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Biochemical and Immunological Characterization of the Duck Hepatitis B Virus Envelope Proteins

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To examine the envelope proteins of duck hepatitis B virus (DHBV), which are encoded by the pre-S/S open reading frame of the viral genome, an antiserum was raised in rabbits against a fusion protein comprising most of the pre-S coding segment. By using this antiserum, viral particles could be precipitated from serum, and two pre-S proteins with molecular sizes of approximately 35 and 37 kilodaltons were detected in the sera and livers of DHBV-infected ducks after Western blotting and after biosynthetic labeling of a primary duck liver cell culture. In serum, the pre-S proteins were shown to exist predominantly in DHBV-DNA-free particles associated with a 17-kilodalton protein which, by N-terminal amino acid sequence analysis, was shown to represent the viral S protein which is encoded by the 3′ proximal segment of the DHBV pre-S/S open reading frame. To compare the immunogenic potential of the S and pre-S proteins, serum particles and gel-purified S protein were used to immunize rabbits. In neither case was a significant immune response against the DHBV S protein observed. However, a good antibody titer against DHBV pre-S was obtained even after immunization with small amounts of the pre-S antigen.

The hepatitis B viruses are enveloped viruses with a distinct liver tropism which are grouped together in the hepadnavirus family (18, 26). So far, four different hepatitis B viruses have been described in detail, namely the human hepatitis B virus (HBV), the duck hepatitis B virus (DHBV), the ground squirrel hepatitis B virus, and the woodchuck hepatitis B virus (25). We have chosen the avian hepatitis B virus DHBV, which infects Pekin ducks, to develop a model system for the study of the virus-host interactions at the molecular level. In comparison to HBV, which infects only humans and chimpanzees, this animal model virus offers the possibility to perform systematic in vivo studies on viral mutants. Before such experiments can be done, however, it is necessary to define the viral gene products which are normally synthesized during infection.

As was shown by sequence analysis (9, 21), DHBV, like all hepatitis B viruses, has a rather compact genome structure with three extensively overlapping open reading frames (Fig. 1). The C frame encodes the viral core protein and overlaps by 242 nucleotides with another open reading frame termed the P frame because the proposed gene product has a homology to reverse transcriptases (21, 27) and is therefore believed to encode the viral polymerase. The P frame completely overlaps with the S frame, which encodes the viral surface proteins. In analogy to the other hepatitis B viruses, the S frame is divided into two parts, which are called the pre-S region (nucleotides 693 to 1283) and the S region (nucleotides 1284 to 1784) (Fig. 1). Of the three major DHBV transcripts which have been mapped (3), two initiate within the S frame (Fig. 1). One of them, starting at about nucleotide 730, encodes a pre-S/S protein with a molecular weight of about 36 kilodaltons (kDa) (for simplicity, designated pre-S protein in this report). The other, starting at about nucleotide 985, encodes an S protein consisting of 167 amino acids, with a calculated molecular size of 18.2 kDa (3, 21).

In this report, the DHBV S-frame gene products found in sera and livers of DHBV-infected ducks are described, and their immunogenicity in rabbits is compared. In addition, we show that the biosynthesis of DHBV proteins can be studied in the natural host cells in vitro.

MATERIALS AND METHODS

Expression of a DHBV pre-S/MS2 fusion protein in *Escherichia coli*. The vector used, pEx33c, is a member of the pEx vector series which allows expression of proteins fused to the N-terminal part of the bacteriophage MS2 polymerase under control of the lambda pL-promoter in *E. coli* (24). It differs from the other pEx vectors only within the polylinker and the MS2-polymerase/polylinker junction. The sequence of the respective region in pEx33c is as follows:

*Eco*RI *Bam*HI *Hin*dIII
MS2-pol<G,GGG,AAT,TCC,GGA,TCC,GGC,CAA,GCT,T>pBR322

For expression of DHBV pre-S sequences, pEx33c was cut with *Bam*HI and *Hin*dIII and ligated with a 409-base-pair *Bst*NI/*Hin*dIII fragment comprising nucleotides 688 to 1097 of the DHBV genome (Fig. 1). The *Bam*HI site of the vector and the *Bst*NI site of the insert then were filled by using *E. coli* polymerase I and ligated. After transformation into *E. coli* W6, which constitutively expresses the phage lambda cI repressor, a clone containing the DHBV fragment in the correct reading frame was selected and used for expression of the MS2/pre-S fusion protein. The expression and purification of such fusion proteins in an *E. coli* strain which contains a temperature-sensitive cI repressor and their use for raising antisera have been described in detail (24).

