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Characterization of a Pre-S Polypeptide on the Surfaces of Infectious Avian Hepadnavirus Particles

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DNA from the pre-S region of the duck hepatitis B virus (DHBV) genome was inserted into an open reading frame vector designed to give high-level expression in *Escherichia coli*. The resulting fusion protein contained the first 8 amino acids of β-galactosidase, 86 amino acids of the DHBV pre-S region, and 219 amino acids of chloramphenicol acetyltransferase at the C terminus (β-gal:pre-S:CAT). Rabbit antiserum against purified β-gal:pre-S:CAT was used to identify pre-S-containing polypeptides in DHBV particles by Western blotting. A dominant species of 36 kilodaltons (kDa) was identified. Antiserum against the major 17-kDa DHBsAg polypeptide also reacted with the 36-kDa protein. This suggests that the DHBV envelope gene polypeptides share the same carboxyl terminus, but differ in the sites from which translation is initiated. N-linked carbohydrate was not detected on either the 17- or 36-kDa envelope proteins. Anti-β-gal:pre-S:CAT abolished infectivity of the virus in an in vitro assay. Thus, the pre-S region is exposed on the surfaces of infectious virions and may be directly involved in binding of virus to host-cell receptors.

Several viruses have recently been identified which share structural and biological properties with the human hepatitis B virus (HBV). This family of hepatotropic viruses, termed the hepadnaviruses, includes the woodchuck hepatitis virus (WHV) (23), the ground squirrel hepatitis virus (GSHV) (10), and the duck hepatitis B virus (DHBV) (11). The hepadnaviruses are related by their morphology, genomic structure, associated pathology, and mode of replication. Cloning and sequencing of viral DNA from isolates of each member of this group has revealed the overall organization of the viral genes (12). DHBV has virtually no sequence homology with the mammalian viruses yet exhibits a very similar genetic organization (8).

For several years it has been known that translation of the 226-amino acid major HBV envelope protein (HBsAg) initiates from the third ATG in the open reading frame (ORF) (12, 18). The region of the ORF upstream from this ATG had coding potential for an additional 174 N-terminal amino acids and was designated pre-S. Recently, polypeptides containing pre-S amino acids have been detected in human hepatitis B virus (5, 22, 25) and WHV and GSHV (20) particles. Pre-S polypeptides are less abundant than the major envelope protein (sAg) in virus particles and have recently been localized to the virion surface (20; D. T. Wong, A. Prince, N. Nath, and J. Sninsky, Abstr. Meet. Mol. Biol. Hepatitis B Viruses, 1985, p. 17, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

The major 17-kilodalton (kDa) DHBV envelope protein (DHBsAg) has previously been identified (9). A minor 38-kDa DHBV protein has been reported and was suggested, from the results of tryptic peptide mapping, to share structural relatedness with the 17-kDa DHBsAg polypeptide (4). The same authors speculated that the 38-kDa species may represent a DHBV pre-S envelope polypeptide. In this study, we used polyclonal antisera generated against bacterially synthesized DHBV pre-S fusion proteins to detect authentic DHBV pre-S polypeptides. This approach has been used previously to identify pre-S polypeptides in the mammalian hepadnaviruses (20, 25).

An in vitro system for infecting cultured primary duck hepatocytes with DHBV was recently described (24). We applied this system to determine the effect of anti-pre-S antisera on DHBV infectivity. Our results indicate that pre-S determinants are present on infectious viral particles.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All plasmids were propagated in either *Escherichia coli* MC1000 (ΔM15, IPOZYx74) (2) or DG101 (*thi-1 endA1 hsdR17 supE44* λ^- *lacI*^{q} *lacZ* ΔM15) (14). Conditions for growth and storage of bacterial cultures were described previously (20).

DNA manipulations and bacterial transformations. Procedures for purification of plasmid DNA, restriction endonuclease and ligation reactions, and bacterial transformation have been previously described (20).

Purification of fusion proteins and generation of antisera. Bacterial strain MC1000 harboring pZL811 or strain DG101 harboring the recombinant plasmid pDpresCAT-1 was grown in 2 liters of Luria broth (15) to a density of 0.6 to 0.8 A_{650} at which time DG101 cultures were induced by the addition of 10 mM isopropyl-β-D-thiogalactopyranoside. Cultures were incubated for 1 h after induction and then centrifuged at 7,000 rpm for 10 min in a JA-10 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The procedures involved in purification of chloramphenicol acetyltransferase (CAT) fusion proteins from the bacterial pellets have been described previously (20). The CAT fusion proteins were recovered at concentrations of ca. 0.1 to 0.5 mg/ml with yields ranging from 5 to 30 mg/liter of bacterial culture. Fusion proteins were stored at 4°C (short term) or −20°C (long term) and used to immunize rabbits by standard procedures (25).

Partial purification of DHBV particles and generation of antisera against the major DHBV surface antigen polypeptide.

