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Expression and Characterization of Hepatitis B Virus Surface Antigen Polypeptides in Insect Cells with a Baculovirus Expression System

ROBERT E. LANFORD,[1]* VERNE LUCKOW,[2,3] RONALD C. KENNEDY,[1] GORDON R. DREESMAN,[1] LENA NOTVALL,[1] AND MAX D. SUMMERS[2,3]

Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, Texas 78284,[1] and Department of Entomology, Texas A&M University,[2] and Texas Agricultural Experimental Station,[3] College Station, Texas 77843

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The baculovirus *Autographa californica* nuclear polyhedrosis virus was used as an expression vector to produce hepatitis B virus surface antigen with and without the pre-S domain. The *S* gene product was expressed as both fusion and nonfusion polypeptides. No difference was observed in the posttranslational modification of the fusion and nonfusion polypeptides. The S proteins were not secreted into the medium but were inserted into the endoplasmic reticulum, glycosylated, and partially extruded into the lumen of the endoplasmic reticulum as 22-nm lipoprotein particles. The oligosaccharide chains on the insect cell-derived S protein were of the N-linked high-mannose form, in contrast to the complex-type oligosaccharides detected on plasma-derived hepatitis B virus surface antigen. The pre-S-S polypeptides were inserted into the endoplasmic reticulum, glycosylated, and modified by fatty acid acylation with myristic acid. A procedure was developed to purify the S protein from cellular membranes by using detergent extraction and immunoaffinity chromatography. The purified S protein was in the form of protein-detergent micelles and was highly antigenic and immunogenic.

A variety of systems have been developed for expression of large quantities of mammalian proteins, including bacterial, yeast, and mammalian cells. Recently, the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has been used successfully as a helper-independent expression vector (55). Foreign genes are expressed under the control of the very strong promoter of the polyhedrin gene (15). Insect cells appear to provide proper processing of mammalian proteins, including secretion (48, 49), glycosylation (7, 22, 49), and nuclear translocation (18, 23, 33), as well as a number of other modifications (reviewed in reference 28). However, many of the proteins that have been expressed in baculovirus either did not require complex posttranslational modifications or were not extensively characterized. This investigation was undertaken as a model system to examine the capacity of insect cells to perform a series of complex modifications on a mammalian protein, the hepatitis B virus (HBV) surface antigen (HBsAg).

The mature HBV virion is composed of a proteinaceous lipid envelope and an inner icosahedral nucleocapsid that contains the partially double-stranded circular DNA genome, a virion polymerase, and a protein covalently bound to the viral DNA (9, 10, 56). The lipid envelope contains the surface antigen (HBsAg) that is also expressed independent of the virion as spherical and filamentous lipoprotein particles with a diameter of approximately 20 nm (42) and is present at high concentrations in the serum of infected individuals. Three partially homologous HBsAg polypeptides are expressed by utilizing three different mRNAs and three in-phase ATG initiation codons (2, 12, 44, 51). The *S* gene encodes the major HBsAg polypeptide, which is 226 amino acids in length. The pres-S_2 domain adds an amino-terminal extension of 55 amino acids to the S polypeptide, and the pre-S_1 region extends the polypeptide and additional 108 to 119 amino acids, depending on the subtype. The S and pre-S_1 polypeptides exist in both glycosylated and unglycosylated forms, while the pre-S_2 polypeptide can be glycosylated at one or two sites (12, 41, 53, 54). The pre-S_1 polypeptide inhibits the secretion of HBsAg particles (3, 4, 31, 35, 39, 50), a property which may be related to its modification by fatty acid acylation with myristic acid (40) and may play a role in virion assembly.

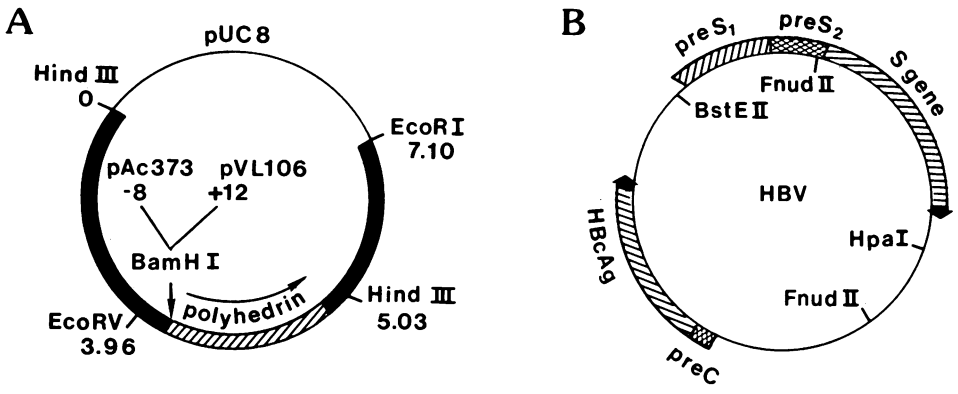
In this investigation, recombinant baculoviruses were isolated that express HBsAg with and without the pre-S domain. HBsAg synthesized in insect cells was glycosylated and partially assembled into lipoprotein particles but was not efficiently secreted. The pre-S_1 polypeptide was both glycosylated and myristylated. A procedure for the purification of highly antigenic HBsAg from insect cells is described.

