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Morphogenetic and Regulatory Effects of Mutations in the Envelope Proteins of an Avian Hepadnavirus

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The envelope gene of the avian hepadnavirus, duck hepatitis B virus, was mutated in order to dissect the functions of the two major envelope proteins pre-S/S and S. Both envelope proteins were found to be required for virus particle assembly and secretion. The placement of stop codons after each of the first three AUG codons in the pre-S region allowed efficient translational initiation at downstream AUG codons to produce novel N-terminally truncated pre-S/S proteins. These proteins could substitute for pre-S/S protein in the production of enveloped virus production, but not in the production infectious virus. A mutant defective in myristylation of the pre-S/S protein produced reduced amounts of enveloped virus, and this virus was not infectious. Mutants defective in the pre-S/S protein accumulated high levels of covalently closed circular viral DNA (cccDNA) compared with the wild type or with a mutant defective in only the S protein. Hyperamplification of cccDNA resulted in high levels of viral RNA, consistent with the proposed role of cccDNA as the transcriptional template. Myristylation of the pre-S/S protein was not required for control of cccDNA amplification, and mutants that produced N-terminally truncated pre-S/S proteins displayed higher levels of cccDNA. We concluded that the pre-S/S protein, but not the S protein, is required for control of cccDNA amplification and persistent infection.

Hepadnaviruses cause persistent infections in the hepatocytes of a number of animal species, including humans (13, 16, 26, 29). Persistent infection of humans with the human hepatitis B virus (HBV) is associated with a high risk for chronic liver disease, including hepatocellular carcinoma (for a review, see reference 1). Epidemiologic evidence supports the contention that resolution of a persistent HBV infection is expected to greatly reduce an individual's subsequent risk for HBV-associated liver disease. Persistent infection of hepatocytes by HBV depends on, among other things, the ability of viral genes to be maintained in the cells without cytopathic consequences to the infected cell; thus, under certain circumstances, virtually all hepatocytes can be productively and persistently infected yet carry out normal hepatocellular function. It seems likely that regulation of virus replication in infected cells would be required in order to prevent accelerated expression of viral genes from occurring at the expense of normal hepatocyte function.

Viral genetic information in persistently infected hepatocytes is found in the nucleus in the form of covalently closed circular DNA (cccDNA) molecules (15, 31, 34), which form a pool of transcriptional templates for the production of RNA copies of the genome (pregenomic RNA). These pregenomic RNAs are packaged into immature viral nucleocapsids together with a virus-encoded reverse transcriptase (27). The encapsidated pregenomic RNA then serves as a template for the synthesis of double-stranded relaxed circular DNA (rcDNA) before assembly of the mature nucleocapsid into a lipid envelope and export of the enveloped virus from the cell (14, 27). cccDNA in infected hepatocytes is initially formed by conversion of a single infecting viral rcDNA molecule into cccDNA (15, 31). Ensuing amplification of the initial cccDNA molecules occurs by transcription of the pregenomic RNA, assembly of the pregenomic RNA into nucleocapsids, cytoplasmic synthesis of rcDNA, and transport of rcDNA to the nucleus (31). Amplification of cccDNA is an intracellular event that is generally limited to the initial stages of infection, until a pool of 10 to 50 copies of cccDNA per nucleus is achieved (31, 34). Further increases in the cccDNA copy number are prevented by inhibition of its formation from cytoplasmic rcDNA (28). Participation of the viral envelope proteins in this inhibition was indicated by the failure of a mutated duck hepatitis B virus (DHBV) genome to inhibit cccDNA amplification (28). The mutant genome, defective in both viral envelope proteins, accumulated large amounts of cccDNA in infected hepatocytes and failed to establish a persistent infection in hepatocyte cultures in vitro. Thus, control of cccDNA copy numbers by one or more viral envelope proteins is one level of regulation that enables productively infected cells to survive virus infection.

The DHBV envelope contains two virus-encoded proteins which result from translational initiation at two different start codons in the envelope open reading frame to produce proteins of 328 (pre-S/S) and 167 (S) amino acids sharing the same carboxy terminus (12, 23, 25, 35). The S envelope protein constitutes 75 to 90% of the protein found in viral envelopes, while the larger pre-S/S protein constitutes 10 to 25%. The pre-S region of the pre-S/S coding sequence contains AUG codons at positions 801, 825, 882, and 957 (11) that are conserved among different strains of DHBV, and it is uncertain which or how many of these potential start codons are utilized to produce the major and any minor pre-S/S envelope proteins. The largest pre-S protein is almost certainly myristylated at its N terminus, since it displays a myristylation signal (Met-Gly) that is conserved in the large pre-S protein of all hepadnaviruses (21), the corresponding HBV pre-S1 protein is myristylated (21), and the N terminus of the DHBV pre-S/S protein is blocked (25). The S protein is translated from a 5′-truncated mRNA lacking the pre-S AUG codons and its translation is initiated from the fifth AUG in the envelope open reading frame at position 1284 (2).

In order to distinguish the roles of the pre-S/S and S

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envelope proteins in the control of viral cccDNA amplification and in viral assembly and secretion, we have constructed a series of mutant genomes which are defective in various aspects of envelope protein expression. After transfecting these mutant genomes into permissive chicken hepatoma cells (4, 28), we tested their ability to support DNA replication, secretion of enveloped DNA-containing particles, and release of infectious virus. We also measured the levels of cccDNA and RNA in primary duck hepatocytes infected with these mutants. We demonstrated that the pre-S/S envelope protein alone could exert full control over cccDNA amplification but that the small envelope (S) protein alone was inactive for such control.

Myristylated pre-S/S protein and S protein were both required for normal assembly and secretion of infectious enveloped virus, but pre-S/S proteins lacking up to 53 amino acids from the N terminus, in conjunction with S protein, were hyperactive in the production of enveloped noninfectious virus. Hyperactive secretion was due to deletion of an amino acid sequence between positions 9 and 28 in the pre-S/S protein.