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DHBV-containing duck serum (8 ml) obtained from 3-week-old animals was pelleted through two 10-ml 10 to 20% (wt/vol) sucrose gradients in 150 mM NaCl–10 mM Tris hydrochloride (pH 7.4) at 25,000 rpm for 16 h at 4°C in a Beckman SW40 rotor. The supernatant was discarded, and the viral particles were resuspended in 0.5 ml of 150 mM NaCl–10 mM Tris hydrochloride (pH 7.4). The viral suspension was centrifuged through a 7-ml 15 to 55% (wt/vol) sucrose gradient with a 1-ml 65% (wt/vol) sucrose cushion at 25,000 rpm for 16 h at 4°C in a Beckman SW40 rotor. Fractions containing virions and surface antigen particles (density, 1.030 to 1.55 g/cm^3) were pooled, and an equal volume of sample buffer was added (200 mM Tris hydrochloride [pH 8.8], 5 mM EDTA, 2% [wt/vol] sodium dodecyl sulfate (SDS), 10 mM dithiothreitol, 0.01% [wt/vol] bromophenol blue). Preparative SDS-polyacrylamide gel electrophoresis (PAGE) was used to purify the major 17-kDa DHBsAg polypeptide. After the sample was denatured at 95°C for 2 min, polypeptides were separated by 12.5% (wt/vol) SDS-PAGE (7). The ratio of acrylamide to *N*,*N*-methylenebisacrylamide in the stock acrylamide solution was 200:1 in the resolving gel and 40:1 in the stacking gel. The use of a low-cross-linking SDS-PAGE facilitates efficient recovery of proteins. After electrophoresis, a test strip was cut from the gel and stained with Coomasssie blue. The region of the unstained gel shown to contain the 17-kDa DHBsAg polypeptide was excised, and the ground gel slice was incubated with 5 ml of 0.1% (wt/vol) SDS for 16 h at 37°C. The supernatant was recovered after brief centrifugation, and the gel was incubated with a further 5 ml of 0.1% (wt/vol) SDS for 2 h at 37°C. The supernatants were pooled, centrifuged at 10,000 rpm in a Sorvall HB4 rotor for 20 min at 10°C, and then precipitated with 8 volumes of acetone at −20°C. The acetone precipitate was dried and suspended in 1 ml of phosphate-buffered saline. The efficiency of recovery and purity of the 17-kDa protein was assayed by SDS-PAGE. This sample was then used to inject two New Zealand rabbits by a standard immunizing procedure (25). Each rabbit received three injections of equal amounts of protein (approximately 10 μg).

Western blot procedures. Partial purification of DHBV for Western blotting was done as previously described for WHV (20). Fractionation of viral polypeptides by SDS-PAGE and details of the Western blotting procedures have also been described previously (6, 20). For preadsorption experiments, undiluted antisera were incubated with 10 μg of hybrid or tribrid protein per ml for 30 min at room temperature, at which time sera were centrifuged at 12,000 × *g* for 4 min. Supernatants were diluted 1:200 in blot wash buffer (20), and hybrid or tribrid protein was added to a final concentration of 10 μg/ml. Diluted antisera and competing antigen were then incubated with protein blots.

Glycosidase experiments. Samples (10 μl each) of 50-fold-concentrated, partially purified WHV and DHBV were brought to a final volume of 30 μl containing 0.5% SDS and 0.1 M β-mercaptoethanol and heated to 100°C for 3 min. The reduced and denatured virion preparations were cooled to 37°C and incubated in a 90-μl reaction mixture containing 0.2 M $NaPO_4$ (pH 8.6), 1.25% Nonidet P-40, and 10 mM 1,10-phenanthroline, with or without 1.2 U of *N*-glycanase (Genzyme) for 4 h. After the 4-h incubation, reactions with or without *N*-glycanase were divided, and equal volumes were fractionated by SDS-PAGE. Viral proteins were electrophoretically transferred to nitrocellulose filters and probed with polyclonal antisera to a WHV pre-S tribrid protein (20) or DHBV pre-S tribrid protein. Proteins were detected by labeling with ^{125}I-protein A followed by autoradiography (see Western blot for details).

In vitro infections of primary duck hepatocytes with DHBV. Primary hepatocyte cultures were prepared from 1-week-old uninfected ducklings by liver perfusion with collagenase. A detailed description of the in vitro experimental infection system is presented elsewhere (24). Dishes of hepatocytes (60-mm diameter) were infected 24 h after plating. DHBV-containing sera from 3-week-old ducklings were diluted in complete medium (L-15 medium [GIBCO Laboratories, Grand Island, N.Y.] containing 5% fetal bovine serum, 15 mM HEPES, [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 300 mg of penicillin per liter, 100 mg of streptomycin per liter, 1 mg of insulin [Sigma Chemical Co., St. Louis, Mo.] per liter, 1.5 mg of glucose per liter, 10^{-5} M hydrocortisone hemisuccinate [Sigma], and 10 U of nystatin per ml). Virus (0.5 ml of a 10^{-1} or 10^{-2} dilution) was preincubated with a 1:20 dilution of the appropriate antiserum for 2 h at 37°C and then used directly to infect a dish of cells. Infection was done at room temperature for 1 h, when virus was aspirated and 4 ml of fresh medium was added to the cells. The cells were incubated at 37°C for up to 12 days, and the medium was changed every 24 h.

Preparation and analysis of DHBV DNA from hepatocyte cultures. Total intracellular viral DNA was prepared from hepatocyte cultures as described previously (24). DNA was electrophoresed in a 1.5% agarose horizontal slab gel in 60 mM Tris hydrochloride (pH 6.9)–20 mM sodium acetate–1 mM EDTA. After denaturation of the gel, DNA was transferred to nitrocellulose by the method of Southern (21). Hybridization was performed at 50°C with a ^{32}P-labeled ribonucleotide probe derived from plasmid pSP6.DHBV5.1 (24). Transcription of this DNA in vitro with SP6 polymerase yields transcripts of plus-strand polarity representing the entire DHBV genome.