MATERIALS AND METHODS

Cells and viruses. The continuous insect cell line Sf9 was derived from *Spodoptera frugiperda* (fall armyworm) (54) and was maintained in TNM-FH medium (13) containing 10% fetal bovine serum. The growth, purification, and assay of AcNPV have been described previously (58, 59). Detailed procedures for the use of AcNPV as a recombinant expression vector system have been published in a manual designed for this purpose (54).

Construction of plasmids. The baculovirus plasmid transfer vectors pAc373 and pVL106 (28) were used in this investigation. Both plasmids contain the pUC8 plasmid with an insert of the polyhedrin-encoding sequences flanked by several kilobases of AcNPV sequences 3′ and 5′ of the polyhedrin gene (Fig. 1). The sequences encoding the *S* gene were obtained by digestion of pN-3 (26) with *Fnu*dII (nucleotides 131 to 1356), and the sequences encoding the pre-S-S

* Corresponding author.



C

	-10 met pro asp tyr	
AcNPV	ACCTATAAAT ATG CCG GAT TAT ...	polyhedrin
	-10	
pAc373	ACC ..... CGAGATCCGCGGATCC	
	BamHI	
	-10 met pro ala arg asp	
pVL106	ACCTATAAAT ATG CCG GCC CGG GAT CC	
	BamHI	

FIG. 1. Structure and partial nucleotide sequence of baculovirus transfer vector and HBV DNAs. (A) Circular map of the baculovirus transfer vectors pAc373 and pVL106. The thick lines represent the 5′ and 3′ sequences flanking the polyhedrin gene, which is represented by the hatched area. The vectors contain a unique *Bam*HI site at −8 (pAc373) and +12 (pVL106) nucleotides with respect to the polyhedrin ATG initiation codon. (B) Functional and structural map of the HBV genome. The positions of the open reading frames encoding the pre-S_1, pre-S_2, and S genes are depicted, as are the restriction endonuclease cleavage sites used to obtain DNA fragments encoding the S gene (*Fnu*DII) and the pre-S-S gene (*Bst*EII and *Hpa*I). (C) Partial nucleotide sequences of AcNPV DNA, the baculovirus transfer vector plasmids, and recombinant plasmids containing the S and pre-S-S genes. The DNA sequence of AcNPV surrounding the polyhedrin initiation codon is compared with the sequences in the baculovirus transfer vector plasmids pAc373 and pVL106 up to the *Bam*HI site used for inserting foreign genes.

gene were derived by digestion of pN-5 with *Bst*EII and *Hpa*I (nucleotides 2824 to 966). Single-stranded termini were filled in by reaction with the Klenow fragment of DNA polymerase I, and the internal *Bam*HI site in the pre-S-S gene was protected by treatment with *Bam*HI methylase. *Bam*HI linkers were added by blunt-end ligation, the linkers were digested with *Bam*HI, and the resulting fragments were cloned into *Bam*HI-digested, alkaline phosphatase-treated pAc373 or pVL106.

Analysis of proteins by SDS-PAGE and immunoblot. Infected Sf9 cells were washed three times in phosphate-buffered saline (PBS), and cellular proteins were solubilized in detergent extraction buffer (50 mM Tris hydrochloride [pH 9.0], 100 mM NaCl, and 1% Nonidet P-40 [NP-40]). In some experiments, cells were divided into soluble and particulate fractions by sonication in PBS and centrifugation at 3,000 × *g* for 20 min prior to detergent extraction. Medium from infected cultures was clarified at 3,000 × *g* for 10 min and adjusted to 1% NP-40. Samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were disrupted in electrophoresis sample buffer containing 2% SDS and 2% 2-mercaptoethanol and heated at 100°C for 5 min. Proteins were separated by SDS-PAGE as described previously (24) and either silver-stained, autoradiographed, or processed for the immunoblot procedure with rabbit antiserum to HBsAg and ^{125}I-protein A (New England Nuclear).

Analysis of HBsAg on CsCl gradients. Sf9 cells were infected with the recombinant virus 106FS and harvested for HBsAg purification at 72 h postinfection. Cells were washed three times in PBS and lysed by sonication in PBS. The cellular lysate was clarified, and HBsAg was pelleted by ultracentrifugation for 70 min at 435,000 × *g* (TL-100 ultracentrifuge; Beckman). The pellet was suspended in PBS and loaded onto a discontinuous CsCl gradient (1.10 to 1.40 g/cm^3). Equilibrium sedimentation of HBsAg was performed by ultracentrifugation for 24 h at 36,000 rpm in an SW41 rotor (Beckman). Fractions (0.5 ml) were collected from the bottom, diluted 10,000-fold, and assayed for HBsAg content by AUSRIA II (Abbott). HBsAg in the peak fraction was examined by electron microscopy following negative staining with phosphotungstic acid.

ConA-agarose chromatography. Sf9 cells infected with the 373S recombinant virus were detergent extracted at 48 h postinfection as described above. The extracts were incubated for 1 h at 37°C with 200 μl of concanavalin A (ConA)-agarose beads (Bethesda Research Laboratories). The supernatant containing unbound proteins was saved for analysis, and unglycosylated HBsAg bound to the resin due to interchain disulfide bonds with glycosylated HBsAg was eluted with 10 mM dithiothreitol. The resin was washed three times with detergent extraction buffer, and bound proteins were eluted with 1 M α-methyl-D-mannoside in 0.1 M borate buffer (pH 6.5) containing 1% NP-40. A portion of each fraction was analyzed by SDS-PAGE and immunoblot as described above.

