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Antibodies to pre-S and X Determinants Arise during Natural Infection with Ground Squirrel Hepatitis Virus

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The DNA sequence of the ground squirrel hepatitis virus (GSHV) genome predicts the existence of several proteins in addition to the major surface (S) and core antigens. These include the pre-S1 and pre-S2 proteins, initiated at sites within the open reading frame preceding and continuous with the coding region for the S gene product, and the X protein, the putative product of an independent reading frame. Using an antibody directed against a peptide predicted by codons 130 to 143 of the pre-S1 reading frame, we identified a 43-kilodalton product of the pre-S1 coding region in preparations of GSHV surface antigen purified from the sera of infected animals. In addition, by immunoprecipitation of S- and pre-S-specific in vitro translation products with ground squirrel sera obtained after GSHV infection, we determined that antibodies arise to both S and pre-S determinants. The antibody response to pre-S includes, in some cases, reactivity to pre-S1-specific domains and is not always associated with an anti-S response. Similarly, by production of the viral X gene product in vitro followed by immunoprecipitation with ground squirrel sera, we showed that antibodies to this viral gene product also arise during infection, indicating that X antigenic determinants are synthesized during viral infection and are recognized by the host immune system.

Ground squirrel hepatitis virus (GSHV) is a small hepatotropic DNA virus closely related to human hepatitis B virus (HBV) (1, 17, 25). Inoculation of ground squirrels with this agent produces both acute and chronic hepatic infection (7) and provides a useful model system for the study of many aspects of HBV replication. The genome of GSHV has been cloned (6, 29), its nucleotide sequence has been determined (27), and the transcripts it engenders in infected liver cells have been mapped and characterized (2). These studies allow a more thorough examination of viral genes and their protein products during natural infection of susceptible animal hosts.

Like the genomes of other mammalian hepadnaviruses, the GSHV genome contains four major open reading frames (28): C, the gene for the viral core (nucleocapsid) proteins; P, the coding region for the viral DNA polymerase; the pre-S/S region, encoding the viral coat proteins (surface antigens); and X, an open reading frame to which no function has been assigned. The products of the viral C and S genes are produced in large quantities in vivo and have been well characterized structurally (3, 4, 8); infected animals regularly display prominent immune responses to these proteins (16). Less is known about the potential products of the GSHV pre-S and X regions, and host responses to these products have not been previously examined.

The organization of the pre-S/S region of GSHV is depicted in Fig. 1A. In HBV, the coding region for the major viral surface antigen is only the distal portion of a much larger open reading frame; the portion 5′ to the S region (referred to as pre-S) contains three in-phase AUG codons and thus could encode several larger S-related proteins. Recent studies of human HBV identified two minor sets of proteins whose synthesis in initiated within the pre-S region; each set is made up of the glycosylated and nonglycosylated derivatives of the same polypeptide (23, 24, 30). One set, termed pre-S2 proteins, is initiated at the third AUG within pre-S, producing S-related proteins with a 55-amino-acid N-terminal extension; this extension includes a binding site for polymerized albumin (13, 14, 22), which has been speculated to play a role in viral infectivity (13, 14). The second set (the pre-S1 proteins) likely originates from the first AUG codon and appears to be present in higher quantities in intact virions than in the more numerous subviral particles, a fact that may indicate a role in virion morphogenesis (9, 32).

Because of such suggestions of biological activities unique to pre-S proteins, there is considerable interest in antibody responses to these proteins and their potential significance in the establishment of protective immunity. Such questions may be more readily addressed in studies of animal hepadnaviruses such as GSHV. Earlier studies (4, 26) detected larger polypeptides that are related to the S proteins in sera of GSHV-infected squirrels, but less is known about these proteins, and serologic responses to them have not been characterized.

Still less is known about the GSHV X gene. The X region represents the smallest of the viral open reading frames (ca. 480 base pairs); its predicted protein product is approximately 15 kilodaltons (kDa). No polypeptide of this size has been identified in purified preparations of viral proteins. Serologic responses to HBV X determinants have been noted in human serum, however (10, 18, 21), and suggestions have been made that such antibodies may be correlated with different stages of infection (18) or pathologic sequelae (21).

The GSHV model provides a convenient system in which to pursue further studies of these antigens and the host responses they elicit. In this paper we present further characterization of the GSHV pre-S and X gene products and outline simple and general methods for examining immune responses to the products of predicted open reading frames such as these. Our results show that in addition to previously recognized anti-S and anti-C responses (16), antibodies specific to GSHV pre-S determinants commonly arise after infection and include antibodies directed against pre-S1-specific epitopes. In addition, we demonstrated that

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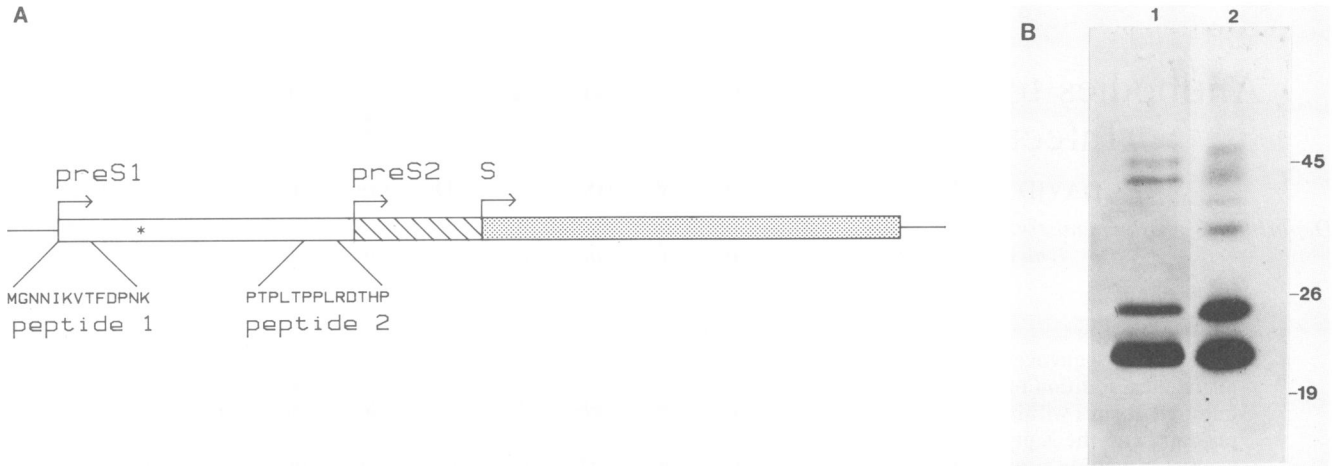