Autoradiography was performed at −80°C with XAR (Eastman Kodak Co., Rochester, N.Y.) film and an intensifying screen.

RESULTS

Construction of plasmid expressing DHBV pre-S sequences. The ORF vector (pZL811) used to express DHBV pre-S sequences has been described previously (25) (Fig. 1). Plasmid pZL811 directs expression of a hybrid protein containing the 8 N-terminal amino acids of β-galactosidase, eight linker-derived codons, and the 219 amino acids of CAT at the C terminus (β-gal:CAT).

A *Sau*3A fragment from the pre-S region of DHBV (nucleotides [nt] 930 to 1185; nucleotide numbering system is that described by Galibert and co-workers [8]) was chosen for insertion into the *Bam*HI site of pZL811 (Fig. 1) because of the complementary 5′ protruding ends generated by these two enzymes and the retention of reading frame between β-galactosidase and CAT. A dimer of DHBV DNA cloned into the *Eco*RI site of pBR322 (16) was digested with endonuclease *Xho*II, and a 1,080-base-pair (bp) fragment (nt 391 to 1471) was initially isolated to simplify purification. The *Xho*II fragment was further digested with endonuclease *Sau*3A, and a 255-bp DNA fragment (nt 930 to 1185) was isolated. The *Sau*3A fragment, having the capacity to encode 85 pre-S amino acids (residues 79 to 164 of a protein initiated from the first ATG of the ORF), was ligated to *Bam*HI-cut, alkaline phosphatase-treated pZL811 (Fig. 1). The ligation reaction was used to transform *E. coli* DG101, followed by selection for transformants on ampicillin-containing agar

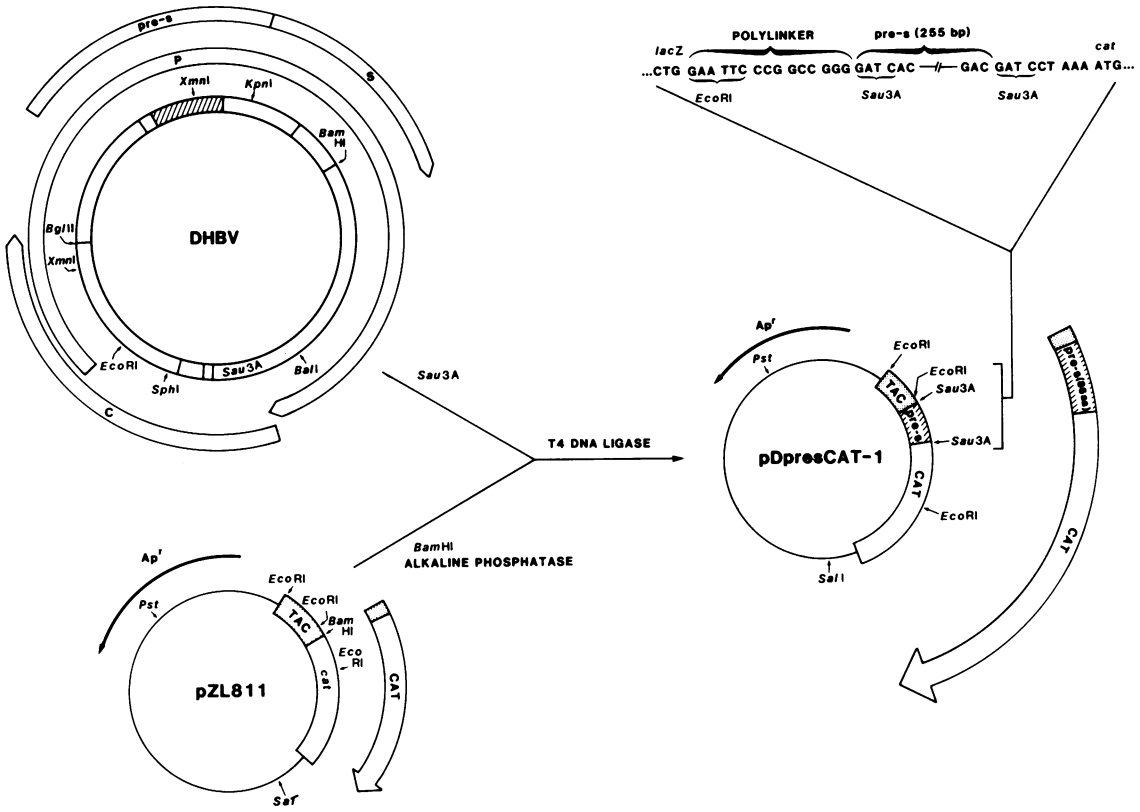

FIG. 1. Construction of β-gal:dpre-S:CAT tribrid protein expression plasmid. The DHBV genomic map shown at the top left is based on the sequence of Mandart et al. (8). Arrows encircling the map represent ORFs. Unique endonuclease sites are indicated for orientation, and *Sau*3A sites are noted within the genomic circular map. Arrows labeled S and C refer to the DHBV surface and core antigen genes, respectively. The segment contiguous with the S gene represents the pre-S portion of the *env* ORF. The arrow labeled P represents the largest ORF. The *Sau*3A fragment containing pre-S sequences, which was inserted into the *Bam* HI site of pZL811, is schematically indicated on the viral map (hatched region) for simplicity, but was actually isolated from a cloned dimer of the viral genome (see Results). The broad arrows associated with pZL811 and pDpresCAT-1 represent the hybrid β-gal:CAT and tribrid β-gal:dpre-S:CAT proteins, respectively. The dotted, hatched, and open portions of the broad arrows represent β-gal, dpre-S, and CAT amino acids, respectively. pDpresCAT-1 encodes a protein containing 86 pre-S amino acids, 85 of which are encoded by the inserted *Sau*3A fragment, and 1 of which is contributed by the vector which encodes the same amino acid (proline) as codon 165 of the pre-S sequence. The thin arrows labeled Ap^r denoted for pZL811 and pDpresCAT-1 represent β-lactamase. The nucleotide sequence noted at the upper right corresponds to the viral DNA-vector junctions.