MATERIALS AND METHODS

Plasmids. Infectious plasmids used for testing of mutant DHBV genomes consisted of either an *Eco*RI dimer of DHBV cloned into the *Eco*RI site of pSP65 (pSPDHBV 5.1Gal2X) (6) or an expression cassette consisting of the DHBV pregenomic RNA-encoding sequences driven by the immediate-early cytomegalovirus promoter (4), cloned into the vector pUC119 (33) (pUC119.DHBV.CMV). The latter construct was used for the production of single-stranded DNA for in vitro mutagenesis. Plasmids used for the production of strand-specific riboprobes (31) consisted of pSPDHBV 5.1Gal2X (for the production of plus-strand riboprobe) or the homologous plasmid in which the DHBV sequences are in the opposite orientation, pSPDHBV 5.2Gal2X (for the production of minus-strand riboprobe).

Construction of mutant genomes. Site-directed mutagenesis was carried out by the methods described by Kunkel et al. (8). Briefly, oligonucleotides containing the desired mutation were annealed to single-stranded uracil-containing pUC119.DHBV.CMV DNA obtained by infection of plasmid-containing *Escherichia coli* CJ236 with M13 strain K07. The primer was elongated with T7 DNA polymerase and ligated with T4 DNA ligase. Following completion of the reaction, double-stranded DNA was transformed into *E. coli* DH5α, and colonies were picked and screened by sequencing. Plasmids containing the appropriate mutations were digested with *Kpn*I (nucleotide 1287) and *Bgl*II (nucleotide 391) to excise the mutated envelope region, and the mutated fragment was subcloned into pUC119.DHBV.CMV, substituting for the corresponding wild-type fragment.

Transfection of LMH cells. Infectious plasmids were transfected into chicken hepatoma cell line LMH (7) by using the calcium phosphate method as previously described (6). Cells were incubated at 37°C for 3 to 7 days following transfection.

Assay of transfected cells for release of DNA-containing enveloped particles. Culture fluids from transfected cells were harvested daily between 3 and 7 days posttransfection, clarified by centrifugation at 1,000 × *g* for 5 min, and stored at 4°C. Portions (10 ml) were precipitated by the addition of 1 g of polyethylene glycol (M_w, 7,000 to 9,000) and incubation at 4°C for 1 h. The precipitates were collected by centrifugation at 10,000 × *g* for 20 min and dissolved in 1.5 ml of hepatocyte culture medium containing 10 mM Mg-acetate. Residual DNA from the original transfection was removed by incubation with 100 μg of DNase I per ml at 37°C for 15 min, and the virus-containing suspension was diluted to 2 ml by the addition of 0.5 ml of a solution of CsCl with a density of 1.845 g/cm^3. Virus was sedimented to equilibrium by centrifugation for 2 h at 80,000 rpm in a Beckman VTi80 rotor, at 20°C. Five fractions were collected and adjusted to contain 40 μg of carrier tRNA, 15 mM EDTA, and 0.2% sodium dodecyl sulfate (SDS) and then precipitated by the addition of 2 volumes of ethanol. Pellets were dissolved in 1 ml of digestion buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.5% SDS, 150 mM NaCl) containing 0.5 mg of pronase per ml. Digestion was carried out for 1 h at 45°C, and nucleic acids were purified by phenol extraction and ethanol precipitation. Each fraction was assayed for viral DNA by agarose gel electrophoresis and blot hybridization.

Assay for viral DNA in transfected cells. At 3 to 7 days posttransfection, cells were lysed by the addition of 1 ml of transfection lysis buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Nonidet P-40) per 60-mm-diameter plate, and the nuclei were removed by Microfuge centrifugation (Beckman) for 1 min. The supernatant was adjusted to 10 mM Mg-acetate and digested with DNase I at a concentration of 100 μg/ml for 30 min at 37°C to remove plasmid DNA derived from the transfection. The lysate was adjusted to 15 mM EDTA–100 mM NaCl–0.5% SDS–0.5 mg of pronase per ml and was further incubated at 37°C for 30 min. Nucleic acids were purified by phenol extraction followed by ethanol precipitation. Viral nucleic acids were assayed in each sample by 1% agarose gel electrophoresis and blot hybridization.

Assay for envelope proteins in transfected cells. LMH cells 2 days posttransfection were lysed by the addition of transfection lysis buffer, and the nuclei were removed by Microfuge centrifugation for 1 min. Proteins were precipitated by the addition of 8 volumes of ethanol, and the precipitate was collected by Microfuge centrifugation for 1 min. The precipitate was dissolved by heating in 200 μl of gel loading buffer (0.1 M Tris-HCl [pH 6.8], 1% SDS, 1% mercaptoethanol, 10% glycerol) at 95°C for 10 min. Proteins (10 μl) were separated by electrophoresis through a 12.5% polyacrylamide gel by the method of Laemmli (10) and electrotransferred to nitrocellulose paper (Hybond-C; Amersham). Immunoblots were developed by blocking the nitrocellulose filter with 10 ml of TNE (Tris-HCl [pH 7.4], 1 mM EDTA, 150 mM NaCl) containing 1% bovine serum albumin and 0.1% Tween 20 for 15 min at room temperature. Following blocking, the filter was incubated with a 1:500 dilution of rabbit anti-pre-S antiserum (kindly provided by J. E. Newbold, University of North Carolina) for 2 h at room temperature and washed with four separate 100-ml changes of TNE containing 0.1% Tween 20, for 15 min each wash. Rabbit immunoglobulin G was localized on the filter by incubating the filter with 10 ml of blocking buffer containing ^{125}I-labeled protein A (3 μCi; New England Nuclear) at room temperature for 1 h. Following four 15-min washes with TNE containing 0.1% Tween 20, the filter was developed by autoradiography.

Infection of primary hepatocyte cultures. Primary hepatocytes were prepared and cultured as previously described (24, 32) from 3- to 7-day-old ducklings obtained from Metzer Farms. Culture supernatants from transfected LMH cells were concentrated by polyethylene glycol precipitation as described above, and the pellets were dissolved in hepatocyte culture medium. Aliquots of virus derived from up to 15

ml of LMH culture fluid were used to infect each 60-mm-diameter dish of duck hepatocytes. Infection of hepatocytes was carried out 2 days after plating by addition of the virus to the culture medium and incubation for 24 h at 37°C. Subsequently, the medium was changed daily for the duration of the experiments.

Assay of infected hepatocytes for viral DNA. Viral DNA replicative intermediates and cccDNA were assayed as previously described (28). Briefly, cells were lysed with buffer containing SDS, and protein-bound replicative forms were precipitated in a protein-detergent complex by the addition of KCl. cccDNA was recovered from the soluble fraction after centrifugation by phenol extraction and ethanol precipitation, while replicative forms were dissolved by digestion of the pellet with pronase and purified by phenol extraction and ethanol precipitation. Viral DNA was assayed by 1% agarose gel electrophoresis and blot hybridization.