Detection of DHBV pre-S proteins in liver and serum by Western blotting, Coomassie blue staining, and autoradiography. (i) Western blotting. For the detection of pre-S proteins in liver, tissue which had been stored at −70°C was homogenized in 4 volumes of phosphate-buffered saline

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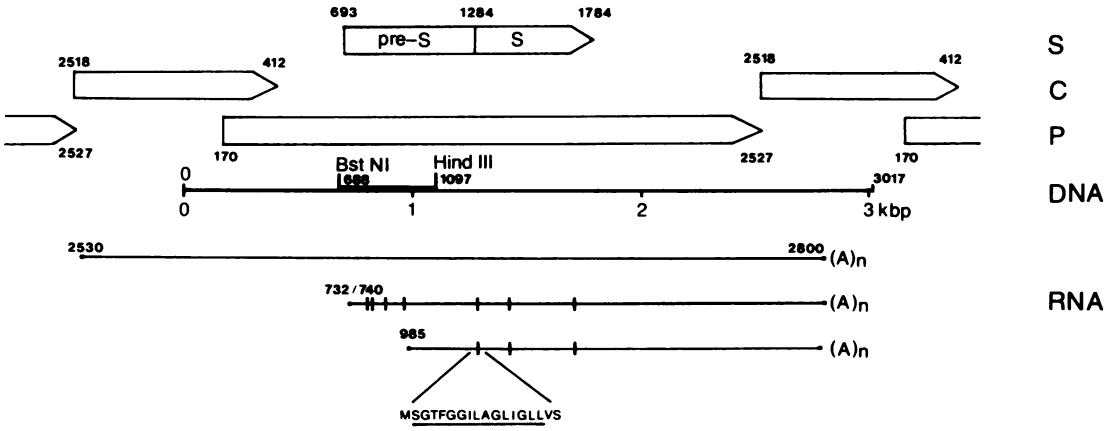

FIG. 1. Linear representation of the DHBV genome with terminal redundancies. In the upper part, the three different open reading frames as defined by respective start and stop codons are shown as open arrows. The confining nucleotide positions are indicated. The line below these arrows represents a genomic unit length. For expression of a MS2/pre-S fusion protein in *E. coli*, the indicated *Bst*NI/*Hin*dIII fragment was used. Beneath this line, the three major transcripts are shown. The in-frame AUGs on both mRNAs which initiate within the S open reading frame are symbolized by vertical bars. The first 18 amino acids of the protein encoded by the mRNA initiating at nucleotide 985 are given. The experimentally determined N-terminal DHBV S amino acid sequence is underlined. Nucleotides are numbered according to reference 9 starting at the unique *Eco*RI cleavage site.

