-15	RRRESSSGGF SQISGRKTSR NHHHSTN-TNS VETATRGSS PGNVQVTR-NA TANP--EPRASR ACNK--VPPRQE EENWVYLRGN TSWPNRITKG LFLVDKNSRN 400
DHBV26	..S..... ..RS..... ..SL..... ..R..... ..ET..... ..D..... ..N..... ..SHIS..... ..A..... ..T..... ..K..... ..SA..... ..DS..... ..P..... ..L..... ..Y..... ..V..... ..DSST..... ..K..... ..I..... ..I..... ..I..... 400
RGHV	G.GRQGPRI..... ..QK..... ..R..... ..E..... ..KG..... ..SH-KST..... ..ENSVGAETRR..... ..SSP..... ..HA..... ..T..... ..VP..... ..SRT---SGTGY..... ..SGHQ..... ..NTESP..... ..F..... ..F..... ..F..... ..F..... 398
HNBV4	..KQKG..... ..VL..... ..QA..... ..EP..... ..E..... ..TRNG..... ..HHRVA..... ..C..... ..T..... ..SVEDFTR..... ..PFT..... ..QSKGAPRYQGT..... ..GTPDQAHG..... ..K..... ..Q..... ..GS..... ..V..... ..R..... ..I..... 401
SGHBV1-15	TPEARLVDF SQFSKGNAM RFPYRWSPNL TLRRILEVPG MPRISLDSQ AFYHLPLNPA SSSRLAVSDG QRVYFRKAP MGVLSPFLL HLFITLALGSE 500
DHBV26	..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... 500
RGHV	..A..... ..A..... ..H..... ..K..... ..S..... ..C..... ..I..... ..H..... ..K..... ..K..... ..K..... ..K..... ..K..... ..K..... ..K..... ..K..... 498
HNBV4	..E..... ..E..... ..C..... ..C..... ..C..... ..C..... ..C..... ..C..... ..C..... ..C..... ..C..... ..C..... ..C..... ..C..... ..C..... 501
SGHBV1-15	ISRRFNWTF TYMDFLLCH PNARHLNSIS HAVCTFLQEL GIRINFDKTT PSPVNEIRFL GYQIDHHYMK IEESRWKELR TVIKKIKGE WYDWKCIQRF 600
DHBV26	..A..... ..A..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... 600
RGHV	..A..... ..A..... ..R..... ..S..... ..V..... ..T..... ..K..... ..L..... ..DKF..... ..DQ..... ..N..... ..Q..... ..QI..... ..K..... ..K..... ..K..... ..K..... 598
HNBV4	..AS..... ..S..... ..S..... ..T..... ..F..... ..M..... ..TT..... ..E..... ..SKQH..... ..N..... ..V..... ..Q..... ..Q..... ..Q..... ..Q..... ..Q..... 601
SGHBV1-15	VGHILFVLFP TKGNIEMLKP MYAAITNKVN FSSFSAVRTL LYKLTMGVCK LQINPKSSVP LPRVATDATE THGAISHITG GSAVFAPSKV RDIHIQELLM 700
DHBV26	..H..... ..H..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... 700
RGHV	..I..... ..I..... ..V..... ..H..... ..V..... ..H..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... ..S..... 698
HNBV4	..I..... ..D..... ..C..... ..HR..... ..A..... ..R..... ..KI..... ..TLD..... ..V..... ..L..... ..L..... ..L..... ..L..... ..L..... ..L..... ..L..... 701
SGHBV1-15	VCLARIMIKP RCILSDSTFV CHKRYQTLFP HFAMYAKQLL SPIQLYFVPS KYNPADGPSR HKPPDWALT YTPLSKAIYI PHRLCGT 787
DHBV26	..KL..... ..T..... ..T..... ..N..... ..VP..... ..S..... ..P..... ..V..... ..V..... ..V..... ..V..... ..V..... ..V..... ..V..... ..V..... 787
RGHV	..A..... ..KL..... ..L..... ..T..... ..I..... ..VL..... ..M..... ..QN..... ..T..... ..T..... ..T..... ..T..... ..T..... ..T..... ..T..... ..T..... 785
HNBV4	..KL..... ..T..... ..Y..... ..RKFSK..... ..TRLT..... ..Y..... ..T..... ..V..... ..V..... ..V..... ..V..... ..V..... ..V..... ..V..... ..V..... 787

