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Characterization of Infectious and Defective Cloned Avian Hepadnavirus Genomes

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The infectivity *in vivo*, replication competence *in vitro*, and expression of viral genes of several molecularly cloned duck hepatitis B virus (DHBV) genomes were investigated. In addition, replication competence, core protein expression, and secretion of viral proteins were investigated for a grey heron hepatitis B virus genome. Except two, all DHBV isolates tested induced a systemic infection in Pekin ducks when injected as cloned viral DNA into the liver. After transfection of chicken hepatoma cells, both defective DHBV genomes expressed intracellular nucleocapsid and pre-S envelope proteins and secreted DHBs/pre-S particles into the medium. One of the defective DHBV genomes and HHBV produced within the cells replicative intermediates encapsidated in core particles and secreted virions, whereas the other defective DHBV genome did not and was unable to efficiently encapsidate the RNA pregenome. Comparative sequence analysis was performed to identify potential amino acid changes in viral proteins of both defective DHBV genomes. The data obtained demonstrate that most cloned avian hepadnaviruses are infectious or replication competent and suggest defects in envelope, polymerase or encapsidation function, respectively, in two cloned DHBV genomes. © 1991 Academic Press, Inc.

INTRODUCTION

Members of the family of hepatitis B viruses (hepadnaviruses) have been isolated, cloned, and sequenced from human (HBV), woodchucks (WHV), ground squirrels (GSHV), Pekin ducks and a goose (DHBV), and from herons (HHBV) (for review see Marion, 1988; and Schödel *et al.*, 1989, 1991). All hepadnaviruses induce acute and chronic infections. Mammalian hepadnaviruses are to various degrees pathogenic whereas those of birds are not (Marion, 1988). Replication of all hepadnaviruses involves synthesis of an RNA pregenome which is reverse transcribed into minus strand DNA which in turn is transcribed into a DNA plus strand by a DNA polymerase (Will *et al.*, 1987). Both the reverse transcriptase (RTase) and the DNA polymerase and in addition an RNase H activity are virus encoded (for review see Schödel *et al.*, 1989, 1991). Similar to retroviruses the RTase probably lacks proofreading activity and this may be one reason why hepadnavirus populations in infected patients and animals are heterogeneous in sequence (Miller *et al.*, 1990; Schödel *et al.*, 1989, 1991 and references therein). For a few cloned HBV, WHV, and one GSHV genomes the infectivity or defectivity were experimentally tested (Sprengel *et al.*, 1984; Will *et al.*, 1985; Seeger *et al.*, 1987, 1989; Girones *et al.*, 1989; Miller *et al.*, 1990), and from some HBV and WHV genomes the defect is obvious because of large deletions in essential genes (Miller *et al.*, 1990; Okamoto *et al.*, 1987). From this limited number of experiments the number of defective mammalian hepadnavirus genomes appears to be rather high and they seem to accumulate in chronic infection.

Avian hepadnaviruses, and in particular DHBV, have served in the past as very useful tools to decipher the life cycle of hepadnaviruses and are increasingly used as model systems for antiviral drug, antibody neutralization, and virus receptor studies (Lambert *et al.*, 1990; Schödel *et al.*, 1989, 1991; Yuasa *et al.*, 1991). HHBV and several genomes of DHBV were isolated, cloned, and sequenced, and a remarkable sequence variation was observed (Uchida *et al.*, 1989; Tong *et al.*, 1990; Mattes *et al.*, 1990; Tong *et al.*, 1991; Sprengel *et al.*, 1991; and some isolates reviewed in Schödel *et al.*, 1991). The significance of this sequence variation is not known since the infectivity of only three cloned DHBV DNA genomes was experimentally demonstrated (Sprengel *et al.*, 1984; Schneider *et al.*, 1991). Naturally occurring defective DHBV genomes were not identified so far.

Here we report the characterization of infectivity and defectivity of several DHBV genomes and one HHBV genome by *in vivo* and *in vitro* assays. The data obtained indicate that most cloned avian hepadnaviruses are infectious or replication competent. Two defective DHBV genomes were identified with possible defects in the pre-S/S and polymerase coding region.