(PBS; 140 mM NaCl, 10 mM phosphate [pH 7.4]), and proteins from samples corresponding to about 1 mg of liver tissue were precipitated with trichloroacetic acid (TCA; final concentration, 10%) for 30 min on ice. The precipitated proteins were recovered by centrifugation and washed once with 1% TCA. After careful removal of residual acid, the proteins were dissolved in 50 μl of sample buffer (3% sodium dodecyl sulfate [SDS], 2% 2-mercaptoethanol, 10% sucrose, 0.1% bromophenol blue, 5 mM EDTA, 200 mM Tris hydrochloride [pH 8.8]), heated for 3 min in a boiling-water bath, and loaded onto a 12.5% SDS-polyacrylamide gel by using the buffer system described by Laemmli (8). For the examination of serum, 5 μl of serum was mixed directly with 45 μl of sample buffer and then treated as described for the liver samples. After separation, the proteins were transferred to a nitrocellulose filter by the method described by Towbin et al. (28). After transfer, unoccupied binding sites were saturated by incubating the filter for 6 h at room temperature (RT) with 1% bovine serum albumin (Miles Laboratories, Inc.) dissolved in PBS. The filter then was incubated with appropriate rabbit antisera diluted 1:2,000 in PBS–1% bovine serum albumin–0.01% sodium azide for at least 16 h at RT. Excess antibody was removed by washing the filter twice for 15 min with PBS containing 0.1% Nonidet P-40 (NP-40; Fluka) at RT. Bound antibody was detected by incubation of the filter with ^{125}I-labeled affinity-purified protein A (Amersham Corp.) at a concentration of 0.05 μCi/ml in PBS–1% bovine serum albumin for 3 h at RT. Unbound protein A was removed by washing with PBS–0.1% NP-40 (30 min, RT). Then the filter was air dried and exposed to X-ray film (XAR-5; Eastman Kodak Co.) at −70°C by using an intensifying screen.

(ii) Coomassie blue staining. After electrophoresis, the gel was stained for at least 1 h with 0.06% Coomassie brilliant blue dissolved in 50% methanol–10% acetic acid. For destaining, the gel was boiled for a few seconds in 7.5% acetic acid–5% methanol in a microwave oven and cooled to RT. After renewal of the destaining solution, this treatment was either repeated or destaining was completed at RT overnight.

(iii) Autoradiography. After electrophoresis, the gels were treated with dimethyl sulfoxide-PPO (2,5-diphenyloxazole) as described by Bonner and Laskey (2). After drying, they were exposed to X-ray film (XAR-5; Kodak) at −70°C.

Biosynthetic labeling of duck liver cells with [^{35}S]methionine. A duck 2 to 6 weeks old, which was shown to be DHBV infected by dot blot analysis of a serum sample as described previously (20), was sacrificed, and the liver was removed. Liver tissue (0.5 g) was minced and incubated at 37°C in 2 ml of 0.25% collagenase (Worthington class 2; Seromed) dissolved in minimal essential medium (GIBCO Laboratories) containing 10% fetal calf serum. After 30 to 40 min, suspended cells were separated from undissociated tissue and pelleted. To lyse the erythrocytes, the cells were suspended in 1 ml of 0.15 M NH_4Cl and incubated for 10 min on ice. Then 2 ml of minimal essential medium was added, and the cells were pelleted, washed twice with methionine-free minimal essential medium, and suspended in 0.5 to 2 ml of methionine-free minimal essential medium.

For labeling, [^{35}S]methionine (specific activity, >800 Ci/mmol; Amersham) was added to a concentration of 0.25 to 0.5 mCi/ml. After 1 to 2 h of incubation at 37°C, the cells were pelleted and lysed in 100 μl of PBS containing 2% SDS, 2% 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. Proteins were denatured by being heated for 3 min in a boiling-water bath, the SDS was diluted to 0.1% by the addition of PBS containing 1% Triton X-100, and undissolved material was removed by centrifugation.

Immunoprecipitation procedure. Rabbit serum (10 μl) in 200 μl of PBS was incubated for 1 h at 4°C with 25 μl of protein A coupled to Sepharose (Pharmacia) preswollen in PBS. The Sepharose then was pelleted, and the supernatant was discarded. The sample solution (usually 200 to 500 μl) was added to this pellet and incubated for 4 to 16 h at 4°C with slight agitation. The Sepharose then was pelleted again, and the supernatant was discarded. The washing procedure varied, depending on the different experimental conditions. If immunoprecipitation was done in the absence of detergent, the pellet was washed four times with PBS. If a nonionic detergent (NP-40 or Triton X-100) had been added before immunoprecipitation, 1% of the respective detergent was added to the PBS for washing. If the sample had been

denatured with SDS, 1% Triton X-100 and 0.1% SDS were added to the PBS for washing. After washing, the samples were either directly dissolved in 50 μl of sample buffer by heating for 3 min in a boiling-water bath and applied to a gel or stored at −20°C.