D

SGHBV1-15	MLNNCYLLSN CTLFLVNTIL LTAHPGTSRL IGQLLHTPLS RKQYIFHIGY VELKNYTPLL -RSCLPRYIY VYIAVVMFDC TFGMYHRL 87
SGHBV	Y..... ..Y..... ..Y..... ..Y..... ..Y..... ..Y..... ..Y..... ..Y..... ..Y..... ..Y..... ..Y..... ..Y..... ..Y..... 87/54
DHBV26	..LP..... ..YH..... ..YRA..... ..I..... ..N..... ..R..... ..R..... ..R..... ..R..... ..R..... ..R..... ..R..... ..R..... ?
HNBV4/A	MYP..... ..I..... ..M..... ..Q..... ..NL..... ..RP..... ..SI..... ..Y..... ..-VSKI..... ..S..... ..I..... ..V..... ..L..... ..DL..... ..PC..... ..C..... 75
HNBV4/C/D	MYP..... ..I..... ..M..... ..Q..... ..IN..... ..RP..... ..NI..... ..Y..... ..-VSKI..... ..S..... ..I..... ..V..... ..L..... ..NL..... ..PC..... ..C..... 75
RGHV	M..... ..Q..... ..INL..... ..H..... ..S..... ..G..... ..I..... ..S..... ..I..... ..F..... ..VF..... ..PC..... ..C..... 66

FIG. 4—Continued

is actually contained in immature core particles released from the transfected cells, as known for HBV, or in virion-like particles. To analyse this, the viral particles pelleted from cell culture medium of cells transfected with genome SGHBV1-15 or with DHBV26 (for comparison) were separated in 10–70% sucrose gradients by ultracentrifugation. When the fractions of these gradients were analysed for the presence of viral DNA by Southern blotting, viral DNA was identified predominantly in fractions with a density of 1.13 to 1.16 g/cm³ indicative for virions (Fig. 7). In the gradient with DHBV particles only signals were seen at the position of RC DNA and linear dsDNA (Fig. 7, right), whereas in the gradient with SGHBV, there were, in addition, strong signals at the position of ssDNA and below. This is consistent with the type of DNA present in the particles used for gradient centrifugation (Fig. 7, input lanes). Analysis of the same fractions by immu-

noblotting using DHBV core protein-specific polyclonal antibodies revealed specific signals in the viral DNA-containing fractions and in fractions with higher density (Fig. 7B). The latter indicates that nonenveloped core particles without viral DNAs are released from both DHBV- and SGHBV-transfected cells and are well separated from virion and subviral particles. This conclusion is corroborated by an analogous analysis of the fractions with a polyclonal antibody against preS envelope protein that revealed reactive antigen almost exclusively in the DNA-containing fractions (Fig. 7C). The data of the DNA and protein analysis imply that SGHBV1-15-transfected cells release virions and virion-like particles with RC and ssDNAs, respectively, as well as core particles without detectable viral DNA. In contrast, in the medium of DHBV26-transfected cells, virtually no virion-like particles were detected. The density of the corresponding fractions is consistent

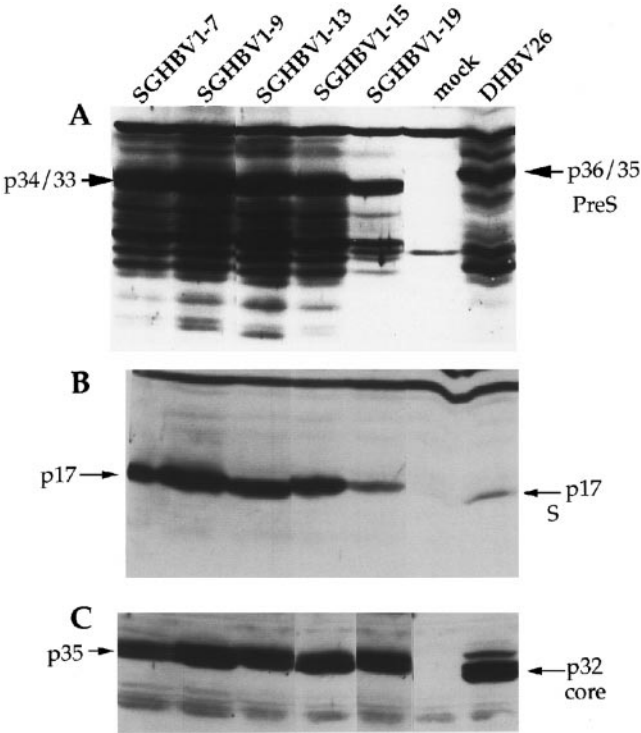


FIG. 5. Expression of envelope and core proteins from SGHBV genomes transfected into LMH cells as determined by immunoblotting. (A) PreS proteins (p34/33) detected with a polyclonal anti-DHBV preS antibody. (B) S protein (p17) detected with a polyclonal anti-DHBV S antibody. (C) C protein (p35) detected with a polyclonal anti-DHBV C antibody.

with this interpretation (Fig. 7D). In conclusion, release of a substantial fraction of virion-like particles with ssDNA is a unique feature of SGHBV.