medium. Plasmid DNAs isolated from 16 transformants were digested with endonuclease *Xmn*I, which cleaves twice in the vector and once within the desired *Sau*3A fragment. Three candidates were identified which contained the expected additional *Xmn*I cleavage site and were further analyzed. The desired 255-bp *Sau*3A fragment contained unique *Hin*dIII and *Sma*I endonuclease recognition sequences, both located asymmetrically within the fragment. Plasmid DNAs from the three candidates containing a new *Xmn*I site were digested with endonuclease *Nco*I, which cleaves once within the CAT gene of pZL811, in combination with either *Hin*dIII or *Sma*I. These analyses indicated that one of the three candidates contained the 255-bp *Sau*3A fragment in the correct orientation for fusion of pre-S sequences to CAT (data not shown). The recombinant plasmid was designated pDpresCAT-1 and was used in all subsequent studies (Fig. 1).

Total cellular protein from bacteria (*E. coli* DG101) harboring plasmid pDpresCAT-1 was fractionated by SDS-PAGE and visualized by Coomassie blue staining. After isopropyl-β-D-thiogalactopyranoside induction, cells containing pDpresCAT-1 synthesized a new protein of ca. 38 kDa, which approximates the molecular weight predicted for a tribrid protein containing 85 pre-S amino acids (β-gal:dpre-S:CAT), and no longer synthesized the 28-kDa polypeptide (β-gal:CAT) encoded by pZL811 (data not shown). In Western blot analysis, anti-β-gal:CAT recognized the 38-kDa protein, in addition to two lower-molecular-weight polypeptides (M_r ca. 30,000 and 22,000) (see Fig. 3, lane 4). The lower-molecular-weight bands appear to be N-terminally truncated molecules as judged by their immunological reactivity (see below).

Generation and characterization of antisera to β-gal:dpre-S:CAT. Sera obtained from rabbits 2 weeks after a secondary injection with β-gal:dpre-S:CAT reacted strongly with the tribrid protein in an enzyme-linked immunosorbent assay (data not shown), as well as on a Western blot. As shown in Fig. 3, lane 6, antiserum to the DHBV pre-S tribrid protein recognized predominantly the two higher-molecular-weight polypeptides produced by bacteria harboring pDpresCAT-1 (38,000 and 30,000), while anti-β-gal:CAT serum, as mentioned above, recognized all three polypeptides efficiently. Since the lower-molecular-weight protein (22,000) contained β-gal:CAT determinants but was lacking pre-S sequences, it is likely an N-terminally truncated product. Whether these N-terminally truncated products result from internal initiation of translation or proteolytic degradation is unclear, although analogous proteins have been observed in similar

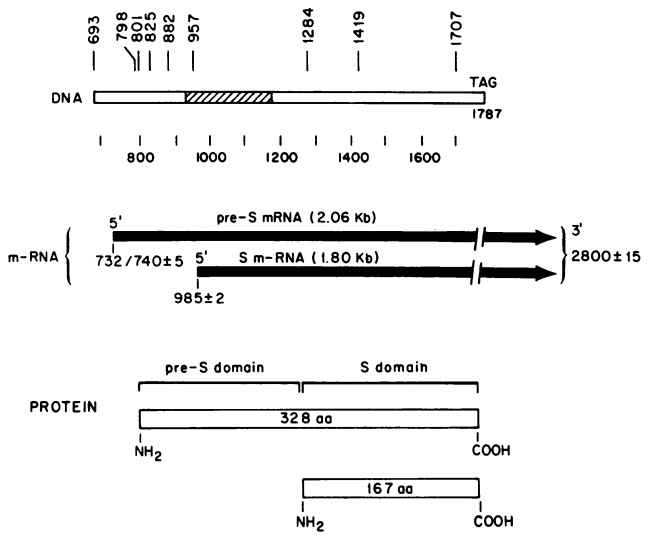

FIG. 2. Schematic representation of DHBV envelope gene expression. The 1,096-bp *env* gene is diagrammed at the top of the figure and numbered according to the system of Galibert and co-workers (8). The positions of the in-frame ATGs in the *env* gene are shown at the top with their map coordinates. The hatched region of the DNA represents the *Sau*3A fragment (nt 930 to 1185) that was inserted into plasmid pZL811 (Fig. 1). The two DHBV transcripts that map to the *env* gene are shown below with their respective sizes and the positions of their 5′ and 3′ termini (1). The two major *env* gene products are detailed at the bottom of the figure. Initiation of translation at the first available AUG in the pre-S mRNA (nt 798) could give rise to the observed 36-kDa polypeptide. Translation of the 17-kDa major surface antigen polypeptide probably initiates at nt 1284. Kb, Kilobases; aa, amino acids.

tribrid constructions (20). These results also suggest that the pre-S amino acids in the tribrid are more immunogenic in rabbits than are the CAT sequences.