Purification of virus and S particles by CsCl equilibrium centrifugation. (i) Small preparation. CsCl (971 mg) was dissolved in a mixture of 0.4 ml of serum and 3.2 ml of PBS (corresponding to a CsCl density of 1.2 g/ml). This solution was centrifuged in an SW60 rotor (Beckman Instruments, Inc.) for 18 h at 50,000 rpm at 20°C. Ten fractions then were collected from the bottom of the gradient and examined for viral DNA by dot blot analysis as described previously (20) and for viral proteins. Before gel electrophoresis, proteins were TCA precipitated as described above. If gradient fractions were used for immunoprecipitation, they were diluted fivefold with PBS.

(ii) Large preparation. Serum was precleared by centrifugation for 10 min at 20,000 × *g*. Then, 28 ml of serum was layered onto a 10-ml cushion of 15% sucrose in 100 mM NaCl–10 mM Tris, pH 7.4, and virus and S particles were pelleted by centrifugation for 4.5 h at 27,000 rpm in an SW28 rotor (Beckman) at 20°C. The pellet was suspended in 3.5 ml of water, and CsCl was added to a density of 1.2 g/ml. The further steps were as described above.

Isolation of DHBV S and DHBV pre-S for immunization and amino acid sequence analysis. S particles purified from serum were TCA precipitated, dissolved in sample buffer, and loaded onto a 12.5% polyacrylamide gel. After slight staining with Coomassie blue, the S and pre-S protein bands were cut from the gel, and the proteins were electroeluted. Protein amounts were estimated from Coomassie blue-stained gels by comparison with known protein standards.

N-terminal amino acid sequence analysis. The electroeluted proteins were subjected to automated gas phase Edman degradation with an Applied Biosystems gas phase sequencer.

Immunization of rabbits with S particles. Undenatured DHBV S particles, purified from serum of infected ducks, were used to immunize a New Zealand White rabbit. The first dose was applied in Freund complete adjuvant (1.5 ml). After 18 days, a second dose was given in incomplete adjuvant (1.5 ml). Each dose contained about 4 μg of S antigen and 1 μg of pre-S antigen as estimated from a Coomassie blue-stained gel in comparison with protein standards. Two weeks after the booster injection, blood was withdrawn, and the serum was tested for antibodies directed against DHBV surface proteins by immunoprecipitation of virus and purified S particles in the absence or presence of detergent as well as by Western blotting.

Immunization of rabbits with gel-purified DHBV S protein. DHBV S protein, obtained from isolated S particles as described above, was used to immunize a New Zealand White rabbit. The first dose was applied in 1.5 ml of Freund complete adjuvant. After 3 weeks, a second dose was given in 1.5 ml of incomplete adjuvant, and 10 days later a third dose in 1.5 ml of incomplete adjuvant was administered. Each dose contained about 20 μg of purified DHBV S as estimated from a Coomassie blue-stained gel. Ten days after the third injection, blood was withdrawn, and the serum was tested for antibodies directed against DHBV-S protein by Western blotting at a serum dilution of 1:1,000, using 20 ng of gel-purified S protein as a positive control.

RESULTS

Expression of a DHBV pre-S polypeptide in *E. coli*. To obtain an antigen for the generation of an anti-DHBV pre-S serum, a 409-base-pair *Bst*NI-*Hin*dIII fragment, comprising nucleotides 688 to 1097 of the DHBV genome (Fig. 1), was cloned into the expression vector pEX33c in frame with the 98 N-terminal amino acids of the MS2-polymerase gene. This construct was used to transform *E. coli* 537, which is a derivative of *E. coli* C600 carrying a temperature-sensitive mutant of the phage lambda repressor gene cI on a kanamycin resistance plasmid. After thermoinduction, a MS2/pre-S fusion protein with the expected molecular size of about 26 kDa was obtained. This protein was purified and used for immunization.