Identification of SGHBV ssDNA in viremic snow goose sera

To examine whether shedding of virion-like particles with viral ssDNA is a unique feature of SGHBV and not a cell culture artifact, we extracted the type of viral DNA from SGHBV viremic sera of snow geese (DW1, DW2, and DW4) and, as a control, from a DHBV-positive duck serum. Analysis of the viral DNA from the snow goose sera and the duck serum by Southern blotting showed strong signals corresponding to RC of various sizes (Fig. 8). However, a discrete band corresponding to the size of ssDNA was obtained in addition with the DNA from all SGHBV sera but not with that from the DHBV-positive duck serum (Fig. 8). Semiquantitative analysis of the blots suggests that the ratio of ssDNA to RC DNA varies in different isolates from approximately 10% to 50%. These data imply that secretion or release of virion-like particles with viral ssDNA is a unique feature of SGHBV that occurs both in transfected cells and *in vivo*.

Infectivity of SGHBV in PDH

The sequence difference between SGHBV and all other known avian hepadnaviruses prompted us to examine whether PDH are permissive for infection with SGHBV. Therefore, similar amounts of SGHBV and DHBV viral particles (Fig. 9B) harvested from the culture medium of LMH cells after transfection either with four cloned SGHBV full-length genomes (SGHBV1-7, SGHBV1-9, SGHBV1-15, and SGHBV1-19) or with a plasmid containing cloned DHBV3 genome were used to infect PDH. Expression of intracellular preS protein in the infected PDH was taken as indication for infection. This was analysed by immunoblotting with a polyclonal anti-DHBV preS antibody. Strong signals for preS protein expression were seen in cells infected with SGHBV genomes SGHBV1-15 and SGHBV1-19 as well as with the DHBV3 genome (Fig. 9A). Cells incubated with viral par-

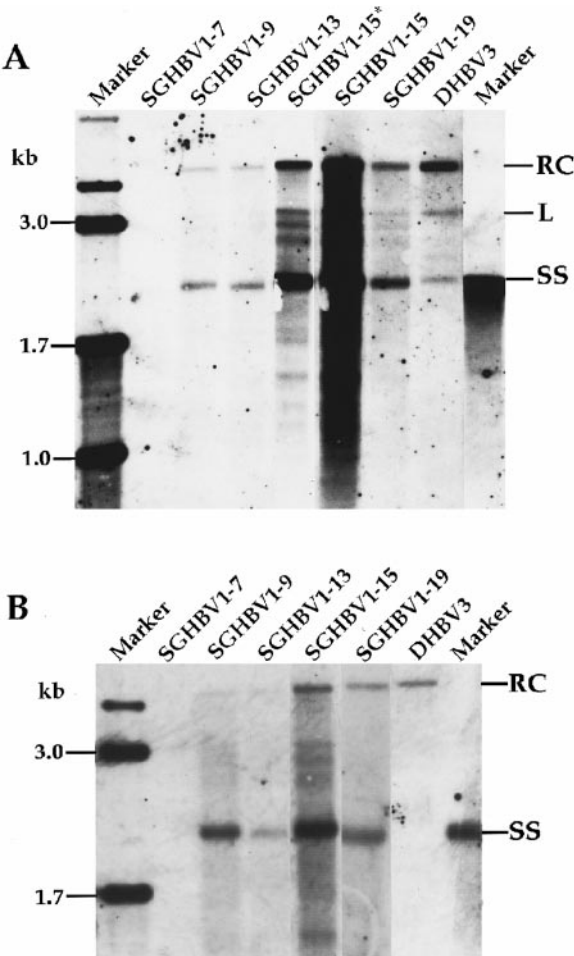


FIG. 6. Southern blot analysis of viral DNA synthesis of SGHBV genomes after transfection into LMH cells. (A) intracellular replicative intermediates. (B) Viral DNA from cell culture media. RC, L, and SS indicate the positions of the RC DNA, of linear dsDNA, and ssDNA minus strands, respectively. SGHBV1-15* is a shorter exposure of lane SGHBV1-15. Full-length DHBV and restriction fragments thereof as well as denatured linear DHBV dsDNA were used as size markers (marker lanes).