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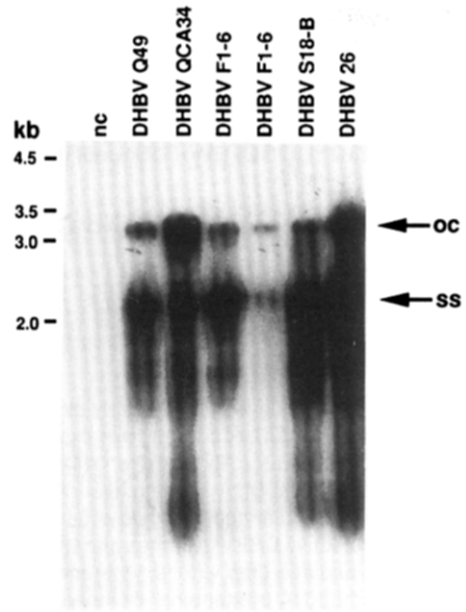


FIG. 1. DHBV replicative intermediates in livers of Pekin ducks transfected with DHBV genomes. nc, mock transfected livers; oc, open circular form of DHBV genome; ss, single-stranded DHBV minus strand DNA.

MATERIALS AND METHODS

DHBV isolates

Cloned avian hepadnavirus genomes were derived from a goose (DHBV 1, Sprengel *et al.*, 1991), Pekin ducks (DHBV 3, 16; Sprengel *et al.*, 1985; Mandart *et al.*, 1984), various other ducks (DHBV 22 and 26; Sprengel *et al.*, 1991; DHBV S18-B, Tong *et al.*, 1990; DHBV F1-6, Mattes *et al.*, 1990; DHBV S5 and S31, Uchida *et al.*, 1989; DHBV QCA34, Tong *et al.*, 1991, and Q49, Tong and Mattes, unpublished), and from a grey heron (HHBV 4, Sprengel *et al.*, 1988). All these genomes were cloned from virions derived from serum of naturally infected animals. Infectivity has been demonstrated so far for DHBV 3, 16, and 26 only (Sprengel *et al.*, 1984; Schneider *et al.*, 1991). The infectivity of the HHBV genome could not be tested in grey herons because they are under conservation protection—in Pekin ducks this genome does not induce viremia presumably due to its heron-specific host range (Sprengel *et al.*, 1988) but a defect in the genome could not be excluded in this study.

Transfection of avian liver tumor cells (LMH)

Chicken hepatoma cells (LMH, Kawaguchi *et al.*, 1987) were grown in petri dishes of 60 mm diameter and transfected by the $Ca_3(PO_4)_2$ method as described (Condreay *et al.*, 1990). The plasmid DNAs were purified by the method of Clewell and Helinski (1969) followed by two isopycnic centrifugations in cesium chloride gradients. All plasmids used contained the corresponding DHBV or HHBV genomes as dimers in a head to tail linked conformation.

About half of the culture medium was changed 3 days after transfection; cells were harvested another 2 to 3 days later. The cells were washed twice with PBS (10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4, 170 m*M* NaCl, 3 m*M* KCl), scraped off and lysed for 1 hr on ice in 100 μl lysis buffer consisting of 150 m*M* NaCl, 20 m*M* Tris/HCl, 10 m*M* EDTA, 1% NP40, 1 m*M* phenylmethylsulfonylfluoride, pH 8. The lysate was sonified for 15 sec before aliquots were used for immunoblotting or DNA isolation for Southern blot analysis as described (Lambert *et al.*, 1990).

Partial purification of viral particles

Viral particles of culture medium of LMH cells (3 × 10^6 cells each) transfected with various DHBV DNAs were concentrated by precipitation with 10% PEG and

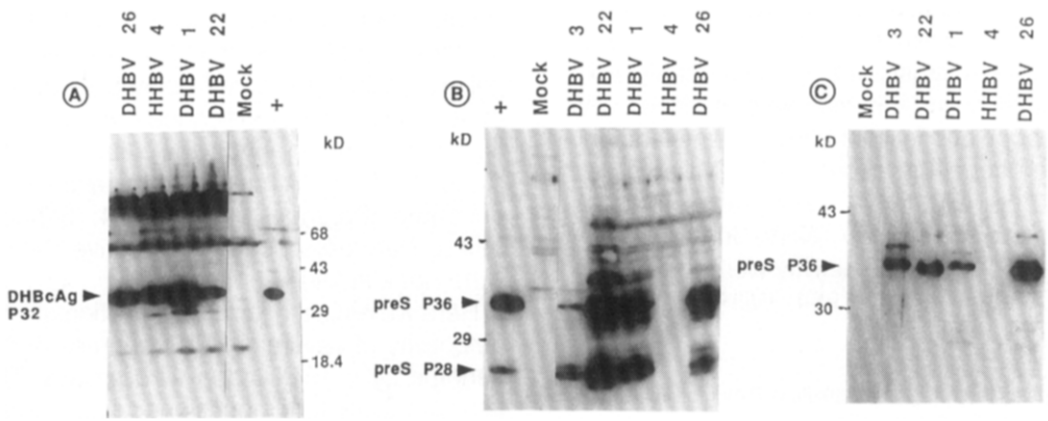


FIG. 2. Detection of nucleocapsid (A) and pre-S (B) protein expression in LMH cells transfected with cloned DHBV and HHBV 4 genomes by immunoblotting with polyclonal antibodies to recombinant DHBcAg and DHBV pre-S protein visualized by autoradiography and ^{125}I-protein A. In the lane designated + a cell extract from an infected duck liver was loaded. (C) Pre-S proteins in virus particles pelleted from the supernatant of transfected LMH cells was detected by immunoblotting as described above.