Cesium chloride gradient fractionation of 5-bromo-uracil deoxyriboside (BUdR)-substituted DNA (28) was carried out in a VTi80 rotor at 65,000 rpm for 15 h at 25°C. The starting density of cesium chloride was 1.75 g/cm^3.

Assay of viral RNA in infected hepatocytes. RNA was purified from infected cells by the guanidine thiocyanate-acid phenol method (3). Each 60-mm-diameter plate was dissolved in 0.5 ml of solution D (6 M guanidine thiocyanate, 0.5% sarcosyl, 0.1% mercaptoethanol, 0.2 M Na-acetate [pH 4.0]) and extracted with 0.5 ml of phenol plus 0.2 ml of chloroform at room temperature. The aqueous layer was recovered after Microfuge centrifugation for 3 min at room temperature and extracted again with phenol-chloroform. RNA was precipitated from the aqueous phase with 1 volume of isopropanol. The precipitate was recovered by centrifugation, washed once with 70% ethanol, washed once with absolute ethanol, and dried. RNA was glyoxylated by dissolving the pellet in 50 μl of dimethyl sulfoxide plus 33 μl of 0.03 M Na-phosphate (pH 7.0) and 17 μl of 6 M deionized glyoxal and heating at 50°C for 1 h. Portions (5 μl) were analyzed by agarose gel electrophoresis (1.5%) using 0.01 M Na-phosphate (pH 7.0) electrode buffer. After electrophoresis, the RNA was transferred to a nylon membrane (Hybond N; Amersham) by blotting, using 0.2 M Tris-HCl (pH 8.9)–1.5 M NaCl as the transfer buffer. RNA blots were hybridized at 60°C in hybridization buffer containing 50% formamide as previously described (6), using a riboprobe specific for the detection of plus-strand viral sequences.

Fluorescent antibody staining. Infected hepatocytes were fixed and stained with rabbit anti-DHB$_c$ as previously described (28).

RESULTS

In order to test which viral envelope proteins were required for control of cccDNA amplification, virus particle assembly, secretion, or infectivity, we constructed a number of mutants by site-directed mutagenesis. Stop codons were created by single base substitutions in the second nucleotide of selected codons in such a way as not to change the coding of the overlapping P gene, which is required for DNA replication. Single stop codons in a series of pre-S/S defective mutants were situated between successive AUG codons beginning with the first conserved AUG (nucleotide 801) in the pre-S/S open reading frame and ending with a stop codon between the fourth and fifth AUG codons. These mutants could allow potential pre-S/S polypeptides to be synthesized from any downstream AUG codons but would terminate translation from upstream AUG codons. In addition, all of the pre-S/S mutants were expected to produce normal S protein, initiating at the fifth AUG in the envelope open reading frame. The mutant genomes were designated by the nucleotide number of the changed base and the letter representing the substituted base (Fig. 1). In addition, we utilized a previously described mutant, 1S, which terminates translation of both pre-S/S and S polypeptides downstream of the fifth AUG. The 1S mutant is defective in control of cccDNA amplification and consequently accumulates large amounts of this species in the nucleus (28). In order to generate a mutation that would eliminate the S protein but not the pre-S/S protein, we changed the uridylic acid of the fifth AUG at position 1285 to cytidylic acid. This mutation resulted in the substitution of a threonine for a methionine in the pre-S/S protein and destroyed the initiation codon for the S protein. Finally, since the pre-S/S protein initiating at the first AUG at position 801 displays a myristylation signal, Met-Gly, at its N terminus, we destroyed this signal (30) by the substitution of cytidylic acid for guanylic acid at position 805 (Met-Gly changed to Met-Ala). We wanted to know whether myristylation was required for any of the functions to be assayed. All mutants were competent to synthesize DNA, as expected, after transfection of the mutated plasmids into LMH cells (Fig. 2).

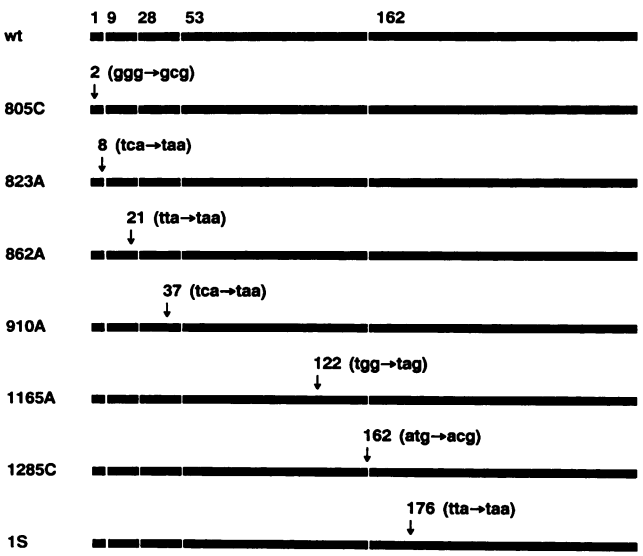

FIG. 1. Point mutations in the DHBV envelope gene. The DHBV envelope gene is depicted by the horizontal lines interrupted at sites at which ATG codons reside. The codon numbers of ATG (wt) or mutant codons (all others) are indicated above the line, and the codon change is indicated. Site-directed mutations were all single base substitutions designed to preserve the coding of the overlapping P gene. Infectious mutant plasmids are designated by the nucleotide number at which the substitution occurred in the DHBV sequence, numbered according to Mandart et al. (11), followed by a letter indicating the substituted base. The envelope mutant 1S was previously described (28) and contained adenylic acid substitutions at positions 1327, 1346, and 1349 to create three in-frame termination codons.