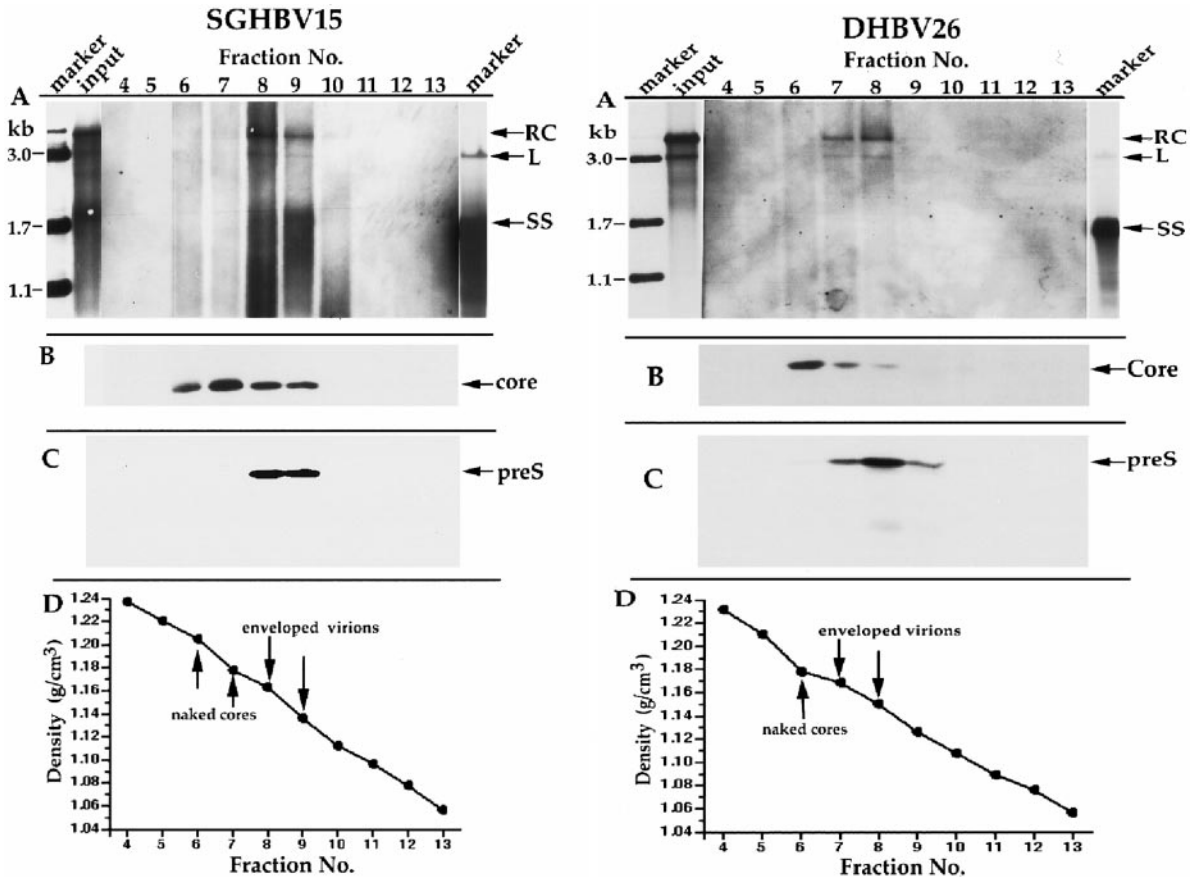


FIG. 7. Analysis of viral particles harvested from LMH cells transfected with genome SGHBV1-15 or DHBV 26 by sucrose gradient centrifugation. The DNA of each fraction was analysed by Southern blotting (A) and the C (B) and preS (C) proteins by immunoblotting using the corresponding polyclonal DHBV antibodies. The SGHBV and DHBV DNAs from the viral particles loaded onto the gradient were also analysed (input lanes). Full-length DHBV and restriction fragments thereof as well as denatured linear DHBV dsDNA were used as size markers (lanes marker). RC, L, and SS denote the positions of the relaxed circular, linear, and viral ssDNA. (D) The sucrose gradient density of the fractions and the position of naked core particles and enveloped particles are indicated.

ticles produced from the replication-defective SGHBV genome SGHBV1-7 and from the replication competent genome SGHBV1-9 did not result in preS protein expression (Fig. 9A). These data indicate that at least two of the four cloned SGHBV genomes are infectious for PDH.

DISCUSSION

In this report, we demonstrated that about 30% of a flock of snow geese were infected with a distinct new member of the avian hepadnavirus subfamily. This virus has unique DNA and protein sequence features and, unlike all other known hepadnaviruses, produces a fraction of virion-like particles with ssDNA minus strands at the expense of RC DNA. Unlike DHBV, but similar to mammalian hepadnaviruses and as recently reported for HHBV and RGHV, SGHBV has an ORF in a position analogous to that of the mammalian hepadnavirus X genes. This implies that all hepadnaviruses, except possibly DHBV, potentially encode a small regulatory protein that may be similar or identical in function to the mammalian hepadnavirus X proteins.

