INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the original text directly from the copy submitted. Thus, some dissertation copies are in typewriter face, while others may be from a computer printer.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyrighted material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is available as one exposure on a standard 35 mm slide or as a 17" × 23" black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. 35 mm slides or 6" × 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

U·M·I
Accessing the World's Information since 1938

300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA

Order Number 8801742

Expression and assembly of hepatitis B virus proteins in cultured insect cells

Mohamad, Adel Abdel-Raouf, Ph.D.

City University of New York, 1987

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

EXPRESSION AND ASSEMBLY OF HEPATITIS B VIRUS PROTEINS IN CULTURED INSECT CELLS

by

ADEL A. MOHAMAD

A dissertation submitted to the Graduate Faculty in Biomedical
Sciences in partial fulfillment of the requirements for
the degree of Doctor of Philosophy, The City University of New York.

1987

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

September 8, 1987.

Date

Chair of Examining Committee

September 8, 1987.

Date

Executive Officer

Dr. Fiorenzo Paronetto.

Dr. Beatrice Pogo.

Dr. Peter Price.

Dr. Robert Neurath.

The City University of New York

ABSTRACT

EXPRESSION AND ASSEMBLY OF HEPATITIS B VIRUS PROTEINS IN CULTURED INSECT CELLS

by

Adel A. Mohamad

Adviser: Professor George Acs.

The baculovirus Autographa californica Nuclear Polyhedrosis Virus (AcNPV) has been used to express various hepatitis B virus (HBV) proteins in cultured insect cells in order to study their properties, interaction and assembly into virus-related particles. Recombinant baculoviruses AcNPV-S, AcNPV-S2S, AcNPV-S1S2S and AcNPV-PCC containing HBV DNA coding sequences for the small (S), middle (M) or large (L) envelope proteins or the precore-core protein respectively under the control of the polyhedrin promoter of the AcNPV have been produced. Infection of cultured insect cells with AcNPV-S or AcNPV-S2S resulted in similarly high levels of expression and secretion of hepatitis B surface antigen (HBsAg) particles made of either the S or M HBV envelope proteins in both glycosylated and unglycosylated forms. The particles had a density of 1.22 g/ml and a size of about 22 nm similar to the HBsAg found in the serum of HBV infected individuals. Infection of the insect cells with AcNPV-S1S2S resulted in expression of the HBV L envelope protein in both glycosylated and unglycosylated forms. The expressed L protein failed to be secreted or assembled into HBsAg particles and was retarded inside the cells in association with the cell membranes. Co-infection of the insect cells with AcNPV-S1S2S along with AcNPV-S and AcNPV-S2S resulted in co-expression of the three HBV envelope proteins and co-assembly and secretion of small amounts of unusual HBsAg particles that were made of all three HBV envelope proteins. These particles had a density of about 1.22 g/ml and were larger than 22 nm by electron microscopy. Infection of the insect cells with AcNPV-PCC resulted in expression of HBV precore-core proteins which were readily secreted into the medium bearing both HBeAg and HBcAg antigenicities. Immunochemical characterization of the secreted precore-core proteins indicated the presence of 26, 22, 19 and 17 kd polypeptides. The cytoplasm of these cells contained only the 26 kd protein. This indicated that expression of the precore sequences, in contrast to the preS sequences, did not inhibit but rather acted as a signal peptide to facilitate the secretion of precore-core proteins which were then processed into smaller core-related proteins. While the expression of the (L) envelope protein inhibited the secretion of the (M) and (S) proteins as HBsAg particles, co-expression of both the (L) envelope protein and the precore-core proteins did not result in any inhibitory effect on the secretion of the precore-core proteins. This indicated the specificity of the interaction between the envelope proteins. On the other hand the co-expression of the precore-core proteins along with various envelope proteins did not affect the secretion, assembly or properties of the HBsAg particles and the precore-core proteins did not seem to associate with these particles during their assembly. This indicates that the co-assembly of HBV envelope and core proteins during the formation of HBV particle may require other factors.

This work provides a unique molecular approach to study the process and the factors involved in the assembly of HBV as well as other viruses.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my professor Dr. George Acs for his generous support and guidance that I will always remember.

I am also grateful to Dr. Peter Price whose teaching and help have been milestones in my scientific training.