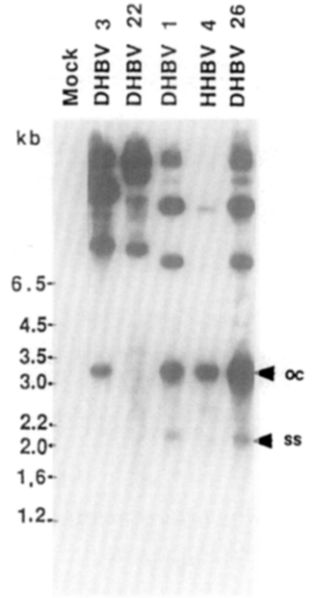


FIG. 3. Southern blot analysis of replicative intermediates extracted from transfected cells. oc, double-stranded open circular DHBV DNA; ss, single stranded DHBV minus strand DNA.

nonencapsidated DNA was digested with DNase I as described (Summers *et al.*, 1991). The pellets were resuspended in CsCl and the particles were separated by ultracentrifugation for 2 hr at 80 krpm in a TLV100 rotor. The gradients were divided into three large fractions (from bottom to top densities were 1.29, 1.27, and 1.24 g/cm^3; core particles (density, 1.35 g/cm^3) pellet under these condition whereas virions stay on top) and were analyzed for virion DNA by Southern blotting after pelleting of the virions with SDS, Proteinase K digestion, phenol and chloroform/isoamylalcohol extraction, and precipitation of the DNA by ethanol as described (Summers *et al.*, 1991).

Detection of viral proteins by immunoblotting

Cell lysates and viral particles pelleted from culture supernatants ($\frac{1}{5}$ of total from each) were applied on

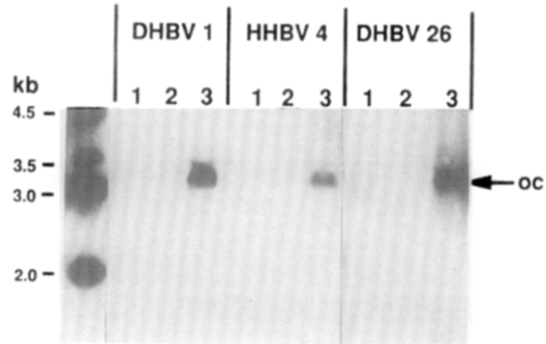


FIG. 4. Southern blot analysis of viral DNA extracted from viral particles purified by CsCl centrifugation (1 to 3 each denote a pool of bottom, middle, and top fractions of the gradient). oc, double-stranded open circular DHBV DNA.

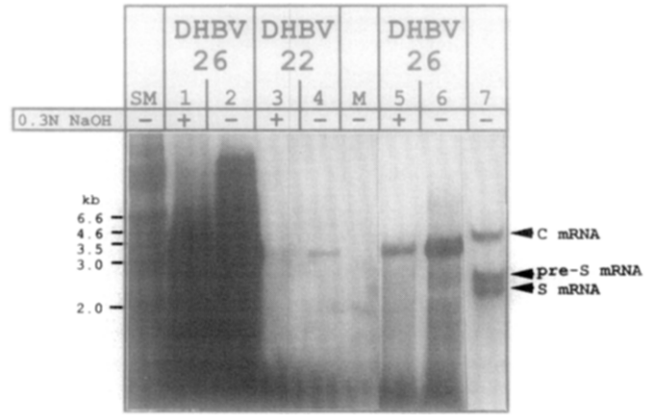


FIG. 5. Analysis of core particle-encapsidated pregenomic RNA in LMH cells transfected with DHBV 26 and DHBV 22 DNA. Lanes 1 to 4: long exposure; lanes 5 and 6: short exposure. Total polyA+ RNA isolated from a DHBV 3 infected liver (lane 7) and DNA restriction fragments (lane SM) were run as size markers. $^+$ and $^-$ denote whether or not the RNA was pretreated with 0.3 *N* NaOH before loading.