FIG. 1. S and pre-S proteins of GSHV. (A) Coding capacity of GSHV pre-S region. Shown are the probable sites of translation initiation for the pre-S1, pre-S2, and S gene products. The first pre-S AUG is designated codon 1. The S region, coding for the major surface protein, is composed of the 222 codons from codons 207 to 429 of the pre-S/S region. It is predicted that the pre-S2 proteins consist of the 222 amino acids derived from the S region plus a 60-amino-acid N-terminal extension derived from the pre-S2 codons 146 to 206. The pre-S1-specific reading frame consists of the first 145 codons beginning at the first AUG of pre-S; the predicted pre-S1 protein is specified by all 429 codons of the pre-S/S region. Also shown are the relative locations and amino acid compositions of the peptides used to generate antipeptide antibodies. Peptide 1 consists of 13 amino acids predicted by codons 1 to 13 of the pre-S/S region. Peptide 2 is predicted by the pre-S1-specific codons 130 to 143. The asterisk indicates the relative position of the second potential translation initiation codon within pre-S. (B) Purification of GSHsAg. GSHsAg was purified by column chromatography and then radioiodinated with Bolton-Hunter reagent. Lane 1, Slab gel electrophoresis of unlabeled GSHsAg in 14% polyacrylamide followed by silver staining; lane 2, the same GHSsAg fractions run in a 14% polyacrylamide slab gel after radioiodination. The numbers (kilodaltons) at the right indicate the positions of the molecular size markers.

anti-X antibodies are elicited, indicating that GSHV X determinants must be expressed during natural infection.

MATERIALS AND METHODS

DNA constructions. The GSHV genome used in our constructions was that sequenced by Seeger et al. (27). Restriction enzymes were obtained from New England BioLabs, Inc. Plasmid pSP24G was constructed by inserting the 1.3-kilobase (kb) *Sac*I GSHV DNA fragment into the *Sac*I site of the pSP65 (Promega Biotech) polylinker region. The orientations of the resultant clones were determined by restriction endonuclease analysis; clones containing the S coding region downstream from the *Salmonella* bacteriophage SP6 promoter and in the correct transcriptional orientation were selected. Plasmid pSP45G was constructed by first isolating the 2.2-kb *Eco*RI-*Bam*HI fragment from a GSHV genomic clone whose only *Pvu*II site was replaced with a *Bam*HI linker. This fragment was then inserted into the polylinker region of pSP65. The recombinants obtained were of the proper orientation because of the location of the target restriction sites in the polylinker region. Plasmid pSPCG was constructed by first isolating the 1.1-kb *Avr*II-*Pst*I fragment containing the GSHV "precore" and core coding region. This fragment was then cloned into the *Xba*I and *Pst*I sites in the polylinker region of pSP65. The clones obtained were in the correct orientation for transcription of the core gene because of the positions of the target restriction sites in the vector. The construction of pSPXG was done by first isolating the 1.1-kb *Sac*II-*Pst*I fragment containing the GSHV X and precore-core open reading frames. This fragment was then inserted into the polylinker region of pSP65. The recombinants obtained were of the proper orientation because of the positions of the target restriction sites in the polylinker region.

In vitro transcriptions and translations. For in vitro transcription, 1 μg of SP6 promoter-containing plasmid was incubated with 20 U of SP6 polymerase in a 50-μl reaction mixture containing 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl, the ribonucleotides ATP, GTP, CTP, and UTP (Pharmacia Fine Chemicals) at 2.5 mM each, 20 U of RNASIN placental ribonuclease inhibitor (Promega), and 10 mM dithiothreitol. The reaction was allowed to proceed for 1 to 1.5 h at 40°C. To produce truncated pre-S-specific transcripts, plasmid pSP45G was cleaved in the pre-S open reading frame with either *Kpn*I or *Fok*I (New England BioLabs). The DNA fragments were purified by phenol extraction and ethanol precipitation before use in in vitro transcription reactions.

For in vitro translation, 1 μg of SP6-directed RNA was added to a 50-μl rabbit reticulocyte lysate translation mix containing 35 μl of nuclease-treated rabbit reticulocyte lysate (Promega), amino acids minus methionine (Promega) at 1 mM each, 10 μl of [^{35}S]methionine (Amersham Corp.), 10 U of RNASIN, and 5 μl of diethylpyrocarbonate-treated distilled H_2O.

Production of antipeptide antibodies. Peptides corresponding to GSHV pre-S1 codons 1 to 13 (MGNNIKVTFDPNK-C) and 130 to 143 (PTPLTPPLRDTHP-C) were kindly synthesized and provided by Lerner. The peptides were coupled to keyhole limpet hemocyanin carrier protein (Sigma Chemical Co.) at a concentration of 1.25 mg of peptide per mg of carrier by a previously described procedure (12). Each of three male New Zealand White rabbits was injected multiple times with 200 μg of conjugated peptide in Freund complete adjuvant (GIBCO Laboratories). After 3 weeks, an equivalent dose in Freund incomplete adjuvant (GIBCO) was administered. Serum was harvested 2 weeks after the last injection.