Identification of pre-S polypeptides from DHBV. The pre-S regions from the mammalian viruses contain two ATGs (three in GSHV), both of which are used to initiate translation (5, 20, 25). In contrast, the DHBV pre-S region contains six ATG codons upstream of and in frame with the surface antigen ATG (Fig. 2) (8). The location of the DHBV pre-S segment of the tribrid protein with respect to the ATGs in the viral pre-S region suggests that the antibody to β-gal:dpre-S:CAT recognizes polypeptides initiated from any of the pre-S ATGs (Fig. 2). Recent analysis of RNA from DHBV-infected duck livers identified a transcript which does not include the first ATG of the pre-S region, but does contain the remaining five ATGs. Translation initiation from the 5′-proximal ATG of this transcript (nt 798, numbering system of Mandart et al. [8]) would give rise to a 328-amino acid polypeptide of ca. 36 kDa.

To identify a pre-S polypeptide(s) from DHBV, we analyzed partially purified viral particles by Western blotting. Using the rabbit polyclonal serum to β-gal:dpre-S:CAT, a 36-kDa protein was identified as the major pre-S species, and a less prominent 34-kDa protein was also occasionally observed (Fig. 3, lane 5). Neither the 36- nor the 34-kDa molecule was recognized by preimmune serum or antiserum to the hybrid protein (Fig. 3, lanes 1 and 3). To assess whether recognition of the two polypeptides was specific, anti-β-gal:dpre-S:CAT was preadsorbed with either the hybrid or the tribrid protein before incubation with the blot. Recognition of the 36- and 34-kDa molecules was eliminated

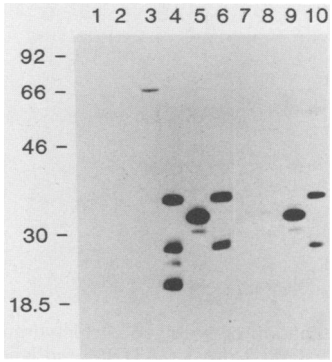

FIG. 3. Western blot identification of DHBV pre-S polypeptides. Odd-numbered lanes contain partially purified DHBV from 0.5 ml of infectious duck serum, and even-numbered lanes contain 0.1 μg of affinity-purified β-gal:dpre-S:CAT fusion proteins. Pairs of lanes were treated with different antisera: lanes 1 and 2, rabbit preimmune serum; lanes 3 and 4, rabbit antiserum to β-gal:CAT; lanes 5 and 6, rabbit antiserum to β-gal:dpre-S:CAT. Lanes 7 and 8 were treated as lanes 5 and 6 except that antiserum was preadsorbed with β-gal:dpre-S:CAT, and lanes 9 and 10 were treated as lanes 5 and 6 except that antiserum was preadsorbed with β-gal:CAT. After incubations with rabbit antiserum, all lanes were probed with ^{125}I-labeled protein A (see Materials and Methods for details). M_rs of protein standards ($\times 10^3$) are indicated at the left.

by preincubation of antiserum with the tribrid protein (Fig. 3, lane 7), but was unaffected by identical treatment with the hybrid protein (Fig. 3, lane 9). These data suggest that the 36- and 34-kDa molecules contain determinants encoded by the pre-S region of DHBV.

Demonstration of surface antigen determinants on pre-S polypeptides. In the absence of an mRNA splicing event, the 36-kDa pre-S translation product is expected to contain 167 amino acids encoded by the DHBV surface antigen gene (Fig. 2). To determine whether the pre-S polypeptide contained surface antigen sequences, we performed Western blotting of DHBV envelope polypeptides and control polypeptides using antiserum to β-gal:dpre-S:CAT (Fig. 4A)

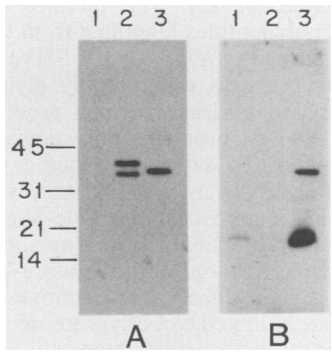

FIG. 4. Lanes 1 and 2 contain 100 ng of β-gal:CAT and β-gal:dpre-S:CAT, respectively. Lane 3 contains polypeptides from partially purified viral particles derived from 0.5 ml of DHBV-positive serum. Polypeptides were resolved in a 12.5% SDS-polyacrylamide gel containing 8 M urea in the resolving gel. After transfer of proteins to nitrocellulose, the duplicate samples were reacted with either a 1:1,000 dilution of rabbit anti-β-gal:dpre-S:CAT serum (A) or a 1:500 dilution of rabbit anti-17-kDa DHBsAg serum (B). Bound antibody was detected by autoradiography after incubation of the filters with ^{125}I-labeled protein A. The position and size in kilodaltons of molecular size standards are shown on the left.