Glycosidase treatment. Sf9 cells were infected with the 373S recombinant baculovirus and pulse-labeled with 250 μCi of [^{35}S]methionine per ml from 24 to 40 h postinfection. Detergent extracts were prepared as described above, and

HBsAg was immunoprecipitated with rabbit antiserum to HBsAg and protein A-agarose beads. HBsAg was eluted from the immune complexes by heating at 95°C for 2 min in the presence of 0.5% SDS and 0.1 M 2-mercaptoethanol. Equal portions of the eluate were used as an untreated control or for digestion with endoglycosidase H (endo H) (Genzyme Corp., Boston, Mass.). Samples were diluted threefold in 50 mM sodium phosphate buffer (pH 5.7) in order to reduce the concentration of SDS and 2-mercaptoethanol, incubated at 37°C for 6 h to permit enzyme digestion, and analyzed by SDS-PAGE and autoradiography.

Analysis of fatty acid acylation. Sf9 cells infected with the recombinant virus 373PS were labeled at 48 h postinfection for 4 h with 25 μCi of [^{35}S]methionine or 1 mCi of [^{3}H]myristic acid (New England Nuclear) per ml. Labeled cells were detergent extracted, HBsAg was immunoprecipitated, and immunoprecipitates were analyzed by SDS-PAGE, fluorography, and autoradiography.

HBsAg purification and analysis of antigenicity. Sf9 cells infected with the 106FS recombinant virus were harvested at 48 h postinfection, washed three times with PBS, and detergent extracted as described above. HBsAg was affinity purified by a modification of a previously described method (60). Affinity columns for the purification of HBsAg were prepared with chimpanzee antiserum reactive with HBsAg. The immunoglobulin G (IgG) fraction of the chimpanzee serum was affinity purified on HBsAg covalently attached to cyanogen bromide-activated Sepharose-4B (Pharmacia, Inc., Piscataway, N.J.). The affinity-purified anti-HBsAg IgG was covalently bound to protein A-agarose by using the bifunctional cross-linking agent dimethyl pimelimidate (43). Detergent extracts containing HBsAg were slowly passed over the affinity resin for 2 h. The resin was washed sequentially with PBS, 1 M NaCl, and PBS. HBsAg was eluted with 3 M ammonium thiocyanate and dialyzed against PBS. The amount of HBsAg polypeptide was quantitated on silver-stained gels by comparison to a known standard of HBsAg purified from human plasma.

RESULTS

Preparation of recombinant baculovirus. This investigation used two different baculovirus transfer vectors for the isolation of recombinant viruses expressing HBsAg. The pAc373 vector contains a *Bam*HI site at nucleotide $^{-}$8 with respect to the polyhedrin initiation codon (Fig. 1A and C). Insertion of a foreign gene into this vector results in the expression of a native protein. The pVL106 vector has a *Bam*HI site at nucleotide $^{+}$12 in relation to the polyhedrin ATG, so that insertion of a foreign gene into pVL106 results in the synthesis of a fusion protein (Fig. 1A and C). The intact 5′ leader sequence of the polyhedrin gene in pVL106 results in the synthesis of higher levels of recombinant protein than are obtained with pAc373 (25, 28).

HBV DNA fragments encoding HBsAg with and without the pre-S region (Fig. 1B) were cloned into the baculovirus transfer vectors as described in Materials and Methods. Insertion of the *S* gene into pAc373 yielded a recombinant transfer vector plasmid, designated p373S, while insertion of the same sequences into pVL106 resulted in p106FS, which encodes a fusion protein with two amino acids derived from polyhedrin, four amino acids encoded by linker sequences, nine amino acids of the pre-S_2 region, and the intact S protein. A DNA fragment containing the entire pre-S-S gene was cloned into pAc373 to obtain p373PS.

Sf9 cells were cotransfected with each of the recombinant transfer vectors and AcNPV DNA. Homologous recombination between the AcNPV sequences present in the transfer vectors and viral DNA gave rise to recombinant viruses that were identified in plaque assays by their lack of polyhedrin occlusions (54).

Expression of HBsAg. Sf9 cells infected with the recombinant baculoviruses 373S, 106FS, and 373PS were examined for the synthesis of HBsAg-related polypeptides at 48 h postinfection. Cellular lysates were prepared with NP-40 extraction buffer and analyzed by SDS-PAGE and immunoblot as described under Materials and Methods. High levels of HBsAg polypeptides were expressed following infection of Sf9 cells with each of the recombinant viruses. 373S induced the synthesis of two polypeptides corresponding in molecular weight to the unglycosylated (p25) and glycosylated (gp29) major HBsAg polypeptides. The fusion polypeptides encoded by 106FS had a discernible reduction in mobility in SDS gels due to the 15 additional amino acids at the amino terminus of the polypeptides and appeared to be present in the glycosylated and unglycosylated forms as well. 373PS induced the synthesis of two polypeptides with apparent molecular weights of 39,000 and 42,000, suggesting that they represented the unglycosylated and glycosylated forms of the pre-S_1–pre-S_2–S polypeptide (p39 and gp42), respectively (data not shown; see Fig. 3 for representative polypeptides).