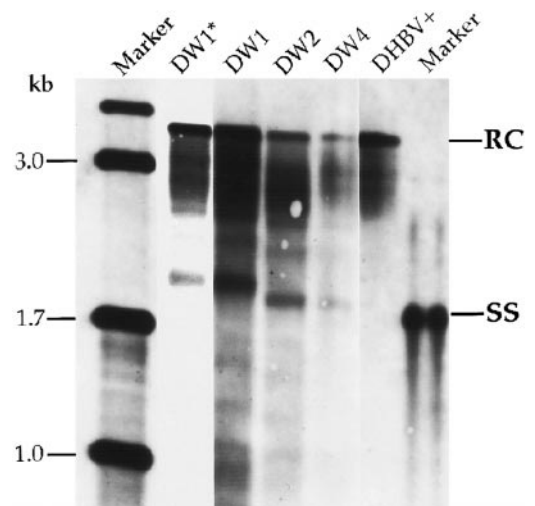


FIG. 8. Southern blot analysis of viral DNA isolated from sera of snow geese (DW) and a duck (DHBV+). RC and SS indicate the positions of the relaxed circular and ssDNA minus-strand DNA, respectively. Lane DW1* denotes a short exposure of lane DW1.

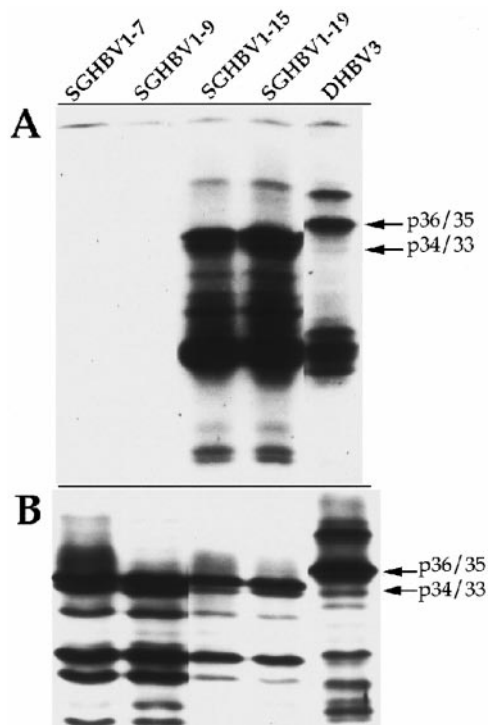


FIG. 9. Testing of the infectivity of SGHBV viral particles produced from transfected LMH cells on PDH. (A) Intracellular preS proteins expressed from the SGHBV genomes 7 days after infection as determined by immunoblotting with an anti-DHBV preS antibody. (B) Detection of preS proteins in an aliquot from the inoculum by immunoblotting shows that similar amounts of viral particles were used for infection.

The snow geese we analysed were housed together with ducks. This raises the possibility that they were horizontally infected by DHBV from ducks. We consider this scenario as very unlikely for several reasons. First, horizontal infection of DHBV, even between ducks, is very rare if it occurs at all (Schödel *et al.*, 1989). Second, none of the numerous DHBV isolates share with SGHBV a putative X-like gene. Third, for none of the DHBV isolates has the synthesis of such a large fraction of virion-like particles with ssDNA ever been reported. Fourth, the comparative sequence analysis indicated sequence features of SGHBV viral proteins shared with HHBV and RGHV. Fifth, all DHBV isolates from ducks in Western countries are 3021 nucleotides long, whereas SGHBV genomes have 3024 nucleotides. DHBV with 3024 nucleotides were so far isolated only from ducks of Chinese origin, and such ducks were not kept together with the snow geese analysed here. Nevertheless, our findings fully exclude neither horizontal transmission of an unusual DHBV isolate to snow geese nor selection of a minor DHBV variant from a heterogeneous virus population after transmission to snow geese. Further studies are also necessary to establish the epidemiological distribution of SGHBV in nature because infected animals were identified in only one of the two flocks tested and both were kept in captivity. Finally, all of the infections in

the animals we analysed may originate from a single infected snow goose that may have transmitted the virus to all of the offspring, as has been shown for DHBV in ducks (Schödel *et al.*, 1989).