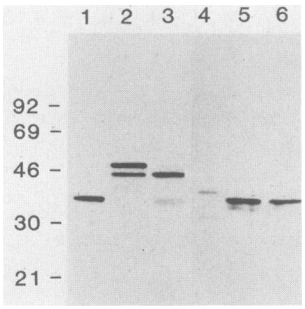

FIG. 5. Determination of N-linked carbohydrates. Lane 1 contains 100 ng of β-gal:WHVpre-S:CAT(20), and lane 4 contains 100 ng of β-gal:dpre-S:CAT. Lanes 2, 3, 5, and 6 each contain a mixture of partially purified WHV and DHBV from 0.4 ml of infectious woodchuck and duck sera. The viral preparations in lanes 2 and 5 were incubated under conditions identical to those in lanes 3 and 6 except for the addition of *N*-glycanase to the latter two. After Western transfer, lanes 1, 2, and 3 were probed with a 1:200 dilution of anti-β-gal:WHVpre-S:CAT(20); lanes 4, 5, and 6 were probled with a 1:200 dilution of anti-β-gal:dpre-S:CAT. After incubation with rabbit antisera, protein blots were incubated with ^{125}I-labeled protein A (see Materials and Methods for details). M_rs of protein standards ($\times 10^3$) are indicated at the left.

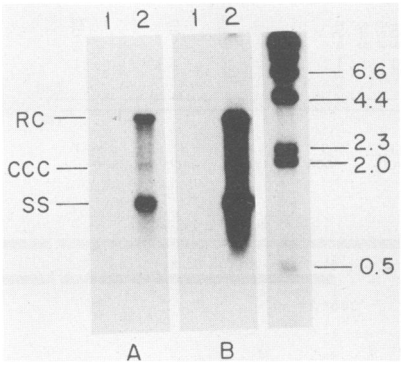

FIG. 6. Effect of anti-β-gal:dpre-S:CAT antiserum on viral infectivity. Virus was incubated with a 1:20 dilution of rabbit anti-β-gal:dpre-S:CAT (lane 1) or preimmune (lane 2) serum for 2 h at 37°C. DHBV-containing serum (0.5 ml of a 10^{-2} [A] or 10^{-1} [B] dilution) was used to infect each dish of hepatocytes (60-mm diameter). Cells were infected 24 h after plating and harvested for nucleic acid preparation 12 days after infection. Equivalent amounts of DNA were loaded on each lane of a 1.5% agarose gel. DHBV DNA was detected by Southern blot hybridization with a ^{32}P-labeled ribonucleotide probe of plus-strand polarity (see Materials and Methods). The positions of relaxed circular (RC), covalently closed circular (CCC), and single-stranded (SS) forms of intracellular viral DNA are shown on the left. The position and size in kilobase pairs of ^{32}P-labeled, *Hin*dIII-digested bacteriophage lambda DNA markers are shown on the right.

and antiserum generated against the major 17-kDa DHBV surface antigen polypeptide (see Materials and Methods) Fig. 4B). This analysis revealed that in addition to reacting with the 17-kDa surface antigen polypeptide to which it was raised, the antiserum also reacted with a 36-kDa polypeptide identical in size to the major species recognized by antibodies to β-gal:dpre-S:CAT (Fig. 4A and B, lanes 3). To demonstrate that the antigen used to generate the anti-17-kDa serum had not contained any of the 36-kDa pre-S polypeptide, we included the tribrid protein in the Western blot (Fig. 4A and B, lanes 2). The failure of the anti-17-kDa serum to react with the β-gal:dpre-S:CAT protein indicated that antibodies that recognize pre-S determinants were not present. Neither antiserum recognized the hybrid protein (Fig. 4A and B, lanes 1).

Determination of N-linked carbohydrates. In HBV the existence of differentially glycosylated pre-S proteins has been demonstrated (5, 22). The number and molecular weight of pre-S polypeptides identified from the other mammalian hepadnaviruses (WHV and GSHV) suggests that these molecules are also differentially glycosylated (20). Unlike the mammalian viruses, which produce four major pre-S proteins, DHBV produces only one major and one minor pre-S species. To determine whether pre-S proteins from WHV and DHBV were glycosylated, we treated virus preparations with *N*-glycanase (peptide-*N*-glycosidase F), followed by Western blot analysis with an antibody to WHV pre-S amino acids (20) or anti-β-gal:dpre-S:CAT serum. *N*-Glycanase is a glycosidase which removes both complex and high-mannose N-linked carbohydrate side chains leaving an aspartic acid at the glycosylation site (3). Before the addition of *N*-glycanase, WHV contained the four pre-S polypeptides previously reported (33, 36, 45, and 47 kDa; p33 was observed upon longer exposure) (20) (Fig. 5, lane 2). After a 4-h incubation with the glycosidase, the levels of two of the pre-S molecules (p47 and p36) were greatly diminished, and the remaining two polypeptides (p45 and p33) were more abundant (Fig. 5, lane 3). These data demonstrate that gp47 and gp36 are glycosylated derivatives of p45 and p33 as previously suggested (20). Western blot analysis of *N*-glycanase-treated DHBV demonstrated that neither the major (p36) nor the minor (p34) pre-S polypeptide from the duck virus was altered in molecular weight after enzyme treatment (Fig. 5, lanes 5 and 6). This indicated that neither the predominant nor the minor pre-S species from DHBV contains N-linked carbohydrates. In addition, after Western blotting of DHBV polypeptides, nitrocellulose filters were reacted with either wheat germ lectin or concanavalin A, both labeled with horseradish peroxidase. Neither of these lectins, which bind efficiently to a wide range of glycoproteins, showed any binding to species of 17 or 36 kDa (data not shown). It is possible that glycosylated forms of DHBV envelope gene polypeptides do exist, but in such small amounts that they were not detected by these methods.