Secretion of HBsAg polypeptides was examined by using clarified medium from the infected cultures for the immunoblot procedure. Cultures infected with the different recombinant viruses were harvested every day for 5 days postinfection. An equal fraction of the cellular extracts and the medium corresponding to 2.5×10^4 cells were analyzed by SDS-PAGE and immunoblot. The same results were obtained with each of the recombinant viruses. The analysis of 373S-infected cultures is depicted in Fig. 2. Maximal levels of intracellular p25 and gp29 were detected 2 to 3 days postinfection. Thereafter, the level of intracellular HBsAg remained constant, but at no time was significant HBsAg detected in the medium.

The level of HBsAg synthesis for 373S, 106FS, and 373PS was determined by comparison of cellular extracts to an HBsAg standard of known concentration purified from human plasma. Cellular extracts were prepared from infected cultures at 48 h postinfection by detergent extraction. Various amounts of the extracts and the HBsAg standard were compared by SDS-PAGE and immunoblot (data not shown). The level of HBsAg synthesis was estimated at approximately 90 mg/liter for 106FS and 6 mg/liter for both 373S and 373PS. These estimates were extrapolated from the amount of antigen derived from cultures grown in 25-cm^2 flasks (4×10^6 cells) to a 1-liter spinner culture (2×10^9 cells).

Intracellular localization of HBsAg in Sf9 cells. The detection of potentially glycosylated forms of HBsAg (gp29 and gp42) in cellular extracts suggested that insertion of the polypeptides into the membrane of the endoplasmic reticulum (ER) had occurred and that secretion of the S polypeptide was blocked at a subsequent step. Unfortunately, accurate methods for fractionating insect cells into subcellular fractions have not been developed. Since HBsAg still in association with the ER should be insoluble in the absence of detergent, and HBsAg in the form of a lipoprotein particle should be soluble, cellular fractionation experiments were performed in an attempt to determine whether the HBsAg polypeptides were in a soluble or insoluble form within the cell.

Sf9 cells were infected with 373S, 106FS, and 373PS viruses and harvested 48 h postinfection. Medium was

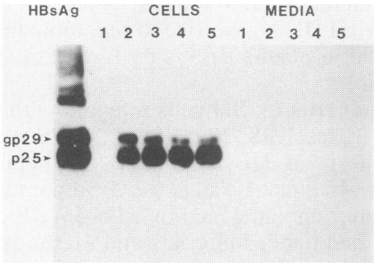


FIG. 2. Lack of secretion of HBsAg synthesized in insect cells. Sf9 cells infected with 373S were harvested every day for 5 days postinfection. The medium was clarified to remove cellular debris, and a cellular extract was prepared by detergent extraction. An equal fraction of the cell extracts and media were analyzed by SDS-PAGE and immunoblot. HBsAg expression was first detected in the cell extracts on day 2 and was approximately equal on days 3 to 5. HBsAg could not be detected in the medium at any time point. The positions of p25 and gp29 from plasma-derived HBsAg are depicted at the left.

clarified to remove cellular debris, and cells were fractionated into soluble and particulate fractions by sonication and centrifugation at 3,000 × *g* for 20 min. Equal portions of medium and soluble and particulate cellular fractions were analyzed by SDS-PAGE and immunoblot. The increased level of HBsAg synthesis obtained with 106FS required that the fractions analyzed for 373S and 373PS be derived from a greater number of cells (2×10^5 cells) than the fractions analyzed for 106FS-infected cultures (1×10^4 cells). The pre-S-S polypeptides (p39 and gp42) encoded by 373PS were localized almost exclusively to the particulate cellular fraction (Fig. 3). The S polypeptides (p25 and gp29) expressed by 373S were distributed approximately equally between the particulate and soluble fractions, whereas the fusion polypeptides derived from 106FS were predominantly localized in the particulate fraction (Fig. 3). Both S and pre-S polypeptides were completely solubilized with 1% NP-40 (data not shown), suggesting that HBsAg in the particulate fraction was membrane found. The percentage of the fusion polypeptides localized to the soluble fraction varied from one experiment to another, with approximately 10 to 20% of the antigen in the soluble fraction. Since a 20-fold greater number of cells were analyzed for 373S than for 106FS, the actual amount of HBsAg polypeptides in the soluble fraction of 106FS-infected cells was greater than or equal to that observed with 373S. These data suggest that formation of a lipoprotein particle by budding of HBsAg into the lumen of the ER may be a rate-limiting step in insect cells. The restriction of p39 and gp42 to the particulate fraction (presumably ER) of insect cells is in agreement with previous observations made with mammalian cells (3, 4, 31, 35, 39).

The assembly of HBsAg into lipoprotein particles in insect cells infected with 106FS was investigated further by equilibrium sedimentation in CsCl gradients and electron microscopy. Sf9 cells were harvested 3 days postinfection with 106FS, and a soluble cellular extract was prepared by sonication in PBS and clarification. HBsAg was concentrated from the clarified extract by ultracentrifugation, and the pellet was suspended and analyzed on a CsCl gradient as described under Materials and Methods. Fractions were collected from the bottom, diluted 10,000-fold, and assayed for HBsAg content by AUSRIA II. Virtually all of the soluble HBsAg activity pelleted in the first ultracentrifugation and banded at 1.19 g/cm^3 in CsCl (Fig. 4A). Examination of the peak fraction by electron microscopy revealed the presence of lipoprotein particles with a diameter of 22.3 ± 3.8 nm (Fig. 4B). These data indicate that the major HBsAg polypeptide synthesized in insect cells is inserted into the ER membrane and at least partially assembled into lipoprotein particles.