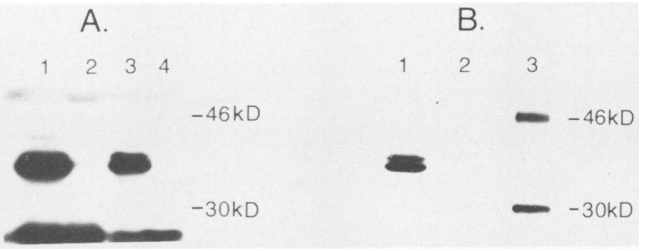

FIG. 2. Detection of pre-S proteins in liver and serum by Western blotting and by immunoprecipitation after biosynthetic labeling. (A) Liver homogenates of a DHBV-positive (lane 1) and of a DHBV-negative (lane 2) duck, corresponding to about 1 mg of liver tissue, and 5 μl of serum of a DHBV-positive (lane 3) and of a DHBV-negative (lane 4) duck were applied to a 12.5% polyacrylamide gel. After being transferred to a nitrocellulose filter, the separated proteins were probed with an antiserum raised against pre-S sequences. (B) About 45 × 10^6 cpm of [^{35}S]methionine-labeled proteins from a DHBV-positive (lane 1) and a DHBV-negative (lane 2) duck liver cell culture were subjected to immunoprecipitation with an anti-pre-S immune serum, and the precipitated proteins were applied to a 12.5% polyacrylamide gel. After PPO treatment, the gel was dried and exposed to X-ray film. Lane 3, Molecular weight marker. kD, Kilodaltons.

Detection of DHBV pre-S proteins in serum and liver of infected ducks. A rabbit was inoculated with the MS2/pre-S fusion protein described above, and the immune serum was used for examination of total serum or liver proteins of DHBV-infected and uninfected ducks. After Western blotting, two dominant bands representing proteins with apparent molecular sizes of about 35 and 37 kDa were detected exclusively in the specimens derived from DHBV-positive animals. (Fig. 2A). The observed molecular size is in good agreement with the expected molecular size, which, as calculated from the known viral sequence, should be 35.7 kDa if the first AUG of the mRNAs starting at about nucleotide 730 is used for initiation of pre-S synthesis.

The ratio between the 35- and 37-kDa pre-S proteins in the sera varied. In most duck sera, i.e., 11 of 15 , the amount of the larger one was about 1/2 to 1/5 that of the smaller protein, in two sera the amount was about 1/10, and in two other sera only the 35-kDa protein could be detected.

Biosynthetic labeling of DHBV pre-S in a primary liver cell culture. To test whether biosynthesis of DHBV proteins can be studied in the natural host cells in vitro, we examined whether the DHBV pre-S proteins can be biosynthetically labeled in a primary liver cell culture. For these experiments, liver tissue was dissociated by collagenase treatment, and cellular proteins were labeled with [^{35}S]methionine in vitro. Newly synthesized DHBV pre-S proteins could be easily detected after immunoprecipitation with the anti-MS2/pre-S antiserum (Fig. 2B).

Immunoprecipitation of virus with anti-pre-S. To demonstrate that proteins containing pre-S sequences are part of

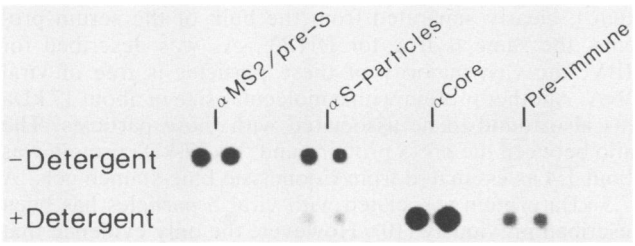

FIG. 3. Immunoprecipitation of virus particles and viral cores. Untreated DHBV-positive duck serum (upper lane) and virus-positive serum, to which 1% NP-40 had been added (lower lane), were subjected to immunoprecipitation with rabbit antisera raised against either an MS2/pre-S fusion protein, DHBV S particles, or bacterially synthesized DHBV core protein. As a control, a rabbit preimmune serum was used. Immunoprecipitated material was dissolved in PBS–2% SDS, and samples were spotted onto a nitrocellulose filter. Viral DNA was detected by hybridization with ^{32}P-labeled cloned DHBV DNA.