17.5% SDS–polyacrylamide gels. Immunoblotting was performed as described (Lambert *et al.*, 1990). Cell lysates and suspensions of pelleted viral particles were boiled in SDS-sample buffer (3% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue, 5 m*M* EDTA, 200 m*M* Tris–HCl, pH 6.8), cleared by centrifugation for 5 min in an Eppendorf centrifuge, and the supernatant was loaded on the gel. After electrophoretic transfer of the proteins from the gel to the nitrocellulose filter, unspecific binding sites were blocked overnight with PBS/5% BSA. For detection of pre-S proteins and viral particles in cellular lysates a polyclonal rabbit anti-pre-SAg serum (anti-pre-S-Kpn; Lambert *et al.*, 1990) and for detection of core proteins an anti-DHBcAg serum (anti-C2989, Schneider *et al.*, 1991) were diluted 1:2000 in PBS/1% BSA and incubated with the nitrocellulose-sheets for 2 hr at RT or overnight at 4°C. The blots were washed 3× with PBS/0.1% Tween before ^{125}I-Protein A (diluted 1:2000 in PBS/1% BSA) was added for 2 hr. After washing 4× with PBS/0.1% Tween the nitrocellulose filters were dried and exposed to an X-ray film.

Analysis of viral DNA by Southern blotting

Replicative intermediates of viral DNA were isolated essentially as described (Summers *et al.*, 1990) using 0.5 mg/ml Proteinase K instead of Pronase. After phenol extraction and ethanol precipitation the DNA was analyzed by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized using a gel-purified *Eco*RI full-length genome fragment of DHBV 26 DNA radiolabeled with ^{32}P-αdCTP to a specific activity of 10^8 cpm/μg.

Isolation and analysis of viral RNA

Transfected cells were lysed in lysis buffer and $MgAc_2$ and DNAse I was added to obtain a final concentration of 6 m*M* and 100 μg/ml, respectively. Core particles were precipitated from these lysates with 6% PEG 8000 in 350 m*M* NaCl, 10 m*M* EDTA by incubation for 30 min at 4° and centrifugation in an Eppendorf centrifuge for 4 min at 4°. The pellet was resuspended in 200 μl of Tris–HCl, pH 8, and treated with nuclease S7 (final concentration: 100 μg/ml nuclease S7, 6 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 50 μg/ml t-RNA) for 20 min at 37°. The reaction was stopped by addition of 10 m*M* EGTA, and proteins were digested with Proteinase K (in 0.1 m*M* Tris–HCl, pH 7.5, 12 m*M* EDTA, 150 m*M* NaCl, 1% SDS) for 30 min at 37°. The RNA was extracted once with phenol and twice with chloroform/isoamylalcohol (24:1 v/v) and after addition of 10 μg of t-RNA precipitated with ethanol. Extraction of total RNA and preparation of polyA+ RNA from a liver infected with DHBV 3 was performed and analyzed by Northern blot analysis as described (Büscher *et al.*, 1985). To ensure that no DNA is present in the RNA isolated from cores an aliquot of the RNA was treated for 5 min at 100° with 0.3 *N* NaOH, neutralized with HCl, and precipitated with ethanol.