Multiple pre-S/S proteins can be synthesized from different AUG codons in the pre-S region. The introduction of stop codons after successive AUG codons in the pre-S region allowed efficient translational initiation from downstream AUG codons, creating in effect N-terminally truncated pre-S/S proteins. Expression of the wild-type envelope gene in

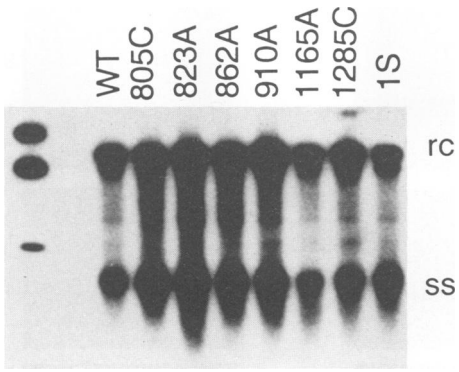

FIG. 2. DHBV DNA synthesis in LMH cells transfected with envelope mutants and wild-type infectious plasmids. Plasmid DNAs (30 μg) containing each of the mutations indicated in Fig. 1 were used for transfection of a 100-mm-diameter dish of LMH cells plated 20 h earlier at a density of 1.3×10^7 cells per dish. The medium was replaced after 15 h and changed 48 and 72 h later. Cells were harvested after 96 h, and viral replicative intermediates were purified and analyzed by agarose gel electrophoresis and blot hybridization, using a riboprobe specific for the detection of viral minus-strand DNA. The migration positions of relaxed circular (rc) and single-stranded (ss) DNA are indicated. WT, Wild type. The left lane shows molecular size standards of 4.6-, 3.0-, and 1.4-kbp double-stranded DNA.

LMH cells resulted in the production of primarily a single polypeptide containing pre-S epitopes (Fig. 3). However, introduction of a stop codon after the first AUG (mutant 823A), presumably the start codon for the major wild-type protein, resulted in the production of three additional pre-S polypeptides seen only in low amounts in cells transfected with the wild-type viral DNA. These polypeptides most likely correspond to proteins produced by translational initiation at the three downstream AUG codons in the pre-S region, since movement of the stop codon to positions downstream of each of the three AUG codons (mutants 862A, 910A, and 1165A) successively eliminated the appearance of the largest truncated pre-S/S polypeptide in each mutant. Introduction of a stop codon after the fourth AUG (mutant 1165A) or after the fifth AUG (mutant 1S) in the envelope open reading frame eliminated all pre-S/S proteins. Mutation of the fifth AUG (mutant 1285C) did not eliminate the major myristylated pre-S/S protein. Since we did not have available an antiserum that detected the S protein on immunoblots, we were not able to confirm the ablation of the S protein in either the 1285C or 1S mutant. We have tentatively designated these various pre-S/S polypeptides as P36, P35, P33, and P30 according to the expected molecular weights, assuming that they were initiated at the four respective AUG codons in the pre-S region. The S protein is designated P18, according to its expected molecular weight. The expected molecular weights agree well with the values estimated from the electrophoretic mobilities of these proteins.

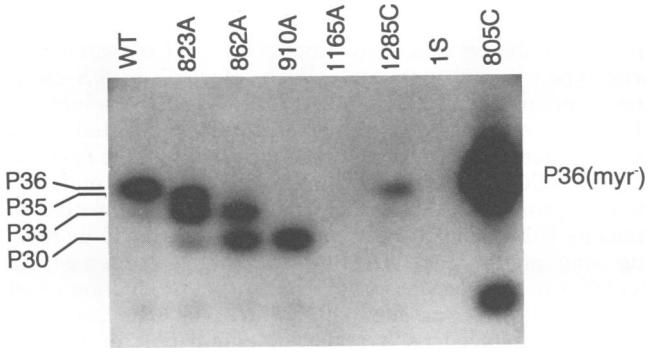

FIG. 3. Pre-S/S-specific envelope proteins in cells transfected with envelope mutant DNAs. LMH cells (60-mm-diameter plates, 3.5×10^6 cells per plate) were transfected with 10 μg of plasmid DNA from each mutant and incubated for 96 h after the DNA was removed. Cell layers were extracted for total Nonidet P-40-soluble proteins as described in Materials and Methods and analyzed by electrophoresis through a 12.5% polyacrylamide-SDS gel. The proteins were electrotransferred to nitrocellulose and probed with a pre-S-specific rabbit antiserum (1:500) followed by ^{125}I-labeled protein A (New England Nuclear Corp.). WT, Wild type; P36(myr$^-$), nonmyristylated P36.

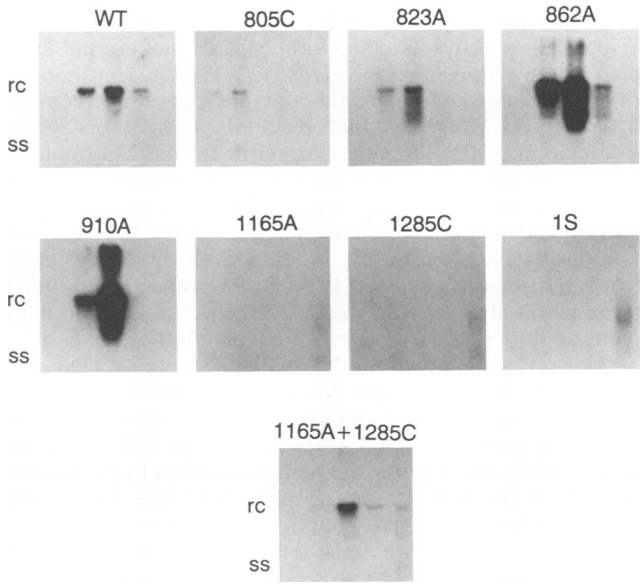

FIG. 4. Cesium chloride gradient purification of enveloped DNA-containing viral particles in supernatants from transfected LMH cells. Culture fluids from the transfection described in Fig. 2 were collected at the 72- and 96-h harvests, concentrated by polyethylene glycol precipitation, and centrifuged to equilibrium in 2 ml of isopycnic CsCl gradients. Total nucleic acids were extracted from each of the five fractions, and virus-specific DNA was assayed by agarose gel electrophoresis and blot hybridization, using a riboprobe specific for the detection of DHBV minus-strand DNA. Enveloped virus banded in the gradient, while free viral cores pelleted. The gel migration positions of relaxed circular (rc) and single-stranded (ss) DNA are indicated. The fractions increase in density from right to left. WT, Wild type.

Destruction of the myristylation signal (mutant 805C) resulted in the accumulation of increased amounts of P36 in the transfected cells. This effect was seen in three separate experiments and was not due to fractionation of the myristylated protein into a Nonidet P-40-insoluble compartment, since SDS extraction of the transfected cells yielded a similar ratio of pre-S/S proteins on immunoblots (data not shown).