the viral envelope, serum derived from a DHBV-positive duck was subjected to immunoprecipitation by using both the anti-MS2/pre-S antiserum and another rabbit serum obtained after immunization with purified viral S particles (see below). Both sera precipitated virus from detergent-free serum, as demonstrated by the presence of viral DNA in the respective immunoprecipitates (Fig. 3). The amount of viral DNA which was precipitated by the anti pre-S antisera was drastically reduced after the addition of 1% NP-40 to the serum. Since this treatment destroys the viral envelope, viral DNA can now be detected in immunoprecipitates obtained with the anticore serum. The slightly positive results obtained with the anti pre-S and the control serum after detergent treatment is caused by an unspecific adsorption of viral cores to protein A-Sepharose, which was also observed in the absence of any serum (data not shown).

Purification of viral S particles from serum and N-terminal amino acid sequence analysis of DHBV-S antigen. To provide further evidence that the DHBV pre-S proteins detected in serum are present in a particulate form, CsCl equilibrium centrifugation was done. The gradient was divided into 10 fractions and examined for viral DNA, total proteins, and pre-S proteins (Fig. 4A to C). As demonstrated by the presence of viral DNA, viral particles were detected mainly in fractions 5 to 7 (density, 1.20 to 1.16 g/cm^3) (Fig. 4A). The major portion of the pre-S proteins, however, was located in fractions 7 to 10 (density, 1.16 to 1.13 g/cm^3), as evidenced by the presence of two protein bands with the expected molecular size of about 35 to 37 kDa after gel electrophoresis (Fig. 4B, left). These proteins were missing in corresponding gradient fractions obtained from serum of an uninfected duck (Fig. 4B, right). Furthermore, they reacted strongly with the anti-MS2/pre-S antiserum as shown by Western blotting (Fig. 4C).

In the same fractions, another protein with a molecular size of about 17 kDa was found specifically in the DHBV-positive serum sample (Fig. 4B, left). To test whether this protein represents the DHBV S antigen, we gel purified this protein and determined its N-terminal amino acid sequence. The sequence elicited was S-G-T-F-G-G-I-L-A-G-L-I-G-L-L, which agrees perfectly with the DHBV S N-terminal protein sequence expected if the first AUG of the mRNA starting at nucleotide 985 is used for initiation of DHBV-S synthesis (Fig. 1) and if the start methionine is removed during biosynthesis. An attempt to determine the N-terminal amino acid sequence of the 35-kDa pre-S protein failed, because the N terminus proved to be blocked.

In summary, the data presented above show that in the serum of DHBV-infected ducks three proteins encoded by the viral S frame can usually be detected. The two larger ones contain the pre-S sequence, whereas in the small one this part is missing. The bulk of these proteins exists in DHBV-DNA-free particles which, because of their lower density, almost completely separate from the viral particles during CsCl equilibrium centrifugation. These particles are referred to as DHBV S particles in this report.

Comparison of the immunogenicity of DHBV pre-S and S antigen. To compare the antigenicity of DHBV pre-S and DHBV S proteins, rabbits were immunized with purified undenatured S particles, which consist of pre-S and S proteins, and the antiserum obtained was tested for S or pre-S antigen-specific antibodies.