Glycosylation of HBsAg. The expression of two S polypeptides by recombinant viruses 373S and 106FS suggested that the polypeptides represented the unglycosylated (p25) and glycosylated (gp29) forms of the small HBsAg polypeptide. However, for unknown reasons, in 373S-infected cells the amount of gp29 in relation to p25 varied considerably between experiments (Fig. 2 and 3). The pre-S-S polypeptides encoded by 373PS also had mobilities on SDS gels that would correspond to the unglycosylated (p39) and glycosylated (gp42) forms of this polypeptide. In order to directly demonstrate the glycosylation of HBsAg in insect cells, the polypeptides were examined for binding to the lectin ConA and for alterations in mobility on SDS gels following treatment with endoglygosidases. For lectin-binding experiments, Sf9 cells were infected with the recombinant viruses 373S, 106FS, and 373PS, and detergent extracts were prepared 48 h postinfection. The extracts were incubated with ConA-agarose beads for 1 h at 37°C, the supernatants containing the unbound proteins were removed, and the resins were sequentially eluted with 10 mM dithiothreitol and 1 M α-methyl-D-mannoside. Portions of each fraction were analyzed by SDS-PAGE and immunoblot. The higher-molecular-weight HBsAg polypeptide from 373S-infected cells was quantitatively bound to the column and eluted with the competing sugar (Fig. 5A), indicating that it represented glycosylated gp29. A portion of the unglycosylated p25 was initially bound to the resin but could be eluted with dithiothreitol, suggesting that it was bound to the column due to interchain disulfide bonds with gp29. Similar results were observed with 106FS- and 373PS-infected cells (data not shown).

The binding of HBsAg polypeptides to ConA demonstrated the presence of α-mannose residues on gp29 and gp42 but did not indicate the type of carbohydrate structure present on the polypeptides. Treatment with endo H was

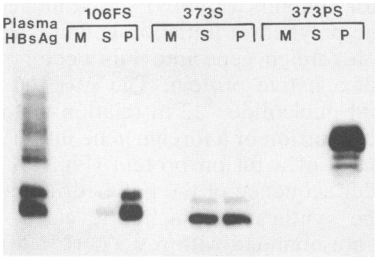


FIG. 3. Comparison of HBsAg synthesized by 373S, 106FS, and 373PS for secretion and solubility of intracellular HBsAg. Sf9 cells were infected with the recombinant viruses 373S, 106FS, and 373PS and harvested at 48 h postinfection. Media (M) were clarified to remove cellular debris, and cellular extracts were divided into soluble (S) and particulate (P) fractions by sonication in PBS and centrifugation at 3,000 × *g* for 20 min. An equal portion of each fraction was analyzed by SDS-PAGE and immunoblot, except that 20-fold fewer cells were used in the analysis of 106FS due to greater levels of HBsAg synthesis obtained with this virus. Secretion of HBsAg could not be detected in any of the cultures. The pre-S-S polypeptides (p39 and gp42) were entirely within the particulate fraction, while the nonfusion and fusion forms of the S polypeptides (p25 and gp29) were partially soluble.

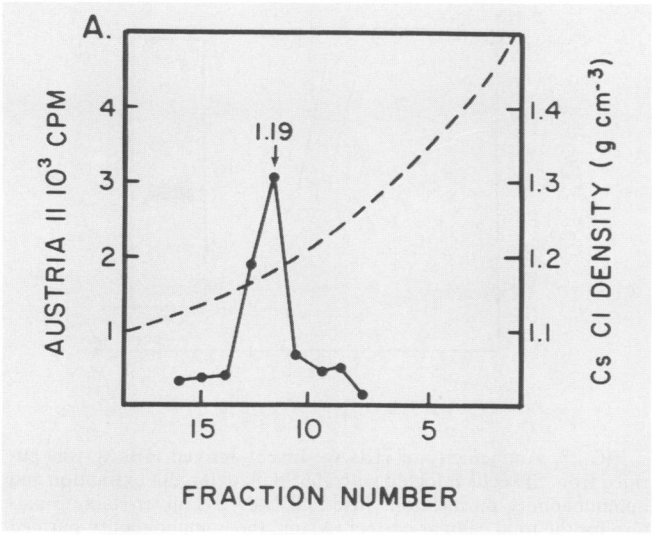


FIG. 4. Assembly of intracellular HBsAg into lipoprotein particles. Sf9 cells infected with 106FS were harvested 48 h postinfection, and a soluble cellular extract was prepared by sonication in PBS and centrifugation at 3,000 × *g* for 20 min. HBsAg was concentrated from the soluble supernatant by ultracentrifugation, and the pellet was analyzed on a CsCl gradient. Each fraction was diluted 10,000-fold and assayed for HBsAg by AUSRIA II. The HBsAg activity banded at a density of 1.19 g/cm^3 (A), and examination of the peak fraction by electron microscopy revealed lipoprotein particles with a diameter of 22 nm (B). Bar, 50 nm.

used to determine the type of oligosaccharides present on gp29, since it removes oligosaccharides of the N-linked high-mannose type (21). Sf9 cells were infected with 373S, labeled with [^{35}S]methionine for 12 h at 24 h postinfection, and detergent extracted. HBsAg was immunoprecipitated from extracts, eluted from the immune complexes with SDS and 2-mercaptoethanol, and treated with endo H as described under Materials and Methods. Treatment of HBsAg derived from 373S-infected cell extracts with endo H resulted in conversion of gp29 to p25, as determined by comigration on SDS gels (Fig. 5B). In contrast, the secreted gp29 from plasma-derived HBsAg was endo H resistant and *N*-glycanase sensitive (1) (data not shown). These results demonstrate that HBsAg derived from insect cells is modified with N-linked high-mannose oligosaccharides, while plasma-derived HBsAg possesses processed N-linked saccharides.