Both pre-S/S and S proteins are required for extracellular production of enveloped DNA-containing particles. Culture supernatants of the transfected LMH cells from Fig. 2 were assayed for the presence of enveloped virions by isopycnic

centrifugation (16) in cesium chloride (Fig. 4). This assay distinguishes between naked viral capsids that are not associated with a lipid envelope (density, 1.35 g/cm^3) and capsids that are enclosed within a lipid-containing envelope (density, 1.14 g/cm^3). Mutations that resulted in the translational termination of P36 after the second or third AUG (mutants 862A and 910A) resulted in greatly increased yields of enveloped virus compared with wild-type virus yields. Release of DNA-containing enveloped particles by these mutants was dependent on the production of the N-terminally truncated pre-S/S proteins, P33 and P30, since translational termination after the fourth AUG (1165A) resulted in complete inhibition of virus release. Elimination of the S polypeptide (1285C) or of both envelope proteins (1S) also completely inhibited virus release. The inhibition of virus release in mutant 1285C (defect in P18 only) was not a result of the missense mutation (Met to Thr) in P36, since the mutated P36 was able to complement a defect in the production of all pre-S/S proteins (1165A) for virus production. Destruction of the myristylation signal for the pre-S/S protein (805C) consistently resulted in a decreased yield of secreted virus. Decreased production of enveloped virion by this mutant may reflect a decreased ability of nonmyristylated P36 to be assembled and secreted, and this defect could account for the increased levels of nonmyristylated P36 observed in LMH cells transfected with the 805C mutant.

All mutants were tested for the release of infectious particles from transfected LMH cells by infectivity assay in primary hepatocyte cultures (24; also data not shown). All mutants were defective in infectious particle production. However, cotransfection of LMH cells with the mutants 1165A and 1285C resulted in the production of infection-competent particles. This result indicates the mutated P36 protein of 1285C is fully functional for infectivity of virions.

Envelope defects resulting in loss of infectivity can be complemented by wild-type envelope proteins. Defects in the production of infection-competent particles could be complemented by cotransfection of the LMH cells with a plasmid that expressed wild-type envelope proteins but which was unable to produce pregenomic RNA that could give rise to infectious virus. The plasmid we used for this purpose contained a linear *Eco*RI dimer of DHBV DNA in which both copies of DR1, the origin for minus-strand DNA synthesis, were precisely deleted. This mutant, ΔDR1, has been shown to be able to provide, in *trans*, replicative and packaging functions to DHBV genomes with lethal mutations in core, P, and envelope genes (6). Thus, successful complementation of envelope defects could be assayed by the appearance of infected cells in hepatocyte cultures exposed to supernatants of LMH cells cotransfected with envelope mutants and ΔDR1.

All cotransfections yielded infection-competent particles, as judged by immunofluorescence staining of the test hepatocyte cultures for viral core antigen (DHB_cAg) (Fig. 5). By counting the number of infected cells in a specified field, we estimated the amount of infectious virus produced by the cotransfected LMH cells. Differences in the abilities of the various mutants to be rescued were reproducible in different experiments. In this experiment, the yield of wild-type virus from transfected cells could be an overestimate, since no measures were taken to prevent secondary infection in the hepatocyte cultures.

Pre-S/S protein but not S protein is required for regulation of cccDNA amplification. We measured the relative levels of replicative-intermediate DNA, cccDNA, and virus-specific RNA produced by the hepatocytes infected by envelope

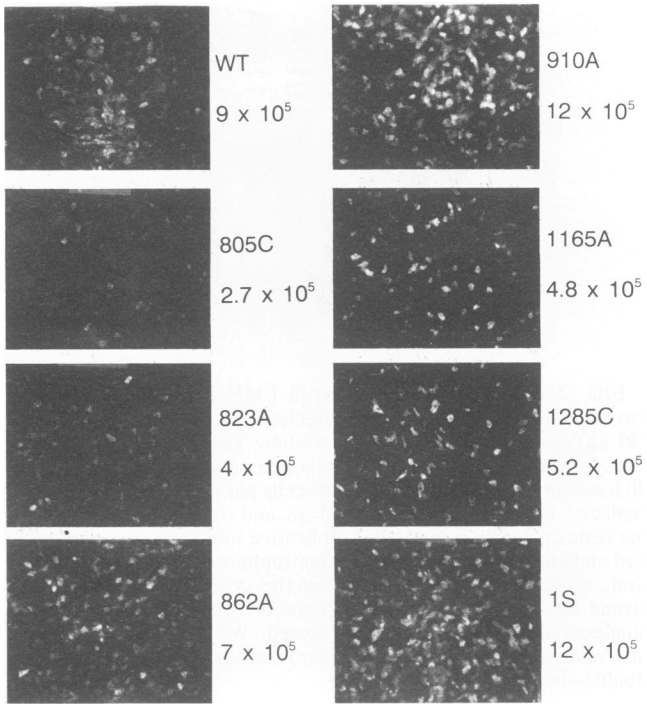

FIG. 5. Fluorescent antibody staining of primary duck hepatocytes infected with viral envelope mutant genomes. Envelope mutant plasmid DNA was cotransfected into LMH cells with the helper plasmid ΔDR1 (15 μg of mutant DNA plus 15 μg of ΔDR1 per 100-mm-diameter plate of LMH cells). Culture supernatants (10 ml) were harvested at 4, 5, 6, and 7 days posttransfection, pooled, concentrated with polyethylene glycol, and used to infect four 60-mm-diameter dishes of primary duck hepatocytes. At 10 days postinfection, cells were fixed and stained with rabbit anti-DHB_c (1:100), followed by rhodamine-labeled goat anti-rabbit immunoglobulin G. Fluorescing cells were counted in a standard field (ca. 80 to 400 cells per standard field), and the number of infected cells per dish was calculated. Infected-cell counts are displayed to the right of each micrograph. Each dish of cells was infected with virus concentrated from 10 ml of pooled medium. WT, Wild type.

mutants in the previous experiment (Fig. 6). Compared with wild type, several mutations which affected pre-S/S envelope proteins (805C, 862A, 910A, 1165A, and 1S) influenced the levels of cccDNA accumulated by the infected cells, while placement of a termination codon between the first and second AUG codons of the pre-S region (823A) or mutation of the S protein AUG (1285C) had no effect. Cells infected by mutants 862A and 910A accumulated progressively increasing amounts of cccDNA, but the largest increment in cccDNA accumulation occurred when the production of all pre-S/S proteins was eliminated (1165A or 1S). Levels of cccDNA produced as a result of elimination of pre-S/S proteins alone (1165A) were comparable to the levels achieved in cells infected by the 1S mutant, previously shown to be unregulated in cccDNA amplification (28). Thus P36, but not P18, is required for cccDNA regulation in hepatocytes.