This anti-S particle serum reacted well with the pre-S proteins after Western blotting, but no reaction with the S antigen was observed (data not shown). An analogous result was obtained after immunoprecipitation. With this antiserum

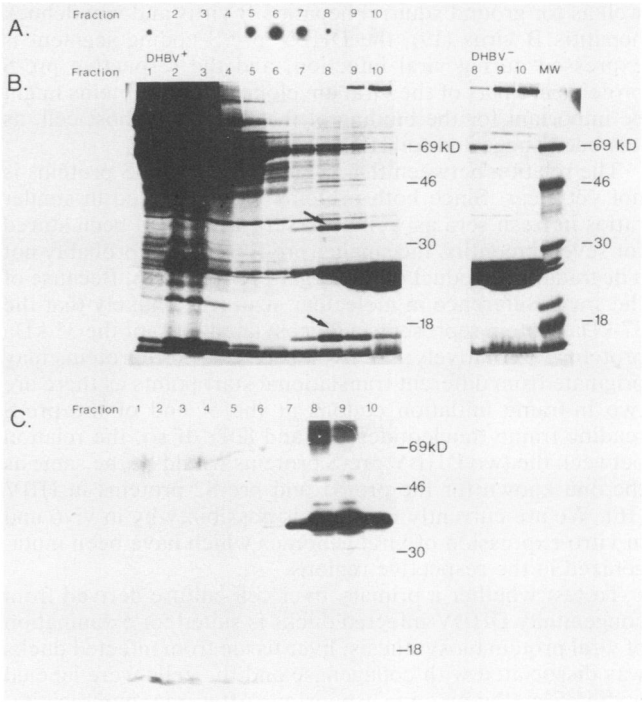

FIG. 4. Separation of virus and viral S particles from serum proteins and detection of viral DNA and pre-S proteins. Duck sera were subjected to CsCl equilibrium centrifugation, and the collected fractions were examined for viral DNA (A), total proteins (B), and pre-S proteins (C). (A) Samples from each gradient fraction were spotted onto a nitrocellulose filter, and viral DNA was detected by hybridization with ^{32}P-labeled cloned DHBV DNA. (B) Proteins, derived from either a DHBV-positive (left) or a DHBV-negative (right) serum sample, were TCA precipitated from gradient fractions, separated on a 12.5% polyacrylamide gel, and stained with Coomassie blue. Only the three top fractions of the DHBV-negative serum sample are shown. The protein bands representing DHBV pre-S (molecular size, about 35-kDa) and DHBV S (molecular size, about 17 kDa) are marked with arrows. MW, molecular-weight marker. (C) Proteins from the gradient fractions shown in panel B were TCA precipitated, separated on a 12.5% polyacrylamide gel, transferred to a nitrocellulose filter, and probed with the antiserum raised against the MS2/pre-S fusion protein.

intact S particles could be precipitated from detergent-free solution. After the addition of a nonionic detergent, however, only pre-S was precipitated (data not shown). These results suggest that after immunization with undenatured S particles, consisting of S and pre-S antigen, the major immune response is directed against the pre-S determinants.

To substantiate this finding, and to test whether DHBV S is a poor antigen per se, a large amount of denatured S antigen was gel purified and used to immunize another rabbit. After two booster injections, the immune serum was tested for its anti-DHBV S titer by Western blotting. Again, no reaction either with the pre-S or the S antigen was observed (data not shown).

DISCUSSION

By using an antiserum raised in rabbits against a fusion protein comprising most of the DHBV pre-S coding region, a 35-kDa protein could be detected after Western blotting in all sera and livers from DHBV-infected ducks tested. With rare exceptions, a 37-kDa pre-S protein was also found. The anti-pre-S serum precipitated both virus and viral S particles. Thus, as has been described for HBV (7, 16, 22, 23) as well as for ground squirrel hepatitis B virus and woodchuck hepatitis B virus (19), the DHBV pre-S coding segment is expressed during viral infection, and the respective pre-S proteins are part of the viral envelope. These proteins might be important for the binding of the virus to its host cell, as has been suggested for HBV (13).