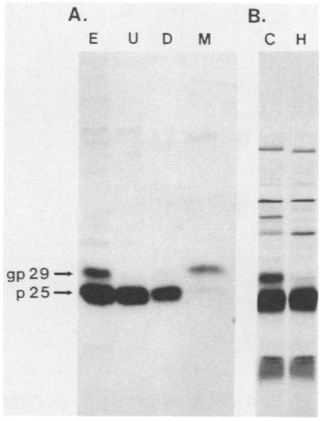


FIG. 5. ConA chromatography and endoglycosidase treatment of HBsAg. (A) Sf9 cells were infected with the recombinant virus 373S, and detergent extracts were prepared 48 h postinfection. The starting extract (E) was incubated with ConA-agarose for 1 h at 37°C, the supernatant containing unbound proteins (U) was removed, and the resin was sequentially eluted with 10 mM dithiothreitol (D) and 1 M α-methyl-D-mannoside (M). A portion of each fraction was analyzed by SDS-PAGE and immunoblot. The higher-molecular-weight glycosylated polypeptide (gp29) was quantitatively bound by the resin. In addition, a portion of the unglycosylated polypeptide (p25) was bound, presumably due to association with the glycosylated polypeptides, and eluted with dithiothreitol. (B) Sf9 cells were infected with the recombinant virus 373S, labeled with [^{35}S]methionine for 12 h at 24 h postinfection, and detergent extracted. HBsAg was immunoprecipitated from the extracts, eluted from the immune complexes with SDS and 2-mercaptoethanol, and either treated with endo H (H) or left untreated as a control (C). The samples were analyzed by SDS-PAGE and autoradiography.

Myristylation of HBsAg. Recently, the modification of the pre-S-S polypeptides of HBsAg by fatty acid acylation (myristic acid) has been demonstrated in mammalian cells (40). In order to determine whether insect cells were capable of performing this modification of HBsAg, 373PS-infected Sf9 cells were labeled with either [^{35}S]methionine or [^{3}H]myristic acid from 44 to 48 h postinfection. HBsAg was immunoprecipitated and examined by SDS-PAGE, fluorography, and autoradiography. Both p39 and gp42 were efficiently labeled with [^{35}S]methionine and [^{3}H]myristate (Fig. 6). Several control experiments were performed to demonstrate the specificity of the labeling. Other cellular proteins were not labeled during the myristic acid pulse, indicating the lack of significant randomization of the isotope. The pre-S polypeptides were not labeled during a pulse with [^{3}H]palmitic acid, and in the same experiments, simian virus 40 (SV40) T antigen was labeled with palmitic acid but not myristic acid in Sf9 cells infected with a recombinant baculovirus encoding T antigen (23). These results indicate that insect cells are capable of the efficient modification of mammalian cell proteins by fatty acid acylation.

Antigenicity and immunogenicity of HBsAg. The lack of HBsAg secretion and the relatively inefficient formation of

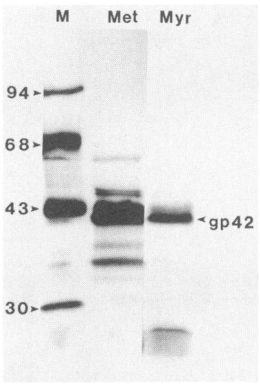


FIG. 6. Myristylation of the pre-S-S polypeptides. Sf9 cells infected with 373PS were labeled with [^{35}S]methionine (Met) or [^{3}H]myristic acid (Myr) for 4 h at 48 h postinfection and then detergent extracted. HBsAg was immunoprecipitated and examined by SDS-PAGE, fluorography, and autoradiography. The positions of molecular weight standards (M) are shown (in thousands) at the left. The pre-S-S polypeptide was efficiently labeled with both methionine and myristic acid.

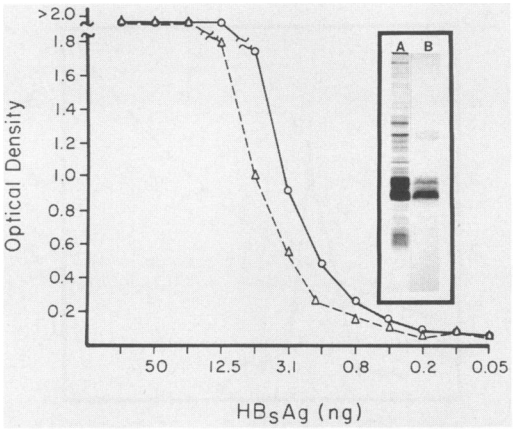


FIG. 7. Antigenicity of HBsAg. Insect-derived HBsAg was purified from Sf9 cells infected with 106FS by detergent extraction and immunoaffinity purification. Inset: SDS-PAGE silver-staining pattern for the total cellular extract (A) and the immunoaffinity-purified HBsAg (B). The insect-derived material was quantitated by comparison to a known standard of plasma-derived HBsAg by SDS-PAGE and silver staining. The plasma HBsAg (○) and the 106FS HBsAg (△) were compared for reactivity in a commercial ELISA for HBsAg (Organon Teknika Corporation; Nuclear Medical Laboratories). The insect-derived material was approximately twofold less reactive than the plasma-derived HBsAg. Both antigens were reactive in the assay at all concentrations greater than 3 ng per assay.