Antipeptide antibody titers were determined by enzyme-linked immunosorbent assay in peptide-coated plastic wells. Briefly, peptides were dissolved in 50 mM sodium bicarbonate buffer (pH 9.5) to a final concentration of 100 pmol/ml.

The peptide solution (200 μl) was added to each well of a 100-well Costar Serocluster plate and allowed to remain overnight. The plates were washed extensively with distilled H_2O to remove free peptide, and serial dilutions of rabbit antipeptide antisera were added to the wells in TBS-CS (50 mm Tris [pH 8.15], 0.85% NaCl, 20% calf serum). Unbound antibody was washed away by repeatedly immersing the plate in a bath of TBS. A 1:1,000 dilution of protein A-β-galactosidase in TBS (100 μl) was added to the wells and allowed to stand for 2 h at room temperature. The wells were washed as before and incubated with 4 mg of *o*-nitrophenyl-β-D-galactoside in TBS containing 10 mg of $MgCl_2$ and 100 mM β-mercaptoethanol. Color was allowed to develop for 40 to 60 min at room temperature.

To further test the reactivity of the antipeptide antisera, plasmid pSP45G was transcribed by SP6 polymerase in the procedure described above. RNA yields were typically in 5- to 10-fold molar excess of the input template. The RNA (1 μg/50 μl of translation mix) was translated in the procedure described above. Samples (10 μl) of the translation mix were immunoprecipitated as described below.

For immunoaffinity purification, 1 mg of peptide was dissolved in 2 ml of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4) and added to 2 ml of Affi-gel 10 (Bio-Rad Laboratories) that had been previously prepared in accordance with the specifications of the vendor. The slurry was incubated on a platform rocker at 4°C for 12 to 16 h. Unbound peptide was removed by washing the gel with 50 column volumes of TBS. Rabbit sera judged to have high antipeptide antibody titers were pooled to a final volume of 1 ml and incubated with the column material for 1 h at room temperature. After unbound material was allowed to flow through, the columns were washed with 50 column volumes of TBS. Bound material was eluted with 50 ml of 100 mM glycine (pH 2.5), neutralized immediately with 2 M Tris buffer (pH 7.5), and concentrated to 0.5 ml in Amicon-30 microconcentrators (Amicon Corp.) before use.

GSHsAg preparation and radioiodination. Serum (2 ml) from a GSHV-infected ground squirrel was layered over a Biogel A5M column (3 by 150 cm; Bio-Rad) and allowed to flow into the column material until it had entered completely. The flow was then stopped, and the column was filled with 15 ml of A5M column buffer (150 mM NaCl, 50 mM Tris [pH 7.5], 0.05% aprotinin [Sigma]) and allowed to run for 48 h at a flow rate of 4 ml/h. Forty 4-ml fractions were collected. Ground squirrel hepatitis virus surface antigen (GSHsAg)-containing fractions were identified by the AUSRIA radioimmunoassay (Abbott Laboratories). Initial GSHsAg-positive fractions from the column were judged to be 90% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining.

For radioiodination, 1 to 2 μg of column-purified GSHsAg was dissolved in 25 μl of 50 mM borate buffer (pH 8.5). ^{125}I-labeled monoiodo derivative Bolton-Hunter reagent (125 μCi; Amersham) was added, and the mixture was incubated for 15 min at 4°C. Unbound label was removed by spin dialysis in a 1-ml column of Sephadex G50 medium (Pharmacia).

Immunoprecipitations and Western blotting. For immunoprecipitation, 10 μl of in vitro translation mix was diluted to 150 μl in RIPA buffer (50 mM Tris [pH 8.15], 0.1% SDS, 0.5% Nonidet P-40, 0.5% sodium deoxycholate). The appropriate antibody (3 μl) was added, and the mixture was incubated for 2 to 12 h at 4°C. A 60% (vol/vol) slurry of protein A-Sepharose (20 μl; Sigma) was added, and the mixture was rocked for 2 h at 4°C. The Sepharose pellets were washed in a small column with 1 ml of RIPA buffer eight times followed by a single wash in isotonic saline. Sample buffer (35 μl; 2% SDS, 50 mM Tris [pH 6.8], 100 mM β-mercaptoethanol, 2 μg of bromophenol blue per ml) was added to the air-dried pellets, and samples were incubated at 90°C for 5 min. The sample buffer was then spun out into Eppendorf centrifuge tubes for SDS-PAGE.

For Western blotting, 10 μg of column-purified GSHsAg was boiled for 5 min in electrophoresis sample buffer and then loaded into a single well of a 2-mm-thick 14% SDS polyacrylamide gel. Samples were electrophoresed at a 20-mA constant current for 4 h. The gel was removed, soaked in blotting buffer (0.192 M glycine, 20% methanol) for 20 min, removed, and then overlaid with nitrocellulose and Whatman 3MM filter paper. Resolved proteins were transferred to the nitrocellulose at a constant 40 V for 4 h. Transfer efficiency was assessed by visualization of the transfer of prestained protein molecular weight markers. The filter was removed, soaked in TBS for 5 min, and incubated in a solution of 5% (wt/vol) nonfat dry milk in TBS (TBS-NFDM) for 30 min to reduce nonspecific adsorption. Affinity-purified antipeptide antibody was diluted to 1:300 in TBS-NFDM and incubated for 8 h at 20°C. The blot was washed three times in 100 ml of TBS-0.05% Tween 20 and incubated with 10 μCi of ^{125}I-labeled affinity-purified protein A (Amersham), diluted to 15 ml in TBS-NFDM, for 2 h at 20°C.