The relation between the 35- and 37-kDa pre-S proteins is not yet clear. Since both proteins were detected in similar ratios in fresh sera as well as in sera which had been stored for several months, the smaller pre-S protein is probably not a degradation product of the larger pre-S protein. Because of the small difference in molecular size, it is unlikely that the 37-kDa protein represents a glycosylated form of the 35-kDa protein. Alternatively, the two observed pre-S proteins may originate from different translational start points as there are two in-frame initiation codons at the 5′ end of the pre-S reading frame (nucleotides 693 and 801). If so, the relation between the two DHBV pre-S proteins would be the same as the one known for the pre-S1 and pre-S2 proteins in HBV (16). We are currently testing this possibility by in vivo and in vitro expression of viral genomes which have been mutagenized in the respective regions.

To test whether a primary liver cell culture derived from congenitally DHBV-infected ducks is suited for examination of viral protein biosynthesis, liver tissue from infected ducks was dissociated with collagenase and the cells were labeled with [^{35}S]methionine in suspension. After immunoprecipitation with the anti-pre-S antibodies, both pre-S proteins could be easily detected. In additional experiments, it has also been possible to detect newly synthesized viral core protein (H. J. Schlicht, unpublished data). In the meantime, similar experiments have been successfully done by using long-term primary liver cell cultures. By using these cultures, it has also been possible to study the synthesis of viral proteins after in vitro infection of virus-negative liver cells. (P. Galle, H. J. Schlicht, and H. Schaller, unpublished data). Recently, a similar tissue culture system was described (29), but viral proteins were not examined in detail.

In the case of HBV, the pre-S proteins detected in serum were shown to be present in lipid-containing particles with low density (16, 23). Since after CsCl equilibrium centrifugation, the respective DHBV proteins were found exclusively in the upper gradient fractions (density, 1.13 to 1.20 g/cm^3), clearly separated from the bulk of the serum proteins, the same is true for DHBV. As was described for HBV, the vast majority of these particles is free of viral DNA. Another protein with a molecular size of about 17 kDa was also found to be associated with these particles. The ratio between the pre-S proteins and this 17-kDa protein was about 1:4 as estimated from Coomassie blue-stained gels. A 17.5-kDa protein associated with viral S particles has been described previously (10). However, the only evidence that this protein represented DHBV S came from a comparison of tryptic peptide maps between HBV S and this 17.5-kDa DHBV protein. To precisely correlate this protein with the viral sequence, from which an S protein consisting of 167 amino acids with a molecular size of 18.2 kDa is predicted, we performed an N-terminal amino acid sequence analysis. The sequence determined is in agreement with a translational start at the AUG at nucleotide 1284, the first AUG on the viral mRNA starting at nucleotide 985. Thus, this viral mRNA encodes the major DHBV surface protein, and in contrast to most secreted or transmembrane proteins (1), there is no signal sequence being cleaved from the S protein during biosynthesis. The HBV S protein also has no cleavable signal sequence (15). In a recent report, it was shown that the translocation of HBV S across the lipid membrane occurs via an internal protein domain (5). The same mechanism may be used by DHBV S.

No evidence could be obtained for the presence of glycosylated DHBV S proteins, which have been described for HBV S. This may be due to the deletion in DHBV S of a hydrophilic region which, in HBV S, forms the hepatitis B surface antigen-subtype antigenic domain (21) and which is also the target of glycosylation (4, 6, 17). Thus, although a glycosylation recognition sequence N-X-S is still present within this region in DHBV S, loss of the hydrophilic environment may render this size inaccessible to the respective modification enzymes.

To compare the immunogenicity of the S and the pre-S antigen, intact DHBV S particles, which are composed of both antigens, were injected into a rabbit. The antiserum obtained reacted well with undenatured as well as with denatured pre-S antigen, but no reaction with the S antigen was observed. Similarly, the injection of large amounts of denatured S antigen failed to elicit a significant antibody titer specific for denatured S antigen. Thus, the S antigen appears to be, at best, only a weak immunogen compared with DHBV pre-S. This finding is reminiscent of the results obtained with HBV S, which is also a poor antigen compared with the HBV pre-S proteins (11, 12, 14). In the case of DHBV S, this difference may be even more pronounced, because the hydrophilic domain against which the HBV S group-specific antibodies are directed (4, 16, 17) is deleted in DHBV S (21).

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