The host range of avian hepadnaviruses has been studied to date only for HHBV and DHBV. According to the currently available information, HHBV infects only grey herons, not ducks (Ishikawa and Ganem, 1995; Sprengel *et al.*, 1988). This host range difference appears to be due exclusively to the drastic difference in preS sequences that affect a step in viral infection after recognition of the putative primary receptor (Breiner *et al.*, 1998). Experimentally, DHBV was shown to infect several duck species but not Muscovy ducks or chickens (Fernholz, *et al.*, 1993; Marion *et al.*, 1984; Pugh *et al.*, 1995; Pugh and Simmons, 1994). Interestingly, DHBV was also shown to infect domestic geese (Marion *et al.*, 1987), although these animals are less closely related to Muscovy ducks. Because DHBV was found to be associated with liver disease when transmitted to domestic geese (Marion *et al.*, 1987) and a single-point mutation in DHBV preS protein can convert the nonpathogenic virus into a pathogenic form (Lenhoff and Summers, 1994), it is conceivable that SGHBV is pathogenic for ducks, provided SGHBV infection can be established in ducks. To our knowledge, naturally occurring infection of geese with an avian hepadnavirus has been found only in Ross geese (Newbold, personal communication). RGHV and SGHBV diverge substantially in sequence from all DHBV isolates known so far. In our study, we have shown that duck hepatocytes are permissive for SGHBV; the corresponding data for RGHV have not been reported. The availability of SGHBV for experimental *in vivo* infections may add important new information on host range and pathogenicity determining factors of avian hepadnaviruses.

The most striking feature that distinguishes SGHBV from all other avian hepadnaviruses is the secretion of a surprisingly large fraction of virion-like particles with DNA of the size of linear DNA minus strands. DHBVs with a similar phenotype (Havert and Loeb, 1997) have previously been created *in vitro* by the introduction of mutations into regions downstream of DR1 (region 3E, nucleotide position 2549–2561), upstream of DR2 (region 5E, nucleotide positions 2205–2462), and in the middle of the genome (region M, nucleotide positions 723–833) (indicated in Fig. 2). These sequences were shown to be important for efficient priming of DNA plus-strand synthesis at DR2 (regions M and 5E) and for circularization of the viral genome (region 3E). This is probably because these sequences play a role in establishment of a specific structure of the DNA minus strand within the core particle that is required for these processes (Havert and Loeb, 1997; Mueller-Hill and Loeb, 1996). No SGHBV-specific mutations compared with all published DHBV isolates are obvious in region 3E, whereas there are four in region 5E (G/T2350A, C2377T, A/C/G2416T, and

A2455G) and five in region M (A/C792A, T796A, C/A830T, G/T831C, and C/G/A832T). Although mutational analysis of the corresponding position in SGHBV is obviously required to investigate whether any of these nucleotide changes are causing the synthesis of a large fraction of virion-like particles with ssDNA, it is reasonable to assume that inefficient initiation of DNA plus-strand synthesis after RNA primer translocation and/or circularization of the viral genome is due to one or several of these nucleotide changes. However, it is also possible that one or several SGHBV core and pol protein-specific mutations hamper DNA plus-strand synthesis due to a defect in core particle maturation or pol function, respectively, as shown for DHBV (Nassal, 1992; Yu and Summers, 1991, 1994). The synthesis of linear viral dsDNA by *in situ* priming of DNA plus-strand synthesis, which occurs naturally in all hepadnaviruses at a frequency of 5–10% (Staprans *et al.*, 1991; Wei *et al.*, 1996; Yang and Summers, 1995) and is strongly increased when RNA primer translocation to DR2 is hampered or blocked (Loeb *et al.*, 1991; Yang and Summers, 1998), is not significantly increased in SGHBV. Therefore, a defect in RNA primer synthesis by incorrect RNase cleavage or its detachment from DR1 is unlikely.

An open question is whether the production of virion-like particles with ssDNA minus strands has any biological relevance *in vivo*. The demonstration of these particles in sera of several animals suggests that it is a genetically stable trait of SGHBV. If the production of these particles did not have a selective advantage, overgrowth of a SGHBV with mainly RC DNA would be expected; because only point mutations can be responsible for this phenotype, it should revert to wild type during replication. We can currently only speculate on a potential biological meaning of the production of these virion-like particles. For instance, provided the ssDNA is converted into RC DNA on a new infection, it could be a less nucleotide-consuming strategy to produce infectious SGHBV than used by DHBV. However, in case fully dsDNA could be produced from these particles on a new infection, the production of virion-like particles with ssDNA minus-strand DNA may also have negative consequences for the host. As speculated previously for linear double-stranded hepadnaviral DNA, minus strands may also have a potential to integrate into chromosomal DNA provided they are transported into the nucleus of cells on a new infection. Although this seems not to be the case in human hepatoma cells (Yeh *et al.*, 1998), such a scenario is well conceivable *in vivo* because nuclear import of the hepadnaviral genome is very inefficient only in human hepatoma cells and not *in vivo*. The use of the SGHBV virion-like particles with ssDNA for infection will make it possible to experimentally study their biological function in an *in vivo* model. Thus the potential pathogenic consequences that may be associated with nuclear viral ssDNA may be uncovered.