RESULTS

pre-S proteins present in GSHV-infected serum. To determine whether pre-S proteins originating from the available translation initiation codons were represented in purified preparations of GSHsAg, we performed a single-step purification of GSHsAg by gel filtration in an A5M column. The initial GSHsAg-containing fractions were GSHV DNA positive and essentially free of detectable contaminating serum proteins, as judged by silver staining. These fractions were pooled and radioiodinated under nondenaturing conditions with Bolton-Hunter reagent. The results of SDS-PAGE of both silver-stained and radioiodinated GSHsAg are shown in Fig. 1B. Silver staining revealed two size classes of GHSsAg-associated proteins: the two predominant S proteins of 21 and 24 kDa and three species in the 40- to 49-kDa range. Upon radioiodination, however, an intermediate class was revealed that migrated at 33 to 36 kDa. These proteins were derived from the pre-S2 region, based on their sizes and their relatedness to the S proteins as shown by protease mapping with both *Staphylococcus* V-8 protease and proteinase K (data not shown). The appearance of these proteins only after radioiodination was probably due to preferential iodination of the lysine residue at position 2 of the pre-S2 region (27); this would have allowed these proteins to be disproportionately labeled relative to their actual proportions in the sample (Fig. 1B).

To examine GSHsAg preparations for products of the pre-S1 region, we first generated antibodies against synthetic peptides derived from the nucleotide sequence of the pre-S1 region. Peptides corresponding to GHSV pre-S1 codons 1 to 13 and 130 to 143 (Fig. 1A) were injected into rabbits, and antibody titers were determined by enzyme-linked immunosorbent assay. All immunized animals generated an antipeptide response. To determine the reactivity of these antibodies to the GHSV pre-S1 region, we synthesized a pre-S protein by in vitro transcription of a recombinant

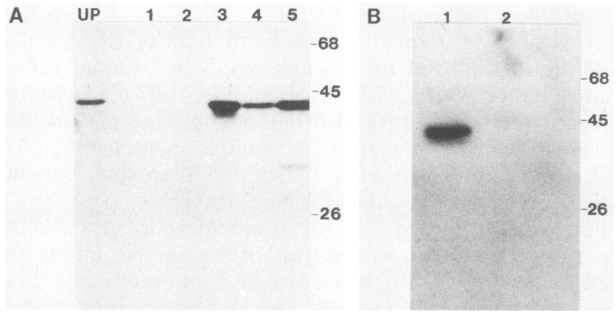

FIG. 2. Identification of GSHV pre-S1 proteins. (A) Immunoprecipitation of a pre-S1 protein made in vitro. A full-length GSHV pre-S1 protein was synthesized by coupled in vitro transcription and translation of pSP45G. SDS-PAGE was done in a 14% polyacrylamide gel. The gel was then fixed, fluorographed, dried, and exposed to Kodak XAR5 film overnight. Lanes: UP, 5% of the unprecipitated translation mix used in each immunoprecipitation reaction; 1, immunoprecipitation protocol done without serum; 2, immunoprecipitation protocol done with preimmune serum; 3, immunoprecipitation with antibody against peptide 2; 4, immunoprecipitation with antibody against peptide 1; 5, immunoprecipitation with anti-HBs. The numbers (kilodaltons) at the right indicate the positions of the molecular size markers. (B) Western blot of GSHV pre-S1 protein. GSHsAg was partially purified by A5M column chromatography, and 20 μg was loaded into a single well of a 12% SDS-polyacrylamide gel (lane 1); corresponding column fractions from an antigen-negative animal were treated identically and loaded into the adjacent well (lane 2). The resolved proteins were transferred to nitrocellulose and probed with affinity-purified anti-peptide 2 antibody. The filter was then washed, incubated with 10 μCi of ^{125}I-labeled protein A, and exposed to film overnight. The numbers (kilodaltons) at the right indicate the positions of the molecular size markers.

plasmid bearing the pre-S/S region downstream of the bacteriophage SP6 promoter; this mRNA was then subjected to in vitro translation as described in Materials and Methods. The synthesis of this protein is initiated at the first AUG within pre-S, producing a principal pre-S1-specific translation product of 43 kDa; some minor products presumed to result from internal initiation at the second and third pre-S AUG were also observed (Fig. 2A, lane UP). The antipeptide antibodies were then used to immunoprecipitate the in vitro translation products. Whereas the serum-free and preimmune serum controls showed no reactivity against the translation products (Fig. 2A, lanes 1 and 2), these proteins were recognized by both antipeptide antisera (Fig. 2A, lanes 3 and 4). As expected, a commercial anti-hepatitis B surface (HBs) antibody was also capable of recognizing the translation products (Fig. 2A, lane 5). The ability of anti-peptide 2 antibody (lane 3), but not anti-peptide 1 antibody (lane 4), to recognize a doublet suggests that the lower band of this doublet may arise from internal initiation at the second AUG codon of the pre-S region.

These sera were then used to detect pre-S1 proteins in preparations of serum-derived GSHsAg. GSHsAg partially purified by column chromatography was concentrated and electrophoresed in a 14% polyacrylamide gel. Corresponding column fractions from a GSHsAg-negative animal were treated in an identical fashion and loaded into an adjacent lane. The resolved proteins were transferred to nitrocellulose and probed with affinity-purified antipeptide antiserum followed by ^{125}I-labeled protein A. Whereas the GSHsAg-negative fraction showed no reactive species (Fig. 2B, lane 2), anti-peptide 2 antibody recognized a polypeptide of 43 kDa (Fig. 2B, lane 1). Surprisingly, anti-peptide 1 antibody, although able to recognize the in vitro translation products, did not identify a protein species when used under identical conditions (data not shown; see Discussion).