The lowest levels of cccDNA were seen in cells infected by the mutant lacking the P36 myristylation signal. This effect is possibly due to an increased level of P36 in the hepatocytes, analogous to that seen in transfected LMH cells. The result would suggest that nonmyristylated P36

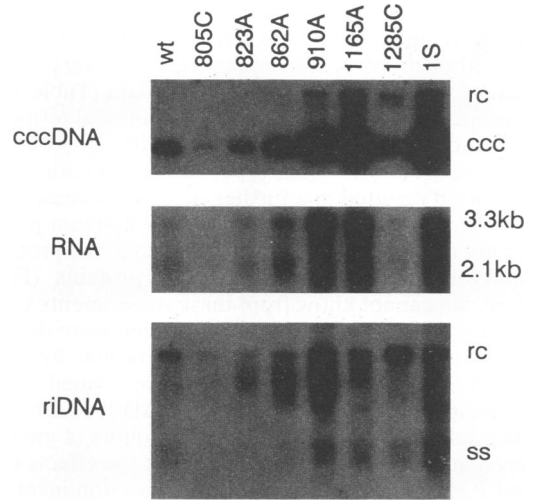

FIG. 6. Viral nucleic acids in primary duck hepatocytes infected with DHBV envelope mutants. Cultures of primary duck hepatocytes from the experiment described in Fig. 5 were harvested at 10 days postinfection and extracted for viral cccDNA, protein-bound replicative intermediates, and total RNA. Nucleic acids equivalent to that obtained from 1/20 of a 60-mm-diameter plate were analyzed by agarose gel electrophoresis and blot hybridization. Viral DNA (cccDNA) and replicative intermediates (riDNA) were probed with a riboprobe specific for the detection of DHBV minus strands, while viral RNA was detected using a riboprobe specific for the detection of DHBV plus strands. The gel migration position of relaxed circular (rc), single-stranded (ss), and covalently closed circular (ccc) DNA and pregenomic (3.3 kb), and envelope mRNAs (2.1 kb) are indicated.

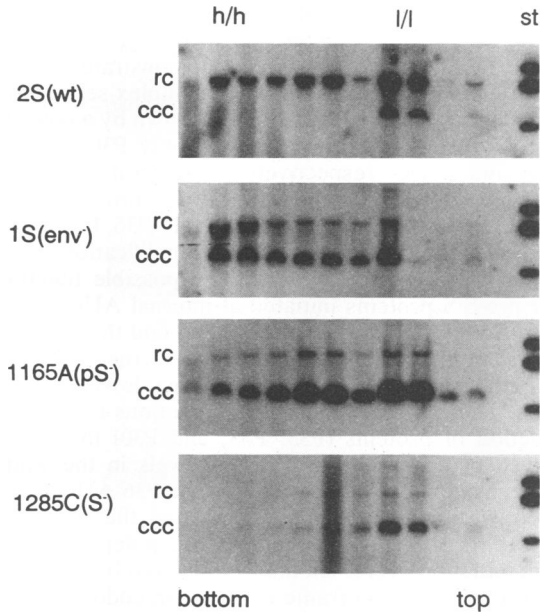

FIG. 7. Density labeling of DHBV cccDNA in primary duck hepatocytes infected with DHBV envelope mutants. Primary duck hepatocyte cultures were infected with DHBV envelope mutants as described in the legend to Fig. 5. At 24 h after infection, suramin (100 μg/ml) was added to the medium and the cultures were maintained in suramin-containing medium during the remainder of the experiment to prevent second rounds of infection by wild-type virus (22). At 4, 5, and 6 days postinfection, the medium was supplemented with BUdR (30 μg/ml). At 7 days postinfection, the infected cultures were harvested and viral cccDNA was extracted. BUdR-substituted cccDNA (h/h) was fractionated from unsubstituted DNA (I/I) by isopycnic centrifugation in a CsCl gradient, and fractions were analyzed for virus-specific DNA by agarose gel electrophoresis and blot hybridization, using a riboprobe specific for minus-strand DHBV DNA. The gel migration positions of relaxed circular (rc) and covalently closed circular (ccc) DNA are indicated. Lane st, Molecular size standards of 4.6, 3.0, and 1.4 kbp.

accumulates in a compartment in which it is active in suppressing cccDNA synthesis. In Table 1, levels of cccDNA have been normalized to the number of infected cells estimated from immunostained cell layers and are consistent with the above interpretations.

Total viral RNA produced by the infected cultures (Fig. 6, RNA panel) was roughly proportional to the total amount of cccDNA produced, rather than to the number of infected cells. The result indicates that amplification of cccDNA results in the increased transcription of viral RNA, as suggested by Tuttleman et al. (31), which is consistent with the proposed role of cccDNA as the transcriptional template (15, 27, 31). Surprisingly, increased levels of RNA did not result in correspondingly large increases in replicative intermediates (Fig. 6, riDNA panel). This result suggests that previously unrecognized controls over the size of the replicative-intermediate pool may compensate for differences in the rate of RNA production.

TABLE 1. DHBV envelope mutation phenotypes

Virus	Envelope proteins[a]	No. of cccDNA/cell[b]	Virion release[c]	Infectivity	
				Without helper	With helper[d]
Wild type	P36, P17	22	1	1	1
805C	P36 (nonmyristylated), P17	19	0.2	0	0.3
823A	P35, P32, P30, P17	25	1.5	0	0.4
862A	P32, P30, P17	43	15	0	0.8
910A	P30, P17	100	25	0	1.3
1165A	P17	630	0	0	0.5
1285C	P36	38	0	0	0.6
1S		420	0	0	1.3

[a] Proteins from transfected LMH cells. Data from Fig. 3.
[b] Primary duck hepatocytes. Data from Fig. 5 and 6.
[c] Release from transfected LMH cells. Data from Fig. 4.
[d] LMH cells cotransfected with ΔDR1. Data from Fig. 5.