RESULTS

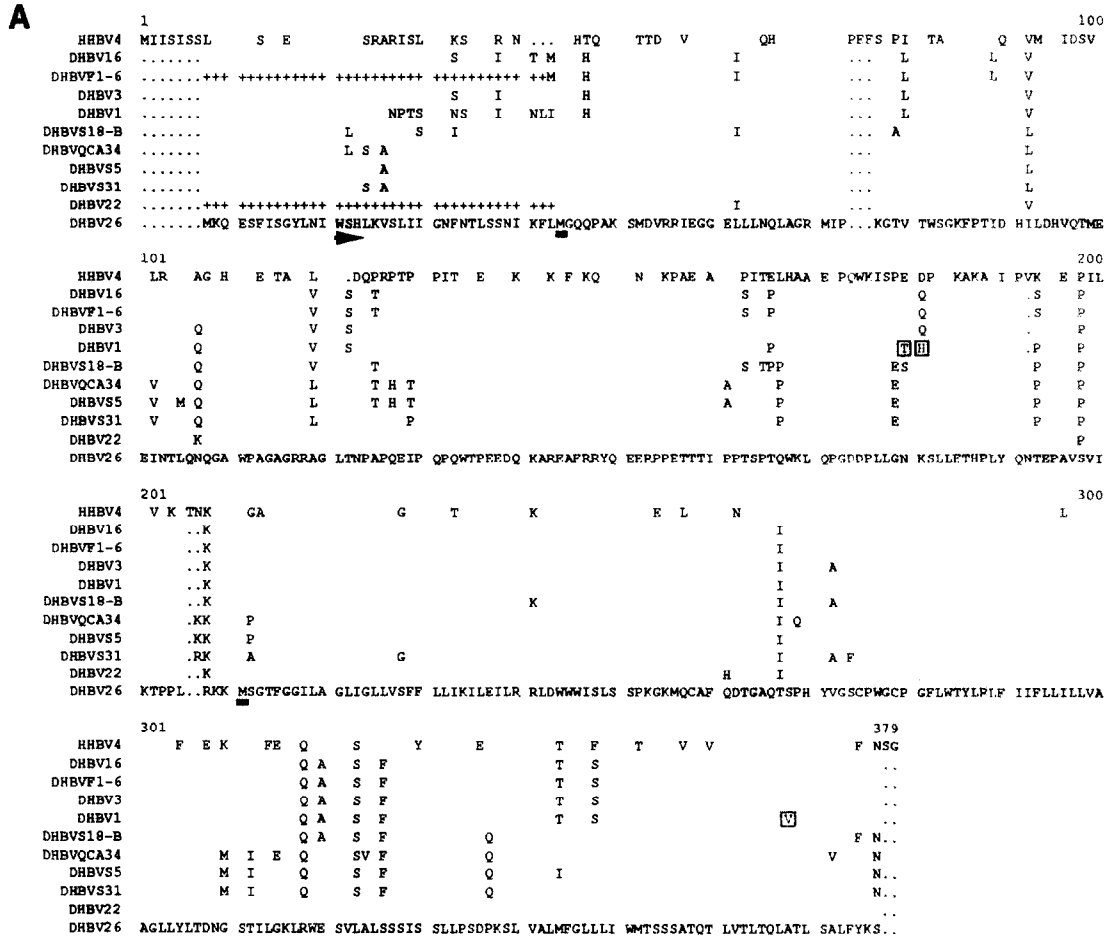
Infectivity of cloned DHBV genomes *in vivo*

The infectivity of 10 cloned DHBV genomes (DHBV 1, 22, QCA34, Q49, S18-B, F1-6, S5, and DHBV 3, 26 and 16 as positive controls) was tested *in vivo* by injecting the cloned double-stranded viral DNA into the liver of 1 to 3 day-old ducklings (five animals for each type of DNA) negative for markers of previous DHBV infection, as previously described (Sprengel *et al.*, 1984). Three weeks after injection of the DNA, the animals were sacrificed and the blood and livers were tested for the presence of replicative intermediates and for DHBcAg and pre-S protein expression in the liver, as well as for viral DNA and pre-S protein in the serum by Southern and immunoblotting, respectively, as described previously (Lambert *et al.*, 1990). Except DHBV 1 and DHBV 22, all other DHBV genomes induced in 3 to 5 animals all viral markers tested. Pre-S protein and DHBV DNA titers were similar to those in animals infected with control virus (data not shown). Representative examples showing the replicative intermediates in the liver of animals transfected with cloned DHBV DNAs are shown in Fig. 1. Except for DHBV S5, serum from animals with these replicative intermediates in the liver was used for infection of two to five animals to further confirm the infectivity of the virus produced. One to 2 weeks after inoculation all these animals had similar high levels of pre-S and core proteins in the serum and in the liver (data not shown) as when inoculated with DHBV of proven infectivity (DHBV 3 or 16) (Schneider *et al.*, 1991). These data indicate that five of the newly analyzed viral genomes with previous unknown infectivity (DHBV QCA34, Q49, S18-B, F1-6, and S5) induce a systemic infection whereas two of the genomes (DHBV 1 and 22) are defective.

Expression of viral proteins from two defective DHBV and one HHBV genome

To test whether the two DHBV genomes and an HHBV genome which were not infectious in Pekin ducks (HHBV was previously tested for infectivity *in vivo;* Sprengel *et al.*, 1988) are defective for expression of viral nucleocapsid and pre-S protein expression, LMH cells were transfected with the cloned viral DNAs. By immunoblotting using an antibody specific for recombinant DHBcAg, strong expression of nucleocapsid protein was visualized in cell extracts prepared from LMH cells transfected with HHBV and all DHBV genomes tested (Fig. 2A). Expression was at least as efficient as that seen with cells transfected with an infectious DHBV 26 genome (Fig. 2A). An analogous experiment using antibodies to a recombinant DHBV pre-S protein revealed in the same cell extracts pre-S proteins qualitatively and quantitatively similar to an infectious DHBV genome (Fig. 2B). The 36-kDa band corresponds to the major pre-S protein, the 28-kDa band is a processing product of P36 (Lambert *et al.*, 1990; Fernholz and Will, unpublished). Minor bands with lower or higher electrophoretic mobilities than pre-S protein P36, which were only seen in transfected LMH cells, are minor pre-S proteins initiated at internal AUGs of the pre-S open reading frame (D. Fernholz and H. Will, unpublished data) and some of them probably also modified and processed forms of P36. The pre-S protein of HHBV, if present in these cell extracts, could not be visualized because DHBV pre-S antibodies do not cross-react with HHBV pre-S proteins due to substantial sequence divergence from DHBV pre-S (Sprengel *et al.*, 1988, Schneider *et al.*, 1991). To test whether the pre-S proteins produced inside the cells are secreted and form virus particles, the medium of