Antibodies to pre-S determinants arise during natural GSHV infection. Recent studies of HBV indicated that at least some pre-S-specific protein determinants may be located on the exterior of sAg-associated particles and virions and that pre-S determinants are highly immunogenic when administered to heterologous hosts in immunization protocols (9, 19, 20). We sought to determine whether antibodies arise to GSHV pre-S protein domains during natural infection of susceptible hosts. To accomplish this, sera from GSHV-infected squirrels were used in immunoprecipitation assays with radiolabeled pre-S proteins prepared by coupled in vitro transcription and translation. Because all pre-S proteins share the S-specific domains, antibodies arising to either S or pre-S would give indistinguishable results; accordingly, we synthesized pre-S-specific protein fragments by truncation of the pre-S template DNA with appropriate restriction enzymes.

Plasmid pSP45G was cleaved with *Kpn*I to produce a linear molecule truncated 113 nucleotides downstream of the putative translation initiation codon for the GSHV pre-S2 proteins (Fig. 3A). The short messages produced by SP6-directed in vitro transcription were translated in an in vitro rabbit reticulocyte lysate system to produce a 21-kDa protein fragment representing all of the pre-S1 region and containing 33 amino acid residues of pre-S2. With this antigen, convalescent-phase sera from a cohort of 20 animals with documented GSHV infections were tested in immunoprecipitation reactions. The immune precipitates were analyzed by SDS-PAGE; sera taken from the animals before the resolution of infection were used as controls. A representative group of such immunoprecipitations is shown in Fig. 3. Whereas none of the preresolution sera was reactive, three of four postresolution sera were capable of recognizing GSHV pre-S1/pre-S2 determinants (Fig. 3A, animals 1, 2, and 4).

To determine whether the presence of anti-pre-S antibodies correlated with an immune response to the products of the GSHV S reading frame, we synthesized GSHV S-specific protein in vitro by using an SP6-derived vector containing the entire coding region for the GSHV S gene (Fig. 3B). Immunoprecipitation of the in vitro translation products directed by this plasmid again showed no reactivity with the preresolution sera. Immunoprecipitation with the postresolution sera, however, showed that two of four animals had anti-S antibodies (Fig. 3B, animals 1 and 2). Surprisingly, one animal with an anti-pre-S response displayed no observable anti-S antibody (Fig. 3B, animal 4).

The data from a similar analysis of a cohort of 19 animals that had resolved documented GSHV infection are presented in Table 1. Of 12 anti-S-positive animals, 6 showed an anti-pre-S response. Of the seven anti-S-negative animals, two were anti-pre-S positive.

Antibodies to pre-S1-specific determinants arise during natural infection. We next sought to determine whether GSHV pre-S1 determinants elicit an antibody response during natural infection. Plasmid pSP45G was cut with the restriction enzyme *Fok*I. One of the resulting fragments is composed of the SP6 promoter and 364 nucleotides of the pre-S1 open reading frame. The transcripts obtained from the cleaved template were translated as described earlier to yield a 121-amino-acid pre-S1-specific protein fragment (Fig. 4A).