HBsAg lipoprotein particles by insect cells dictated that a purification procedure other than CsCl gradients be used. The efficient detergent solibilization of HBsAg from 106FS-infected cells and previous reports that HBsAg detergent micelles were highly antigenic (16, 46, 47) suggested that affinity purification of detergent-extracted HBsAg might yield a suitable antigen. To this end, Sf9 cells infected with 106FS were extracted 48 h postinfection with PBS containing 1% NP-40 and the HBsAg was affinity purified as described in Materials and Methods. Analysis of the cellular extract and the purified HBsAg by SDS-PAGE and silver staining demonstrated that p25 and gp29 had been purified to near homogeneity (Fig. 7). Examination of the preparation by electron microscopy confirmed the presence of micelles with diameters ranging from 20 to 50 nm.

The antigenicity of the purified recombinant HBsAg was examined by comparison with plasma-derived HBsAg in a commercial enzyme-linked immunosorbent assay (NML [HBsAg] ELISA; Organon Teknika Corporation; Nuclear Medical Laboratories). Prior to assay, the concentration of the recombinant HBsAg was determined by comparison to a known standard of HBsAg purified from human plasma by SDS-PAGE and silver staining. The two preparations could not be compared by absorbance at 280 nm, since one was a detergent micelle and the other was a lipoprotein particle. The two forms of HBsAg were compared in the ELISA at concentrations ranging from 0.05 to 100 ng per assay. Both antigens yielded positive reactions at all concentrations above 3 ng per assay (Fig. 7). The reactivity of the recombinant HBsAg in the ELISA was approximately equivalent to that of the plasma-derived HBsAg, indicating that a highly antigenic form of HBsAg can be purified to near homogeneity in large quantities from insect cells infected with recombinant baculoviruses. In addition, the immunogenicity of insect cell-derived HBsAg was examined in BALB/c mice. Five mice were immunized intraperitoneally with two injections (5 μg each) of alum-precipitated HBsAg (20) at a 2-week interval. Serum was obtained 14 to 21 days following the second immunization, and the anti-HBsAg response was determined in a solid-phase ELISA that used a solid-phase coat of insect cell-derived HBsAg. The results demonstrated that HBsAg purified from insect cells can induce a vigorous in vivo anti-HBsAg response in mice (mean titer, 20,000).

DISCUSSION

The synthesis, assembly, modification, and secretion of HBsAg lipoprotein particles by mammalian cells represent a complex series of posttranslational modifications. Bacterial and yeast expression systems are not capable of properly processing HBsAg. The use of the baculovirus AcNPV as an expression vector system has recently found widespread acceptance (28, 49, 54). Proper posttranslational modification has been demonstrated for many of the proteins expressed in insect cells (reviewed in reference 28). However, most of the mammalian proteins synthesized in insect cells have not been extensively characterized or do not normally require a series of complex processing steps. This investigation was performed to examine the fidelity of modification of highly complex mammalian proteins by insect cells as a model system. No difference in modification was observed between fusion and nonfusion forms of HBsAg. Both forms of the S protein were inserted into the ER, partially modified by glycosylation, complexed by interprotein disulfide bonds, and partially extruded into the lumen of the ER as lipoprotein particles. Significant levels of HBsAg were not detected in the medium. Production of intracellular lipoprotein particles was confirmed by purification on CsCl gradients. HBsAg reactivity banded at a density of 1.19 g/ml, and electron microscopy documented the presence of particles with a diameter of approximately 22 nm. Cellular fractionation experiments demonstrated that a large percentage of the HBsAg polypeptides were in a particulate fraction. The solubility of the HBsAg in the particulate fraction with nonionic detergents suggested that the polypeptides were membrane associated (presumably in the ER). Sophisticated procedures for ER membrane purification have not been developed for insect cells. However, endoglycosidase treatments also suggested that HBsAg lipoprotein particles were in the ER due to the presence of N-linked high-mannose

oligosaccharide chains. In mammalian cells, N-linked high-mannose oligosaccharides are present within the ER, but these structures are generally processed to a complex oligosaccharide structure following the transition of the polypeptide from the ER to the Golgi as part of the pathway for secretion (8, 27). Although insect cells do not modify N-linked oligosaccharide side chains to the complex form, they are capable of trimming high-mannose side chains to a trimannosyl core (17). HBsAg synthesized in Sf9 cells had not been processed to the trimannosyl core stage, since this structure is endo H resistant (21). Recently, a defect in the processing of oligosaccharide chains on the epidermal growth factor receptor in Sf9 cells was observed, even though the epidermal growth factor receptor was transported to the cell surface (11). Due to the lack of HBsAg secretion in insect cells, it was not possible to evaluate the ability of insect cells to process the carbohydrate side chains.