FIG. 6. Comparative analysis of all published avian hepadnavirus sequences of the pre-S/S (A), nucleocapsid (B), and pol (C) proteins. Indicated are the translation initiation sites (underlined) and transcription initiation sites (arrows). Amino acid changes unique to the defective DHBV 1 (A and B) and DHBV 22 (C) proteins are enboxed.


A
HHBV4
DHBV16
DHBVF1-6
DHBV3
DHBV1
DHBVS18-B
DHBVQCA34
DHBVS5
DHBVS31
DHBV22
DHBV26
B
HHBV4
DHBV16
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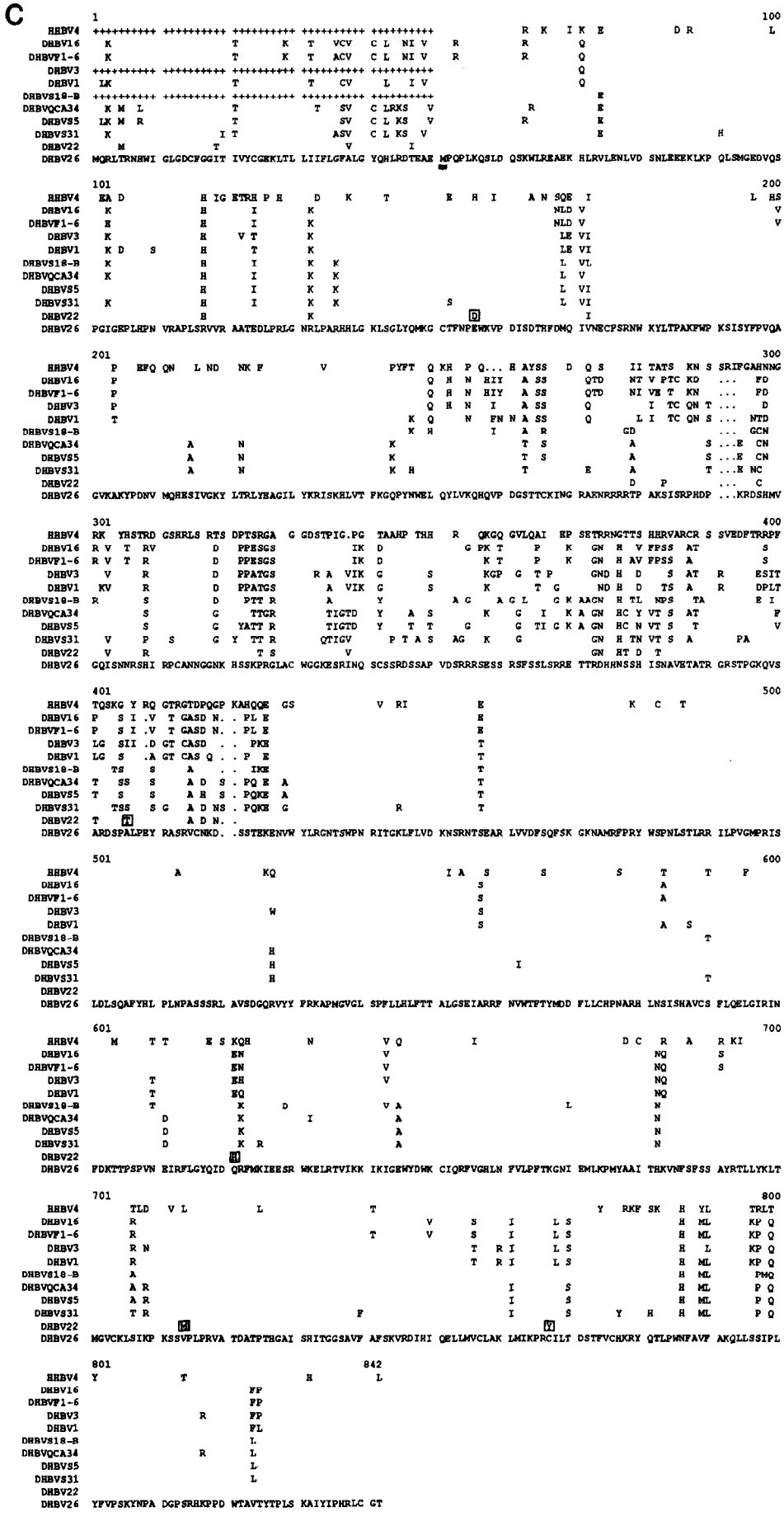

FIG. 6—*Continued*

by other viral proteins produced from coinfecting DHBVs. It can, however, not be excluded with certainty that the defect arose during cloning of the DHBV genomes.