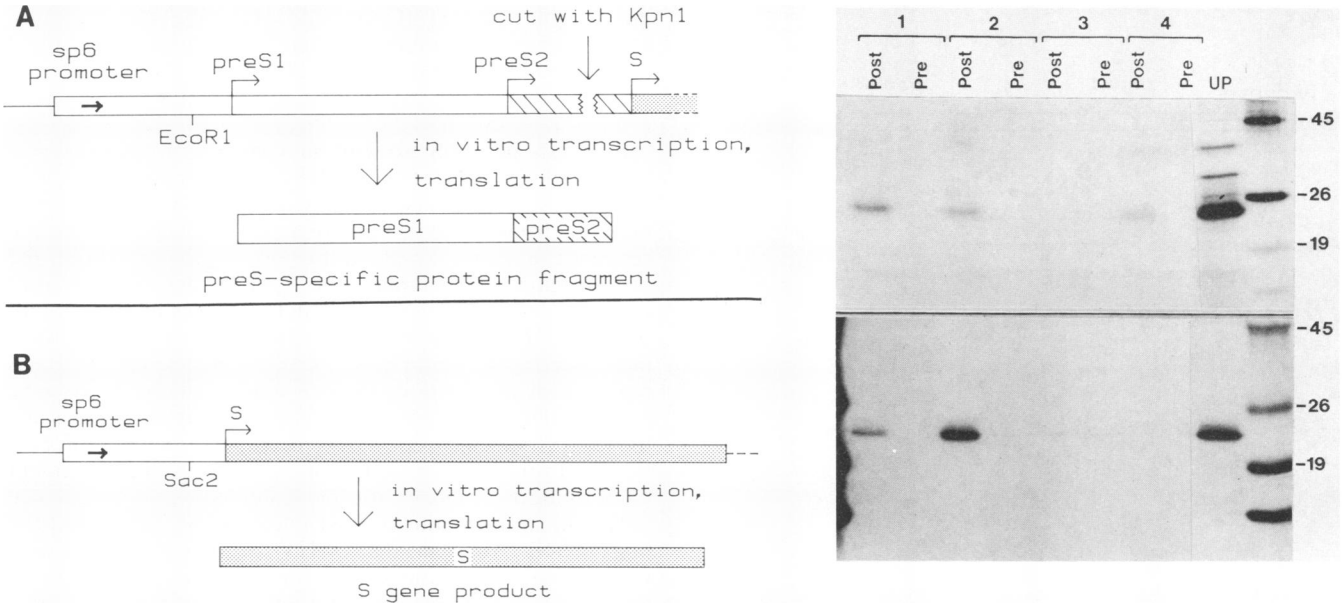

FIG. 3. Antibodies to S and pre-S proteins in GSHV infection. (A) Detection of pre-S-specific antibodies in sera of animals after GSHV natural infection. A pre-S-specific polypeptide was synthesized by in vitro transcription of a pre-S construction truncated by *Kpn*I followed by in vitro translation in a rabbit reticulocyte lysate (shown diagrammatically in the left-hand panel). The translation mix was divided into aliquots and immunoprecipitated with sera obtained from ground squirrels before and after resolution of viral infection as measured by GSHsAg radioimmunoassay followed by SDS-PAGE in a 14% polyacrylamide slab gel. The gel was then fixed, fluorographed, dried, and exposed to film overnight. Immunoprecipitations were done with post- and preresolution sera from the same animal (indicated by the number above each pair of wells). Lane UP contained 5% of the unprecipitated translation mix used in each immunoprecipitation reaction. (B) Detection of S-specific antibodies in sera of animals after natural infection. The same sera and procedure as those used in panel A were used for immunoprecipitation of the GSHV S protein made in vitro (left-hand panel). Lanes vertically aligned with those in panel A contained post- or preresolution serum from the same animal. The numbers (kilodaltons) at the right indicate the positions of the molecular size markers.

The results of immunoprecipitation with ground squirrel sera that were earlier shown to be reactive to the pre-S1/pre-S2 fragment are shown in Fig. 4B. Four of eight animals with detectable anti-pre-S1/pre-S2 reactivity had anti-pre-S1 reactivity (Fig. 4B, lanes 3, 4, 6, and 10; Table 1). Of these, two were anti-S negative and two were anti-S positive. This demonstrates that the GSHV pre-S1 domains are immunogenic in some animals and that an anti-pre-S response is not necessarily associated with a detectable anti-S response.

Antibodies to determinants specified by the C and X genes arise during natural GSHV infection. We were interested to know whether the above approach could be extended to detect antibodies to putative viral proteins which have not been directly identified. Principal among these is the product of an open reading frame, termed X, which is predicted to be a 15-kDa protein. Accordingly, we performed coupled in vitro transcription and translation of SP6-derived plasmids bearing the intact X open reading frame. Sera obtained from 18 animals after resolution of primary GSHV infection were screened for their ability to immunoprecipitate the radiolabeled X protein. As an internal control, radiolabeled core antigen produced in a similar fashion was included in each precipitation mix; earlier studies with both HBV- and GSHV-infected hosts demonstrated that the majority of infected individuals produce anti-C antibody (32). Of the 12 anti-C-positive sera, 4 recognized the X gene product at levels significantly above the background levels (Fig. 5, lanes 13, 14, 15, and 19); two additional sera were weakly reactive (lanes 2 and 11). Some sera also precipitated minor translation products of 18 to 20 kDa; these species may represent products of internal translation initiation or premature termination within template GSHV RNA.

TABLE 1. Anti-S and anti-pre-S reactivities of ground squirrel sera after resolution of GSHV infection[a]

Reactivity pattern			No. of animals
Anti-S	Anti-pre-S	Anti-pre-S1	
+	+	+	2
+	+	−	4
+	−	−	6
−	+	+	2
−	−	−	5

[a] Sera from 19 animals were screened for anti-S and anti-pre-S antibodies by using the methods outlined in the text. Anti-S reactivity was determined by immunoprecipitation of an in vitro product of the entire S coding region. Anti-pre-S reactivity was assayed by immunoprecipitation of an in vitro translation product from the pre-S reading frame prematurely truncated with *Kpn*I; the primary product was a pre-S-specific polypeptide composed of the entire pre-S1 region and 33 amino acids of pre-S2. Anti-pre-S1 reactivity was determined by the ability of sera to immunoprecipitate a pre-S1-specific polypeptide produced by coupled in vitro transcription and translation of the GSHV pre-S reading frame truncated by *Fok*I; this polypeptide contains 121 amino acids of the pre-S1 region. +, Positive reactivity; −, negative reactivity.

DISCUSSION

Identification of GSHV pre-S proteins. Our studies confirmed the presence of two classes of GSHV pre-S-derived proteins, one likely originating from the pre-S2 AUG codon and resulting in proteins of 33 to 36 kDa and a larger class originating from the pre-S1 region. Despite the presence of multiple species of 40 to 45 kDa observed after silver