Effect of antiserum to β-gal:dpre-S:CAT on in vitro infectivity. A system for infecting cultures of primary duck hepatocytes with DHBV was recently described (24). We applied this system here to examine the role of pre-S polypeptides in viral infectivity. Before infection a dilution of virus was incubated with a 1:20 dilution of anti-β-gal:dpre-S:CAT or preimmune serum. After infection, cells were maintained in culture for 12 days when they were harvested for DNA preparation. Southern blot analysis was used to assay for the presence of replicative forms of viral DNA resulting from a productive in vitro infection (Fig. 6). No DHBV was detected in cells infected with virus preincubated with anti-β-gal:dpre-S:CAT (Fig. 6A and B, lanes 1). Cells infected with virus treated with preimmune serum contained large amounts of all forms of replicative DHBV DNA normally found in infected tissue (Fig. 6A and B, lanes 2) (24).

The anti-17-kDa DHBsAg polyclonal serum did not affect infectivity when preincubated at 1:20 dilution with a similar amount of virus. Filter binding assays indicate that equivalent amounts of native DHBV surface antigen bind approximately 100-fold less antibody from identical dilutions of anti-17-kDa protein serum as compared with anti-β-gal:dpre-S:CAT serum (data not shown). We suspect that the low titer

of antibodies to native DHBsAg in the anti-17-kDa protein serum may account for the observed lack of effect on viral infectivity.

DISCUSSION

This study identified a minor DHBV envelope polypeptide of approximately 36 kDa which contains determinants encoded by both the pre-S and S regions of the DHBV *env* gene. A DHBV polypeptide of the same relative size and abundance was identified in a previous study (4). We believe that translation of the 36-kDa protein initiates at the second available ATG in the pre-S region (nt 798) and terminates at the stop codon of the major surface antigen gene (nt 1787). This conclusion is based on the size of the polypeptide determined by SDS-PAGE, fine mapping of the DHBV mRNA transcripts (1), and data obtained here by using antisera against specific *env* gene products. There are currently no amino acid sequence data available for either the 17-kDa or the 36-kDa polypeptide.

The overall arrangement of the DHBV *env* gene is similar to that previously determined for HBV (5, 25) and WHV and GSHV (20), though some features of *env* gene expression do appear to differ. In HBV and WHV there are only two ATGs in the pre-S region, and both appear to be utilized yielding two pre-S-containing polypeptides (5, 20). There are six in-frame ATGs in the DHBV pre-S sequence. The first ATG (nt 693) maps upstream from the 5′ end of the putative pre-S mRNA (Fig. 2). The next three ATGs are clustered between nt 798 and 825 and could all potentially be utilized to initiate translation of a 36-kDa polypeptide. Translation of the minor 34-kDa pre-S polypeptide may initiate at one of the downstream ATGs. The 34-kDa species was not detected in all DHBV-containing serum samples examined, suggesting that it is not always synthesized at the same level.

A second difference was observed in the transcription of the DHBV *env* gene. Infection by the three mammalian hepadnaviruses produces two major polyadenylated viral RNA transcripts. One of these is a larger-than-genome-length molecule, and the other initiates in the middle of the *env* gene (12). Both RNAs share the same 3′ end and exhibit some heterogeneity at their 5′ termini. The *env* gene transcript is thought to direct expression of both the major surface antigen protein and the shorter of the two pre-S polypeptides (12). An mRNA has yet to be assigned as the template for translation of the large pre-S protein. There is in vitro evidence, however, for a message which initiates upstream of the HBV *env* gene (19). DHBV transcribes three major mRNAs, two of which are analogous to those of the mammalian viruses (1). The third mRNA initiates at the 5′ end of the *env* gene and is coterminal with the two other major transcripts (Fig. 2). Translation of this additional RNA could give rise to the 36-kDa polypeptide, as mentioned above. Although the DHBV transcripts which initiate within the *env* gene are equally abundant in infected liver (13), the relative abundance of the two major *env* gene products in viral particles suggests that the 17-kDa DHBsAg is produced at much higher levels than the 36-kDa pre-S polypeptide (Fig. 4B, lane 3). The factors controlling the differential expression of the DHBV *env* gene products are not understood.

A third difference between DHBV and the mammalian hepadnaviruses was observed in the posttranslational modification of the viral *env* gene products. Neither the 17-kDa nor the 36-kDa DHBV envelope polypeptide appears to contain N-linked carbohydrate. This is in contrast to the mammalian hepadnavirus particles which contain N-linked glycosylated forms of both the pre-S and sAg proteins (5, 20).

We used a tissue culture system for infecting primary duck hepatocytes with DHBV (24) to examine the role of pre-S determinants in viral infectivity. Polyclonal rabbit antiserum against purified DHBV pre-S fusion protein was incubated with virus immediately before infection of cells. Incubation with the antiserum abolished viral infectivity, since no intracellular replicative forms of viral DNA were later detected. These data demonstrate that pre-S determinants are present on particles responsible for infectivity. The block to infection may be due to a steric factor caused by antibody aggregation of viral particles, rather than being attributable to specific interference with the interaction between pre-S polypeptides on the virus and receptor molecules on the host-cell surface. Further experiments will be required to demonstrate whether DHBV pre-S determinants interact specifically with cellular receptors.