Indications as to the type of defect that renders genomes DHBV 1 and 22 noninfectious were obtained by studying synthesis of viral DNA, expression of viral proteins, and secretion of viral particles. The data obtained *in vitro* and *in vivo* suggest that genome DHBV 1 is fully functional except in the early step of the infection process. Possible candidate mutations which are responsible for DHBV 1 defectivity could be located in the envelope (pre-S/S) proteins which are believed to bind to cell specific virus receptors and mediate virus entry into the cell (reviewed in Alberti *et al.*, 1990). To further support this speculation a comparative sequence analysis of pre-S/S protein sequences, as deduced from all cloned viral DNAs, was performed (Fig. 6). Three amino acid changes unique to the predicted DHBV 1 pre-S/S proteins were observed (enboxed in Fig. 6A). It is therefore conceivable that DHBV 1 pre-S/S proteins have an altered structure which does not allow attachment to the cells or interfere with one of the other early steps of virus cell-penetration or uncoating. A gross structural change in the DHBV 1 pre-S/S proteins induced by the observed mutations is unlikely because particle formation and secretion is unaltered. A definite answer will require studies of the interaction of the DHBV 1 pre-S/S protein with the DHBV receptor protein. Two amino acid changes unique to DHBV 1 are also present in the nucleocapsid protein; one is at a position where the HHBV core protein has also an amino acid altered, and the other is a conservative change from leucine to isoleucine (enboxed in Fig. 6B). Perhaps these mutations alter the interaction of pre-S with core protein which is involved in regulation of the copy number of intracellular ccc-DHBV DNA and of virion secretion (Summers *et al.*, 1990, 1991).

The most likely defect of the DHBV 22 genome which expressed all viral proteins tested *in vitro* and led to secretion of viral particles is a defect of the polymerase protein(s). The comparative sequence analysis of all known DHBV pol-proteins revealed five mutations unique to DHBV 22, four of them in highly conserved regions (enboxed in Fig. 6C). Three of the mutations are located at the carboxyterminal end of the pol-protein which encodes the RNase H domain (Schödel *et al.*, 1988; Kudyakov and Makhov, 1989; Radziwill *et al.*, 1990). Similar mutants were shown to be in part replication competent and to produce DHBV minus strand DNA (Radziwill *et al.*, 1990). Southern blot analysis with DNA from DHBV 22-transfected cells revealed only a weak smear in the region of replicative intermediates but not a clear band of minus strand DHBV DNA. The smear could represent degraded plasmid DNA and/or a small amount of heterogeneous replicative intermediates. Because of these technical difficulties we can therefore currently not decide whether DHBV 22 is a RNase H-defective mutant. One of the unique DHBV 22 mutations is located in the highly conserved amino-terminal end of the pol-protein which is believed to represent at least part of the genome-linked protein (Bartenschlager and Schaller, 1988; Khudyakov and Makhov, 1989). The second possibility is, therefore, that DHBV 22 is defective because of a nonfunctional genome-linked protein. As the polymerase protein has an essential role in encapsidation of the RNA pregenome (Bartenschlager *et al.*, 1990) and is a RNA binding protein (Köchel *et al.*, 1991) this mutation could also prevent efficient RNA pregenome encapsidation. All these possibilities are not mutually exclusive and the combination of DHBV 22 specific mutations could also play a role. Mutational and functional analysis will provide us with answers to these questions.

In a previous report we have shown that the HHBV 4 genome is not infectious in Pekin ducks and have speculated that this is due to a heron-specific host range (Sprengel *et al.*, 1988; Sprengel and Will, 1988). Our current study has demonstrated the replication competence of the cloned HHBV 4 genome and efficient expression of nucleocapsid protein of the predicted size. Although pre-S/S proteins could not be visualized because a corresponding antibody is not available, the observation that viral particles were shed into the medium as efficiently as from infectious DHBV genomes suggests that both pre-S and S proteins are expressed, form viral particles and are secreted. Whether or not the HHBV DNA is also infectious in herons remains unknown.