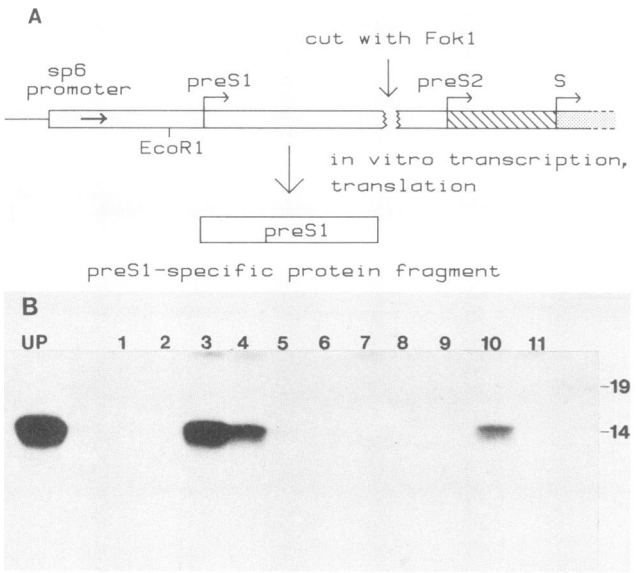

FIG. 4. Anti-pre-S antibodies against pre-S1-specific determinants. (A) Production of pre-S1-specific polypeptide. A pre-S1-specific polypeptide was produced by in vitro transcription of a pre-S reading frame truncated by *Fok*I followed by in vitro translation, as described in the text. (B) Immunoprecipitation of pre-S1-specific polypeptide with sera obtained from animals after natural infection with GSHV. Sera from animals previously demonstrated to have anti-pre-S reactivity were used in immunoprecipitations of the pre-S1-specific polypeptide followed by SDS-PAGE in a 14% polyacrylamide gel. The gel was then fixed, fluorographed, dried, and exposed to film overnight. The numbers (kilodaltons) at the right indicate the positions of the molecular size markers. Lane UP, Unprecipitated translation mix (the amount loaded represented 4% of the amount used in each immunoprecipitation reaction); lanes 7, 9, and 11, preresolution controls; lanes 1 through 6, 8, and 10, immunoprecipitations with postresolution sera. A longer exposure revealed that the serum used in lane 6 was also reactive against the pre-S1-specific peptide.

staining, we observed only one strongly reactive pre-S1 species in immunoblots of preparations of GSHsAg. In both HBV and woodchuck hepatitis virus, two pre-S1 proteins exist that differ in the degree of glycosylation (5, 26); the failure of our antipeptide sera to recognize more than one GSHV protein may have been due to a reduced affinity for modified forms of the protein or to other technical factors.

We were unable to determine with certainty whether (as seems likely) the GSHV pre-S1 protein arises by translation initiation at the first AUG of the pre-S region. An antibody raised against peptide 1 (representing the first 14 amino acids of pre-S) failed to recognize viral protein in the immunoblot procedure, although this antibody did precipitate an in vitro pre-S protein translated from the first AUG (Fig. 2). Initiation of translation at the next available AUG would result in the synthesis of a protein with a molecular weight of only 38,000, significantly smaller than the protein observed by immunoblotting with anti-peptide 2 sera. Other less direct evidence also suggests that the first pre-S1 AUG of hepadnaviruses is used: the nucleotide position of the second pre-S1 AUG is not conserved among HBV subtypes (31); the second AUG appears to be absent in woodchuck hepatitis virus (5, 15); and whereas the nucleotide context surrounding the first and third AUGs of GSHV pre-S (A--AUGG and A--AUGA, respectively) makes them potentially efficient initiators of translation, the second AUG lies in an unfavorable context (C--AUGA) (11). We are currently attempting to determine whether the lack of reactivity of anti-peptide 1 antibody against GSHV proteins made in vivo is due to underrepresentation of these domains in the viral protein, posttranslational modification, or technical factors.

Synthesis of pre-S proteins in vivo. Our group recently mapped the 5′ ends of the GSHV poly(A)$^+$ RNAs (2). Two size classes of RNAs were identified: 3.5-kb transcripts initiating from the region upstream of the core antigen open reading frame and 2.3-kb transcripts originating within the pre-S region. Interestingly, the 5′ ends of both classes of mRNAs are heterogeneous and in both cases bracket potential translation initiation codons. The 2.3-kb transcripts most likely encode the major viral coat proteins encoded by the S region and, by virtue of their 5′ heterogeneity, the pre-S2 proteins. However, no subgenomic transcript encompassing the pre-S1-specific region has been identified in infected livers, despite the presence of a pre-S-proximal element conforming to a eucaryotic promoter consensus sequence beginning at nucleotide 978, 81 nucleotides upstream of the first pre-S1 AUG in the GSHV genome (R. Colgrove, unpublished observation). It is formally possible that these proteins are synthesized from mRNAs directed by this promoterlike region; low-level expression from such a promoter might explain the difficulty in observing such a message in infected livers. Alternatively, these proteins might be synthesized by internal translation initiation from the genomic-length message whose first translatable reading frame encodes the core antigen (see above).

Naturally occurring antibodies to pre-S proteins. We have outlined the use of the SP6 system to produce labeled GSHV pre-S proteins in vitro to examine sera from ground squirrels for the presence of specific circulating antibodies resulting from GSHV infection. Using this approach, we demonstrated that GSHV infection in its natural host generates an antibody response to pre-S protein determinants in addition to an immune response to the major viral proteins of the S reading frame. This response includes antibodies to pre-S1-specific protein domains. An anti-pre-S response is not always associated with an anti-S response; two animals demonstrated an anti-pre-S1/pre-S2 response with no detectable anti-S antibodies.

Our data are of interest in light of the recent demonstration by Milich et al. (19) that pre-S2 proteins of HBV are more immunogenic in normal mice than are the products of the S domain; in addition, inbred strains that are genetically incapable of responding to the S domain can respond to the pre-S2 proteins. However, since mice are not susceptible to HBV infection, these studies were performed by immunizing animals with large quantities of recombinant or serum-derived antigen. Our data indicate that responses to pre-S proteins can be observed in susceptible homologous hosts recovering from an authentic viral infection; such anti-pre-S responses were even demonstrable in occasional animals lacking detectable anti-S responses. Further studies are required, however, to determine whether such responses contribute to the resolution of infection or immunity to reinfection.