Two steps in HBsAg secretion appeared to be blocked in insect cells, both of which involved the processing of a lipoprotein particle. The first step was the efficient formation of HBsAg particles. Although this is a highly specialized process, HBsAg lipoprotein particles are produced and secreted by a number of undifferentiated mammalian tissue culture cell lines. The fact that insect cells were capable of modifying a major percentage of HBsAg into lipoprotein particles indicates that the cells are not totally devoid of this process. The extremely high level of HBsAg synthesis in insect cells with the baculovirus expression vector system may have surpassed the capacity for any cell type to produce lipoprotein particles, so that it became a rate-limiting step. A second block in the secretory process occurred at the level of transport of lipoprotein particles through the cellular secretory pathway. HBsAg synthesized in mammalian (CHO) cells also appears to accumulate in the ER prior to transport to the Golgi, since essentially all of the intracellular HBsAg is endo H sensitive and the half-time for secretion is unusually long (37, 38). Under certain conditions, low levels of HBsAg have been detected in the medium of infected insect cultures. The detection of HBsAg in the medium was highly erratic, and what was detected was probably the result of cell death. In a recent report, Kang and co-workers (19) suggested that insect cells could secrete HBsAg when grown in spinner culture but not when grown as adherent cells. Insect cells can be readily grown in spinner culture, but considerable caution is required to prevent cell rupture in the late stages of infection, when the cells become very fragile. We have been unable to detect a significant level of HBsAg secretion in spinner culture when sufficient care was used.

Despite the lack of secretion, the level of processing of HBsAg observed in insect cells is substantially better than in yeast cells. The S protein is not glycosylated in yeast cells (6, 14, 30, 33, 57, 61), and the limited amount of particle formation that has been detected may represent an artifact of the extraction process (6). The lack of particle formation with the pre-S-S polypeptides with a fractionation procedure identical to that used for S suggests that particle formation is not occurring during extraction in our system. Inefficient secretion of HBsAg has been observed in other cell systems as well. Following infection of monkey kidney cells with recombinant SV40 (35) and adenoviruses (5) that express HBsAg, 60 and 22% of the HBsAg remained cell associated, respectively. Even in CHO cells, in which secretion of HBsAg is considered efficient, 22 h is required to completely secrete newly synthesized HBsAg (37). Only 2.5% of the S protein synthesized in *Xenopus* oocytes was secreted during a 48-h period (50). In *Xenopus* oocytes, the major block in secretion of HBsAg appears to be a defect in the formation of soluble lipoprotein particles in the lumen of the ER (45). The exceptionally low level of secretion of the S protein observed in *Xenopus* oocytes and insect cells may be attributable to the reduced temperatures at which these cells must be maintained. However, the efficient secretion of HBsAg may be a property unique to the differentiated hepatocyte, which is dedicated to the production of serum lipoprotein particles.

The lack of secretion initially presented a major problem in the purification of HBsAg from insect cells. This problem was overcome by detergent extraction and affinity purification, in which HBsAg was purified to near homogeneity in a single step. Although detergent extraction disrupts the HBsAg lipoprotein particles, HBsAg readily forms micelles following detergent treatment, and the resulting HBsAg detergent micelles are highly antigenic and immunogenic in comparison to HBsAg lipoprotein particles (16, 46, 47). The reactivity of our purified material was comparable to that of plasma-derived HBsAg, as evaluated by a commercial HBsAg assay, and was highly effective when used as a solid phase for anti-HBsAg assays as well. The baculovirus-derived HBsAg induced a vigorous in vivo immune response in mice. The specificity of the immune response to insect cell-derived HBsAg in comparison to the response against plasma-derived HBsAg is currently under further investigation.

A recombinant baculovirus was also constructed that expressed the pre-S-S gene open reading frame. The current data would suggest that the pre-S-S polypeptides (p39 and gp42) are modified in an identical manner in insect and mammalian cells, including insertion into the ER, glycosylation, myristylation (40), and lack of secretion (3, 4, 31, 35, 39). The use of insect cells offers a unique advantage over mammalian cells because pre-S-S can be expressed in the absence of the S polypeptide due to the inability of insect cells to recognize the S promoter that is within the pre-S coding domain.

The level of HBsAg synthesis attained by using the baculovirus expression vector system was exceedingly high. The system promises to be one of the most cost-effective means for producing recombinant antigens. The improvement of expression vectors and culture conditions should yield even higher levels of synthesis. During the course of this investigation, the importance of sequences immediately 5′ to the polyhedrin ATG initiation codon became apparent (25, 28, 29). The expression of HBsAg was approximately 15-fold lower with the recombinant virus 373S (6 mg/liter of cells) than with 106FS (90 mg/liter). The vector pAc373 is missing an AT-rich stretch from nucleotides −8 to −1 with respect to the polyhedrin initiation codon (Fig. 1), while the fusion vector pVL106 contains an intact 5′ untranslated region (28). Analysis of HBsAg mRNA levels revealed that the differences in the level of protein expression were directly proportional to the amount of HBsAg-specific mRNA detected in 373S- and 106FS-infected cells (25). The sequences immediately upstream of the polyhedrin initiation codon are apparently crucial for maximum transcriptional activity from this promoter. A series of nonfusion vectors have been constructed in which the 5′ untranslated region remains intact and the initiation codon has been altered to prevent the synthesis of a fusion protein (V. Luckow, manuscript submitted). We recently used one of these vectors (pVL941) for the expression of SV40 large T antigen (23) and HBsAg and obtained levels of recombinant antigen

comparable to those obtained with the fusion vectors. The use of these vectors will greatly increase the yield of recombinant protein in future investigations with the baculovirus expression vector system.

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