It is not known how the 17- and 36-kDa proteins are arranged on the envelope of mature DHBV viral particles. A relative hydrophilicity profile of the predicted amino acids of the DHBV *env* gene protein (E. Schaeffer, unpublished data) suggests that the hydrophobic S domain is embedded in the lipid bilayer, while the hydrophilic pre-S domain would be exposed on the outer surface. The results presented here are consistent with the pre-S domain of the 36-kDa protein being exposed on the exterior of infectious viral particles.

Recent studies have shown that antisera against HBV pre-S determinants can protect against viral infection in vivo (17; Wong et al., Abstr. Meet. Mol. Biol. Hepatitis B Viruses 1985). Such experiments involve the immunization of chimpanzees. Consequently, the immune-mediated protective effect is difficult to dissect and could involve humoral or cell-mediated immunity or a combination of the two. The application of an in vitro system for assaying infectivity of DHBV will allow us to test directly the involvement of humoral immunity directed against specific epitopes in viral neutralization.

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LITERATURE CITED

1. **Buscher, M., W. Reiser, H. Will, and H. Schaller.** 1985. Transcripts and the putative RNA pregenome of duck hepatitis B virus: implications for reverse transcription. Cell **40**:717–724.
2. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**:179–207.
3. **Elder, J. H., and S. Alexander.** 1982. Endo-β-N-acetylglucosaminidase F: endoglycosidase from *Flavobacterium meningosepticum* that cleaves both high-mannose and complex glycoproteins. Proc. Natl. Acad. Sci. USA **79**:4540–4546.
4. **Feitelson, M. A., P. L. Marion, and W. S. Robinson.** 1983. The nature of polypeptides larger in size than the major surface antigen components of hepatitis B and like viruses in ground

squirrels, woodchucks and ducks. Virology **130**:76–90.
5. **Heerman, K. H., V. Goldmann, W. Schwartz, T. Seyffarth, H. Baumgarten, and W. H. Gerlich.** 1984. Large surface proteins of hepatitis B virus containing the pre-S sequence. J. Virol. **52**:396–402.
6. **Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder.** 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Technol. **1**:3–8.
7. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
8. **Mandart, E., A. Kay, and F. Galibert.** 1984. Nucleotide sequence of a cloned duck hepatitis B virus genome: comparison with woodchuck and human hepatitis B virus sequences. J. Virol. **49**:782–792.
9. **Marion, P. L., S. S. Knight, M. A. Feitelson, L. S. Oshiro, and W. S. Robinson.** 1983. Major polypeptide of duck hepatitis B surface antigen particles. J. Virol. **48**:534–541.
10. **Marion, P. L., L. S. Oshiro, D. C. Regnery, G. H. Scullard, and W. S. Robinson.** 1980. A virus in beechey ground squirrels which is related to hepatitis B virus of man. Proc. Natl. Acad. Sci. USA **77**:2941–2945.
11. **Mason, W. S., G. Seal, and J. Summers.** 1980. Virus of Pekin ducks with structure and biological relatedness to human hepatitis B virus. J. Virol. **36**:829–836.
12. **Mason, W. S., J. M. Taylor, and R. Hull.** 1987. Retroid virus genome replication. Adv. Virus Res. **32**:35–96.
13. **Mason, W. S., J. M. Taylor, G. Seal, and J. Summers.** 1982. An HBV like virus of domestic ducks, p. 107–116. *In* W. Szmuness, H. J. Alter, and J. E. Maynard (ed.), Viral hepatitis. Franklin Institute Press, Philadelphia.
14. **Meselson, M., and R. Yuan.** 1968. DNA restriction enzyme from *E. coli*. Nature (London) **217**:1110–1114.
15. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 433. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Molnar-Kimber, K. L., J. Summers, and W. S. Mason.** 1984. Mapping of the cohesive overlap of duck hepatitis B virus DNA and of the site of initiation of reverse transcription. J. Virol. **51**:181–191.
17. **Neurath, A. R., S. Kent, K. Parker, A. Prince, N. Strict, B. Brotman, and P. Sproul.** 1986. Antibodies to a synthetic peptide from the pre-S 120-145 region of the HBV envelope are virus neutralizing. Vaccine **4**:35–37.
18. **Peterson, D. L., I. M. Roberts, and G. N. Vyas.** 1977. Partial amino acid sequence of two major component polypeptides of hepatitis B surface antigen. Proc. Natl. Acad. Sci. USA **74**:1530–1534.
19. **Rall, L. B., D. N. Strandring, O. Laub, and W. J. Rutter.** 1983. Transcription of hepatitis B virus by RNA polymerase II. Mol. Cell. Biol. **3**:1766–1773.
20. **Schaeffer, E., R. L. Snyder, and J. J. Sninsky.** 1986. Identification and localization of pre-S encoded polypeptides from woodchuck and ground squirrel hepatitis viruses. J. Virol. **57**:173–182.
21. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
22. **Stibbe, W., and W. H. Gerlich.** 1983. Structural relationships between minor and major proteins of hepatitis B surface antigen. J. Virol. **46**:626–628.
23. **Summers, J., J. Smolec, and R. Snyder.** 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc. Natl. Acad. Sci. USA **75**:4533–4537.
24. **Tuttleman, J. S., J. C. Pugh, and J. Summers.** 1986. *In vitro* experimental infection of primary duck hepatocyte cultures with duck hepatitis virus. J. Virol. **58**:17–24.
25. **Wong, D. T., N. Nath, and J. Sninsky.** 1985. Identification of hepatitis B virus polypeptides encoded by the entire pre-S open reading frame. J. Virol. **55**:223–231.