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the transfected cells was subjected to ultracentrifugation to pellet viral particles. The pellets were analyzed for pre-S proteins by immunoblotting. Pre-S proteins were identified in the pellets of all supernatants (Fig. 2C). These data demonstrate that both defective DHBV virus genomes did not only express pre-S proteins but they were also secreted as viral particles with similar efficiencies as observed with infectious DHBV genomes.

Replicative intermediates and virion DNA produced from LMH cells

For both defective DHBV 1 and DHBV 22 genomes and one HHBV genome, the replication competence and the ability to produce and secrete virions were analyzed by searching for intracellular replicative intermediates of viral DHBV DNA and virion-encapsidated DHBV DNA in the medium of transfected cells. The DNA of partially enriched core particles from lysed LMH cells transfected with plasmids containing head to tail linked dimers of these genomes, and DNA of virus particles secreted from these cells and purified by CsCl centrifugation were extracted and subjected to Southern blot analysis using a ^{32}P-labeled DHBV DNA probe. In the enriched core particles, major bands of open circular and minor bands of DHBV minus strand DNA (in addition to various forms of higher molecular weight input plasmid DHBV DNA) that are representative for replicative intermediates were observed with all tested DHBV genomes (except DHBV 22) and with the HHBV genome (Fig. 3). With DNA extracted from CsCl-purified virions from the medium of LMH cells transfected with DHBV 1, DHBV 26, and HHBV 4 a single band of approximately 3.3 kb corresponding to virion DNA was detected (Fig. 4). In contrast, no signal was detected in medium of cells transfected with DHBV 22 (not shown) consistent with the absence of replicative intermediates in enriched core particles. These data also indicate that the defective DHBV 1 and the HHBV genomes are not only replication competent but can form and secrete virions similar to infectious DHBV genomes.

Pregenomic RNA produced from DHBV 22 is inefficiently encapsidated

Expression of similar amounts of pre-S and core proteins from the DHBV 22 genomes and absence of replicative intermediates and virions in experiments with LMH cells suggested a defect in DHBV 22 either in polymerase protein function, RNA pregenome encapsidation, or both. To test whether the DHBV 22 RNA pregenome is encapsidated, core particles from LMH cells transfected with the DHBV 22 genomic DNA or with an infectious DHBV genome as a positive control were partially purified, the RNA extracted and probed by Northern blotting for encapsidated pregenomic RNA. A strong band of about 3.3 kb corresponding to the DHBV RNA pregenome was seen in core particles derived from the infectious DHBV 26 genome (Fig. 5, lanes 5 and 6) whereas using the same exposure time (2 hr, room temperature, no intensifier screen) no signal was obtained with particles derived from the DHBV 22 genome (data not shown). After longer exposure (14 hr with intensifier screen), however, a very minor signal corresponding to the RNA pregenome was also seen with cores from DHBV 22 (Fig. 5, lanes 3 and 4). The signal intensity is, however, drastically weaker than that obtained with DHBV 26 (Fig. 5, lanes 1 to 4). These data suggest a major defect of DHBV 22 in encapsidation of pregenomic RNA.

DISCUSSION

In this study the infectivity of eight out of 10 DHBV genomes tested was demonstrated. One of two DHBV genomes (DHBV 1) and one HHBV (HHBV 4) genome which are not infectious in Pekin ducks were characterized *in vitro* as replication competent and were shown to produce and secrete virions *in vitro* whereas one DHBV genome (DHBV 22) appeared replication incompetent and produced only virus particles containing little RNA pregenome. Taken together, previously published data (Sprengel *et al.*, 1984; Schneider *et al.*, 1991) and the results of this study indicate that most cloned avian hepadnavirus genomes are infectious. The replication competence of HHBV and its ability to produce and secrete virions *in vitro* and defects in cloned DHBV genomes have so far not been reported.

The percentage of defective cloned DHBV genomes from the panel of genomes tested here is lower than that of WHV and HBV which occasionally outnumber genomes without overt defects and appear to accumulate during chronic infection (Miller *et al.*, 1990 and references therein). Possible explanations could be that virtually all defective mammalian hepadnaviruses reported so far are from long-term chronic carriers whereas all DHBV genomes identified in our study were obtained from young or only a few years old animals. In addition, defective chromosomally integrated viral WHV and HBV DNA frequently found in chronic carriers (reviewed in Matsubara and Tokino, 1990) which can give rise to defective virion DNA by complementation is only seldomly observed in chronic DHBV carrier ducks (Yokosuka *et al.*, 1985; Sprengel and Will, 1988). For DHBV, efficient synthesis of virions with defective viral DNA by complementation had been recently experimentally demonstrated *in vitro* (Horwich *et al.*, 1990). It is likely that the defective genomes identified in our study are due to the infidelity of the reverse transcription process and were complemented

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