The significance of the X-like ORF in SGHBV genomes is currently not clear. The presence of a similar X-like ORF in all isolates of HHBV as well as in RGHV argues that all avian hepadnaviruses, except possibly DHBV, potentially encode and express similar proteins from these ORFs. Note, an X-like protein could even be expressed from all DHBV isolates if one assumes translation initiation at a nonconventional AUG codon. This could be one strategy to keep expression of the protein low. The absence of a typical TATA box upstream of the X-like ORFs in avian hepadnavirus genomes and the failure to detect an X-like transcript in DHBV may indicate that transcription of the X-like genes is similarly low as known for mammalian hepadnaviruses (Yen, 1996). Furthermore, similar to the X protein of mammalian hepadnaviruses (Yen, 1996), the SGHBV X-like protein, if expressed, appears to be dispensable for viral replication and *in vitro* infectivity. This can be indirectly deduced from our experiments with SGHBV1-19 that could express only a severely truncated X-like protein due to a stop codon mutation. The absence of a significant sequence similarity between the X proteins of the mammalian hepadnaviruses and the putative X-like proteins of avian hepadnaviruses (data not shown) does not exclude similar or related functions. In fact, the interaction of X protein mainly with cellular proteins would make a strong sequence similarity surprising because many of these cellular proteins in mammals and birds probably also differ drastically in sequence. Preliminary experiments that strongly suggest that X-like proteins are indeed expressed by avian hepadnaviruses and have similar biological functions corroborate our hypothesis.

MATERIALS AND METHODS

Sera

Serum samples from snow geese (*Anser caeruleus*) were obtained from the Tierpark, Berlin Friedrichsfelde ($n = 13$), and from Tierpark Hellabrunn, Munich ($n = 2$), Germany.

Amplification and cloning of complete viral genomes from virions

Viral DNA was amplified as recently published (Netter *et al.*, 1997). Briefly, serum samples were boiled for 10 min and centrifuged, and the supernatants were used for PCR. For amplification of the complete viral genome, primers were chosen that anneal to the nick region of DHBV: primer P1, 5'-AAACTGCAGCTCTTCAATTACACCCCTCTCCTTCGGAGC-3'; and primer P2, 5'-AAACTGCAGCTCTTCTAATTCTTAAGTTCCACATAGCC-3'.

Each primer contains an heterologous sequence at its 5' end with restriction sites for *Pst*I and *Sap*I. Cleavage with *Sap*I releases linear viral genomes devoid of any heterologous primer sequences. For cloning purposes, the PCR

products were purified by using a PCR purification kit (Qiagen, Germany) and then inserted into a linearized pUC18 vector with a single 5'-thymidine-nucleotide overhang.

Direct sequence analyses of the PCR-amplified SGHBV DNA and of cloned SGHBV genomes

PCR-amplified SGHBV DNA was directly sequenced after purification. In addition, five plasmids with full-length SGHBV DNA inserts obtained by cloning were sequenced. The sequencing reaction was performed with 0.4 pmol of fluorescent labeled primers and by using the Sequitherm Long Read cycle Sequencing Kit (Epicentre Technologies, Madison, WI). The primers for sequencing were specific for vector sequences closely adjacent to the inserts (M13 and M13rev) and specific for conserved regions within the avian hepatitis B genomes (P3–P8): M13 (5'-GTTTCCCAGTCAC-GAC-3'), M13rev (5'-CAGGAAACAGCTATGAC-3'), P3 (positions 178–199, 5'-ACCATTGAAGCAATCACTAGAC-3'), P4 (positions 314–292, 5'-GAACGTTCTTCTCCCATAGACAAC-3'), P5 (positions 649–672, 5'-GATGCAACATGAATCAATAG-TAGG-3'), P6 (positions 1071–1090, 5'-GAAGAAGATCA-GAAAGCTCG-3'), P7 (positions 1327–1305, 5'-CCGATTAG-GCCAGCTAGTATTCC-3'), and P8 (positions 2189–2210, 5'-GCAATATCCCATATCACCGGCG-3').