Naturally occurring antibodies to other viral proteins. Nucleotide sequence comparisons of the mammalian hepadnaviruses show the preservation of four major open reading frames. The smallest potential coding region (termed X), a reading frame of ca. 460 nucleotides, could encode a protein estimated at 15 kDa. No viral gene product in this size range has been identified that corresponds to this region, but Moriarty et al. recently identified antibodies to two

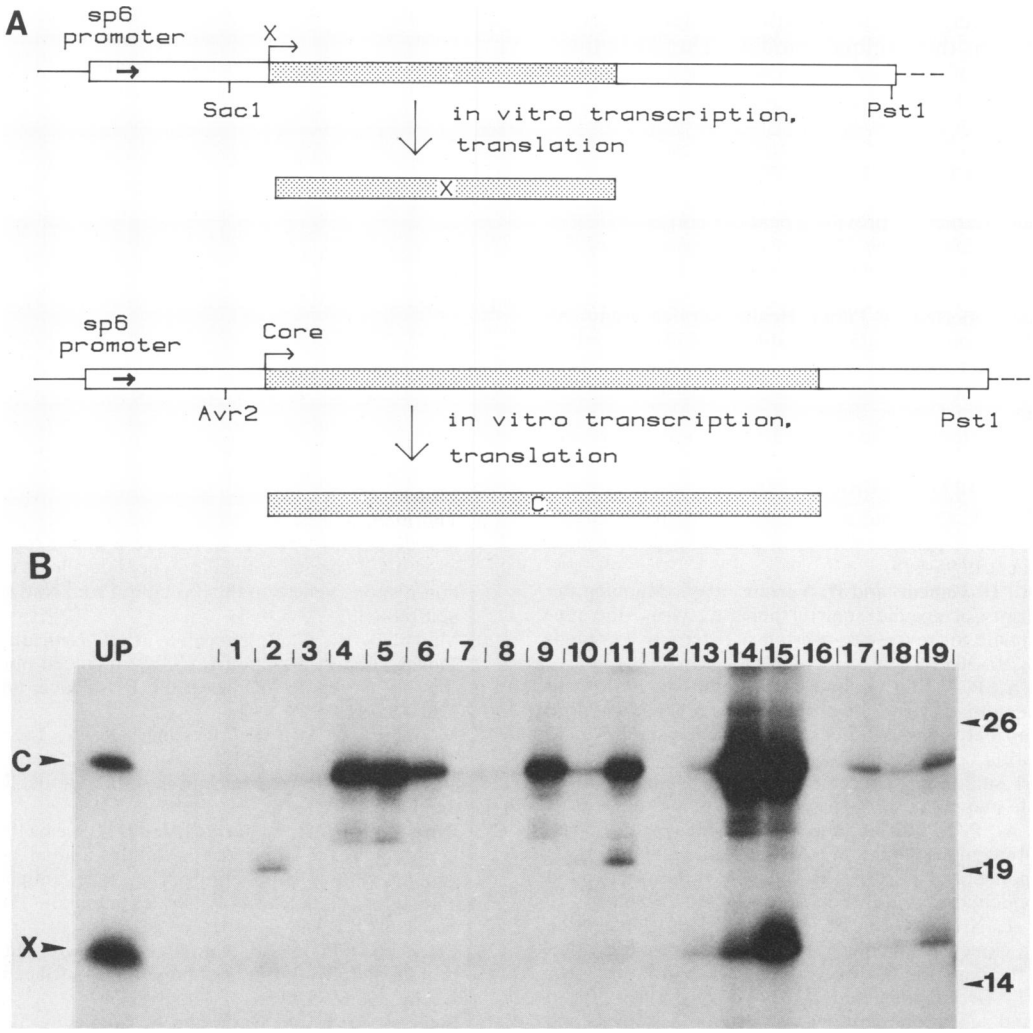

FIG. 5. Antibodies to the product of GSHV X region arising during natural infection. (A) Production of in vitro translation products of GSHV C and X open reading frames. The GSHV C and X proteins were produced by coupled in vitro transcription and translation as described in the text. The upper panel diagrams the production of the X gene product. The lower panel shows production of the C gene product. (B) Immunoprecipitation of GSHV C and X gene products with sera obtained from animals after natural infection with GSHV. Sera from 18 animals obtained after documented GSHV infection were used in immunoprecipitations of the mixed translation products of the C and X reading frames, followed by SDS-PAGE in a 14% polyacrylamide gel. The gel was then fixed, fluorographed, dried, and exposed to film overnight. The numbers (kilodaltons) at the right indicate the positions of the molecular size markers. Lane UP, Unprecipitated translation mix (the amount loaded represented 5% of the amount used in each immunoprecipitation reaction); lane 16, immunoprecipitation with a preinoculation serum used as a negative control; lanes 1 through 15 and 17 through 19, immunoprecipitations with postresolution sera. A longer exposure revealed that the sera used in lanes 2 and 11 were also reactive against the X-specific region.

peptides predicted by the HBV X coding region in sera of patients infected with HBV (21). Furthermore, these investigators suggested a serologic correlation between an anti-X response and the presence of liver cirrhosis or hepatocellular carcinoma or both. Recent studies (10, 18) with bacterial fusion proteins containing X sequences also showed that anti-X antibodies are present in sera from HBV patients.

We demonstrated that in a cohort of 18 animals which had recovered from primary GSHV infection, 6 generated an antibody response to the GSHV X protein and this response was usually associated with a high anti-C titer. This number is likely to represent a minimal estimate for the prevalence of such antibodies in convalescent-phase sera because in the same experiment only ca. 70% of the infected animals displayed anti-C responses with this technique; Marion and colleagues previously demonstrated that virtually all infected squirrels have anti-C antibodies when tested by radioimmunoassay (16). This disparity may be due to the nature of the test antigen or to the requirement in our system for protein A binding by the antibody. Interestingly, in a comparable study of anti-X antibody in human HBV infection, Meyers et al. (18) observed anti-X responses in one-third of HBsAg-positive sera but only rarely in convalescent-phase sera after resolution of HBs antigenemia. These differences may reflect different assay techniques or species-specific features of viral infection.

Our data provide strong circumstantial evidence for the synthesis of GSHV X determinants at some stage of viral infection. The frequency with which anti-X antibody arises after uncomplicated GSHV infection suggests that it is

unlikely to be uniquely associated with persistent infection or its sequelae in this animal model. The structure, biogenesis, and role of the X protein in the natural history of hepadnaviral infection remain to be determined.

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