Pre-S/S protein alone controls amplification by inhibition of cccDNA synthesis. In order to determine whether the low level of cccDNA found in cells infected by the 1285C mutant was the result of inhibition of cccDNA synthesis, we measured the rate of incorporation of a density-labeled precursor of DNA, BUdR, into cccDNA in 1285C-, 1165A-, 1S-, and wild-type-infected cells. Incorporation of BUdR was assayed by fractionation of the cccDNA through isopycnic CsCl gradients and assay of each fraction for virus-specific DNA by agarose gel electrophoresis and blot hybridization (Fig. 7). Between 4 and 7 days postinfection, incorporation of BUdR into cccDNA was greatly reduced in wild-type and 1285C-infected cells compared with that in cells infected with pre-S/S defective mutants 1165A and 1S. This result indicates that the pre-S/S protein alone is sufficient for inhibition of cccDNA synthesis.

DISCUSSION

The experiments described here demonstrate that hepadnavirus envelope proteins display a complex set of morphogenetic and regulatory functions. As shown by a comparison of the properties of mutants lacking only P36 or only P18 (1165A and 1285C, respectively), both viral envelope proteins were required for the extracellular production of enveloped DNA-containing particles. Only P36, however, was required for the control of cccDNA amplification.

When we attempted to assess the possible functions of minor pre-S/S proteins initiated at internal AUG codons in the pre-S region, we were surprised to find that termination of translation by ribosomes initiating at the P36 AUG allowed efficient initiation at at least three downstream AUG codons. These novel translational initiations resulted in the production of proteins (P35, P33, and P30) that were not observed or observed only at low levels in the wild-type DNA-transfected cells. Presumably the P36 AUG would be utilized with equal efficiency in all of the mutants and therefore its ability to interfere with cap-dependent downstream initiation events should be unaltered by the introduction of a premature in-frame termination codon. Two alternative explanations may account for this increased downstream initiation. First, ribosomes terminating translation of P36 may reinitiate efficiently at downstream AUG codons. Reinitiation has been observed to occur in eukaryotic bicistronic mRNAs containing the coding regions for xanthine-guanine phosphoribosyl transferase and dihydrofolate reductase (18). In this case the efficiency of reinitiation is influenced by the relative positions of the termination codon for the upstream reading frame and the AUG of the downstream reading frame. The distances between the termination codons in the various mutants and the downstream AUG codons that were utilized to produce novel pre-S/S proteins are consistent with this interpretation.

Alternatively, the downstream AUG codons for P35, P33, and P30 may be in a suitable context for internal, cap-independent translation initiation (19) but are normally masked by ribosomes that are traversing the region translating P36. Such a model has been recently proposed to account for the expression of the HBV P protein by translational initiation at an internal AUG in the core mRNA, which is translated inefficiently, but not the precore mRNA, which is translated efficiently (17). If the usage of downstream AUG codons were indeed dependent on the density of ribosomes engaged in translation through the region, it would be possible for regulation of translational initiation at the first AUG (that for P36) to influence the complement of pre-S/S proteins produced by wild-type-infected cells. Whether such regulation might occur in vivo warrants further investigation.

Since both pre-S/S and S proteins are required for the release of DNA-containing enveloped particles from transfected cells, these envelope proteins must perform separate functions. Our assays do not distinguish between a defect in the assembly of nucleocapsids into envelopes and a defect in the secretory pathway for enveloped virus. In mammalian hepadnaviruses, the S envelope protein is apparently sufficient to direct its own assembly into empty envelopes and their secretion from the cell (5, 20). It is tempting to assign the function of envelope formation and secretion to the analogous DHBV S envelope protein, P18. In this model, the role of targeting the envelope to the mature nucleocapsid for assembly would fall to P36.

Whatever the function of P36 in enveloped virus production, this same function could be performed by pre-S/S proteins with up to 53 amino acids missing from their N termini, although with efficiencies that are very different from that of wild-type P36. Quantitative data (Table 1) from the experiment in Fig. 4 shows that translational termination of P36 at the 862A mutation increased virus production by 15-fold compared with wild type, and termination at the 910A mutation resulted in a further twofold increase. Translational termination of P36 at various downstream positions resulted not only in the loss of P36 but also in the production of a different complement of pre-S/S proteins (Fig. 3). Therefore, we cannot know from these experiments whether the effects on virus release that we observed were due to the production of alternative pre-S/S proteins that function in lieu of P36 or due to unique functions performed by these novel proteins. In cotransfections with ΔDR1, all envelope mutants produced roughly equivalent amounts of enveloped particles (data not shown), indicating that the effects of P35, P33, and P30 on virus production were not dominant.

The increased production of enveloped DNA-containing particles by mutants 862A and 910A relative to the wild type and 823A was associated with the absence in P30 and P33 of a domain that was present in P36 and P35. Such a domain would therefore lie between amino acids 9 and 28 of P36. Kuroki et al. (9) have described a domain near the N terminus of the HBV pre-S1 protein that causes retention of envelope proteins in the endoplasmic reticulum when the pre-S1 protein is overexpressed relative to the pre-S2 and S proteins. The effect we observed may similarly be due to differences in the rate of maturation or release of enveloped particles, dictated by the presence or absence in pre-S proteins of some sort of retardation signal. This explanation presumes that the amount of P36 produced in a wild-type transfection is sufficient to retard movement of viral particles from the endoplasmic reticulum further into the secretory pathway.

P36 appeared to be the only envelope protein that was active in regulating cccDNA amplification. Mutants that produced N-terminally truncated pre-S/S proteins (862A and 910A) accumulated higher levels of cccDNA than wild-type virus but not levels as high as those achieved by mutants totally defective in pre-S/S proteins (1165A and 1S). These intermediate levels of cccDNA copy numbers could result either from partial destruction of the pre-S domain required for control of cccDNA amplification or from increased turnover (degradation or secretion) of fully active mutant proteins. In either case, compensating higher rates of synthesis would be required to attain equivalent regulatory levels of mutant pre-S/S proteins and these higher rates of synthesis would be achieved by the accumulation of higher levels of cccDNA. The current experiments do not distinguish between these possibilities.