Sequence analysis was performed with the software provided by MacVector (Oxford Molecular Group, UK) and by the package provided by the Wisconsin Genetics Computer Group (Devereux *et al.*, 1984). The SGHBV sequences were aligned to different DHBV, HHV, and RGHV sequences available from GenBank (NCBI, Bethesda, MD): DHBV1 [accession number (AC): X58567], DHBV3 (Sprengel *et al.*, 1991), DHBV22 (AC: X58568), DHBV26 (AC: X58569), DHBV f1-6 (AC: X12798), DHBVQCA34 (AC: X60213), DHBV16 (AC: K01834), DHBV S18-B (AC: M21953), DHBV 1 (AC: X74623), DHBV S-5 (AC: M32990), DHBV S-31 (AC: M32991), DHBV P2-3 (AC: M60677), RGHV (AC: M95589), and HHV-4 (AC: M22056). The sequences of SGHBV clones have been deposited in GenBank: SGHBV1-7 (AC: AF110999), SGHBV1-9 (AC: AF111000), SGHBV1-13 (AC: AF110996), SGHBV1-15 (AC: AF110997), and SGHBV1-19 (AC: AF110998).

Amplification of a subgenomic DNA fragment from the nick region of SGHBV genomes

Viral DNA was isolated from a snow goose serum (DW1) and the nick region was amplified by using primers P9 (positions 2147–2163, 5'-CCTTTGCCACGTGTAGC-3') and P10 (positions 257–236, 5'-CCACGAGGTTT-TCTAGTAGCC-3') that anneal upstream and downstream of this region. The amplified fragment, about 1.1 kb in length, was directly sequenced with primer P11 (positions 2499–2478, 5'-GCTTTCGAGAGAGGGGTGTATG-3') to verify the sequence of the nick region.

Transfection of monomeric SGHBV DNA

Full-length SGHBV DNA inserted into pUC18 vector was released by restriction enzyme cleavage with *SapI*. The linearized DNA was circularized by incubation with T4 DNA ligase (4 units/ml added to 3 pmol of DNA) at 14°C overnight. After ligation, the DNA was extracted with phenol and phenol–chloroform, precipitated with 2-propanol, washed with 70% ethanol, dissolved in water, and used for transfection.

Transfection of chicken hepatoma cells

Chicken hepatoma cells (LMH) (Kawaguchi *et al.*, 1987) were grown in DMEM/F-12 medium and transfected by the $\text{Ca}_3(\text{PO}_4)_2$ method as described (Condreay *et al.*, 1990). Culture medium was changed 1 day after transfection. The supernatant was collected after 2 and 4 days, and the cells harvested at day 4 after transfection. Viral DNA was purified from intracellular core particles and from extracellular viral particles as published elsewhere (Günther *et al.*, 1995). The intracellular replicative intermediates and viral particle-derived DNAs were analysed by Southern blotting using for hybridisation Dig-labeled (Boehringer Mannheim, Germany) DHBV and SGHBV probes.

Infection of primary hepatocytes

Viral particles of cell culture medium of LMH cells transfected with DHBV or SGHBV DNAs were clarified by centrifugation at 3000 rpm for 15 min. The supernatant was then laid on top of a 20% sucrose cushion, adjusted with PBS, and centrifuged at 25,000 rpm (SW28 rotor; Beckman) for 8 h. The viral pellet was then resuspended in PBS. The viral genome DNA was isolated and analysed by Southern blotting. Isolation and infection of PDH were performed as described previously (Bruns *et al.*, 1998). The cells were harvested at day 7 after infection. Productive infection was tested by demonstration of intracellular preS protein expression by immunoblotting using an anti-DHBV preS polyclonal antibody.

Separation of viral particles by sucrose gradient centrifugation

The pellets from the culture medium of LMH cells transfected with a plasmid containing a tandem of genome SGHBV1-15 were resuspended in PBS, loaded onto a 10–70% sucrose gradient, and separated by ultracentrifugation at 45,000 rpm in an SW50.1 rotor (Beckman). After centrifugation, 300- μl fractions were collected. Each fraction was analysed for viral DNA by Southern blotting and for surface antigen (preS protein) and nucleocapsid protein (C protein) by immunoblotting.

Immunoblotting

For preparing the samples, the transfected cells were lysed directly in SDS loading buffer. The sera were di-

luted 1:100 with TE buffer, and SDS loading buffer was then added. The samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane or PVDF membrane. The blots were incubated with a rabbit anti-serum raised against recombinant DHBV preS or S protein (the latter kindly provided by Dr. H. Schaller, University of Heidelberg, Germany), and a rabbit antiserum specific for DHBV C protein. The proteins were then visualized by using a peroxidase-coupled anti-rabbit IgG antibody and by a chemiluminescence substrate.

DNA and protein sequence analyses

Sequences of DNAs and proteins were aligned using the program Pileup from GCG-package (UWGCG) or MacVector (Kodak, New Haven, CT). The split composition analysis was performed with the SplitsTree program (Huson, 1998).

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