The mechanism behind the essential role of myristylation of P36 in the virus life cycle was partially clarified by these experiments, since myristylation of P36 was shown to be required for the infectivity of extracellular enveloped virus. Ancillary effects of destroying the P36 myristylation signal are probably related to the requirement for myristylation in infectious particle production in as yet unexplained ways. Nonmyristylated P36 accumulated to higher levels in transfected cells than myristylated P36, suggesting a defect in its assembly into envelopes or in the secretion of envelopes containing nonmyristylated P36. In agreement with this interpretation, lack of myristylation of P36 resulted in a decreased rate of release of enveloped DNA-containing particles. The lack of infectivity of these particles might be

explained by a requirement for myristylated P36 in the correct assembly of particles with an envelope structure that can mediate infection. In addition, the presence of myristylated P36 itself in viral envelopes may be necessary for virus attachment or uptake into hepatocytes.

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REFERENCES

1. **Beasley, R. P.** 1988. Hepatitis B virus. The major etiology of hepatocellular carcinoma. Cancer **61**:1942–1956.
2. **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.
3. **Chomczynski, P., and N. Sacchi.** 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
4. **Condreay, L. D., C. E. Aldrich, L. Coates, W. S. Mason, and T.-T. Wu.** 1990. Efficient duck hepatitis B virus production by an avian tumor cell line. J. Virol. **64**:3249–3258.
5. **DuBois, M., C. Pourcel, S. Rousset, C. Chany, and P. Tiollais.** 1980. Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA. Proc. Natl. Acad. Sci. USA **77**:4549–4553.
6. **Horwich, A. L., K. Furtak, J. Pugh, and J. Summers.** 1990. Synthesis of hepadnavirus particles containing replication-defective duck hepatitis B virus genomes in cultured HuH7 cells. J. Virol. **64**:642–650.
7. **Kawaguchi, T., K. Nomura, Y. Hirayama, and T. Kitagawa.** 1987. Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH. Cancer Res. **47**:4460–4464.
8. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–383.
9. **Kuroki, K., R. Russnak, and D. Ganem.** 1989. Novel N-terminal amino acid sequence required for retention of a hepatitis B virus glycoprotein in the endoplasmic reticulum. Mol. Cell. Biol. **9**:4459–4466.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **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.
12. **Marion, P., S. Knight, M. Feitelson, L. Oshiro, and W. Robinson.** 1983. Major polypeptide of duck hepatitis B surface antigen particles. J. Virol. **48**:534–541.
13. **Marion, P., L. Oshiro, D. Regnery, G. Scullard, and W. Robinson.** 1980. A virus in Beechey ground squirrels that is related to hepatitis B virus of humans. Proc. Natl. Acad. Sci. USA **77**:2941–2945.
14. **Mason, W., C. Aldrich, J. Summers, and J. Taylor.** 1982. Asymmetric replication of duck hepatitis B virus DNA in liver cells (free minus strand DNA). Proc. Natl. Acad. Sci. USA **79**:3997–4001.
15. **Mason, W., M. Halpern, J. England, G. Seal, J. Egan, L. Coates, C. Aldrich, and J. Summers.** 1983. Experimental transmission of duck hepatitis B virus. Virology **131**:375–384.
16. **Mason, W., G. Seal, and J. Summers.** 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J. Virol. **36**:829–836.
17. **Ou, J.-H., H. Bao, C. Shih, and S. M. Tahara.** 1990. Preferred translation of human hepatitis B virus polymerase from core protein- but not from precore protein-specific transcript. J. Virol. **64**:4578–4581.
18. **Peabody, D., and P. Berg.** 1986. Termination-reinitiation occurs in the translation of mammalian cell mRNAs. Mol. Cell. Biol. **6**:2695–2703.
19. **Pelletier, J., and N. Sonnenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature (London) **334**:320–325.
20. **Persing, D., H. Varmus, and D. Ganem.** 1985. A frameshift mutation in the preS region of human hepatitis B virus allows production of surface antigen particles but eliminates binding to polymerized albumin. Proc. Natl. Acad. Sci. USA **82**:3440–3444.
21. **Persing, D., H. Varmus, and D. Ganem.** 1987. The preS1 protein of hepatitis B virus is acylated at its amino terminus with myristic acid. J. Virol. **61**:1672–1677.
22. **Petcu, D., C. Aldrich, L. Coates, J. Taylor, and W. Mason.** 1988. Suramin inhibits *in vitro* infection by duck hepatitis B virus, Rous sarcoma virus, and hepatitis delta virus. Virology **167**:385–392.
23. **Pugh, J., J. Sninsky, J. Summers, and E. Schaeffer.** 1987. Characterization of a pre-S polypeptide on the surfaces of infectious avian hepadnavirus particles. J. Virol. **61**:1384–1390.
24. **Pugh, J., and J. Summers.** 1989. Infection and uptake of duck hepatitis B virus by duck hepatocytes maintained in the presence of dimethyl sulfoxide. Virology **172**:564–572.
25. **Schlicht, H., C. Kuhn, B. Guhr, R. Mattaliano, and H. Schaller.** 1987. Biochemical and immunological characterization of the duck hepatitis B virus envelope proteins. J. Virol. **61**:2280–2285.
26. **Sprengel, R., E. Kaleta, and H. Will.** 1988. Isolation and characterization of hepatitis B virus endemic in herons. J. Virol. **62**:3832–3839.
27. **Summers, J., and W. Mason.** 1982. Replication of the genome of hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell **29**:403–415.
28. **Summers, J., P. M. Smith, and A. L. Horwich.** 1990. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. J. Virol. **64**:2819–2824.
29. **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.
30. **Towler, D., J. Gordon, S. Adams, and L. Glaser.** 1988. The biology and enzymology of eukaryotic protein acylation. Annu. Rev. Biochem. **57**:69–99.
31. **Tuttleman, J., C. Pourcel, and J. Summers.** 1986. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell **47**:451–460.
32. **Tuttleman, J. S., J. C. Pugh, and J. W. Summers.** 1986. In vitro experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. J. Virol. **58**:17–25.
33. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153**:3–11.
34. **Wu, T.-T., L. Coates, C. Aldrich, J. Summers, and W. Mason.** 1990. In hepatocytes infected with duck hepatitis B virus, the template for viral RNA synthesis is amplified by an intracellular pathway. Virology **175**:255–261.
35. **Yokosuka, O., M. Omata, and Y. Ito.** 1988. Expression of preS1, preS2, and C proteins in duck hepatitis B virus infection. Virology **167**:82–86.