TABLE OF CONTENTS

III.RESULTS

LIST OF TABLES

LIST OF FIGURES

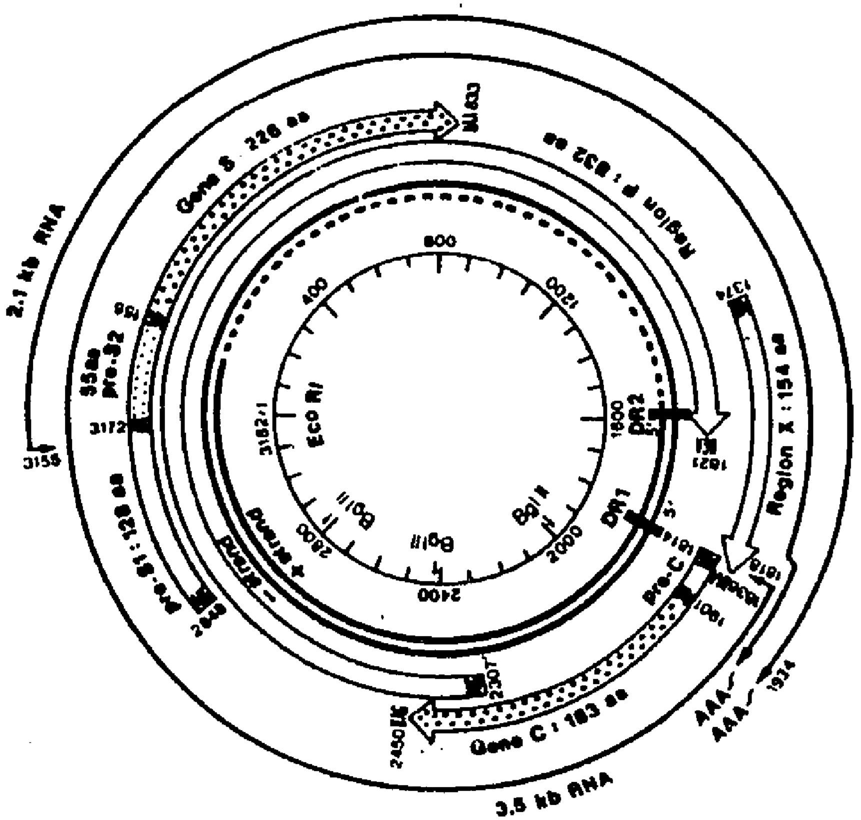
INTRODUCTION

1.Background:

The fast progress in molecular biology and recombinant DNA technology has allowed in a great detail understanding of the basic structure and organisation of HBV DNA (Tiollais et al, 1985). HBV (fig. 1) is a partially double-stranded DNA virus and belongs to a group of recently recognised animal viruses, the Hepadna Viridae (Robinson et al, 1982). Other viruses of this group are the Woodchuck hepatitis virus (Summers et al, 1978), the Beechy ground squirrel hepatitis virus (Marion et al, 1980) and the Pekin duck hepatitis virus (Mason et al, 1980). All these viruses have a common structure, are mainly hepatotropic and lead to persistant virus infection. In addition to acute infection, HBV infection may progress to chronic liver disease, hepatic cirrhosis and probably primary liver cancer. The virus is transmitted by parenteral inoculation of contaminated body fluids like blood as well as by close personal contact during which body fluids may be transfered from a carrier of the virus to an otherwise healthy individual.

Sera of infected individuals contain two major forms of viral particles (Dane et al, 1970). The 22 nm particles are found in either a spherical form or as a tubular filaments and consist mostly of dimers of HBV envelope proteins embeded in a lipid bilayer. These particles carry a predominant HBV antigen, HBsAg, and hence called HBsAg particles. They are empty and contain no core proteins or nucleic acids (Robinson and Lutwick, 1976). Continuous presence of these particles after resolution of the acute illness indicates a carrier status. The other less abundant

Figure 1. Structure and genetic organisation of the HBV genome (subtype ayw). The broad arrows surrounding the genome represent the four large open reading frames of the long strand transcript. The number of amino acids (a.a.) encoded by the coding sequences are indicated. The two thin arrows surrounding the broad arrows represent the two major HBV mRNA's. The partial restriction map and the numbering of the nucleotides indicated on the inner circle correspond to the ayw genome. The positions of initiation and stop codons of the coding sequences and the 5' and 3' ends of the two major mRNAs are indicated.


2.1 kb RNA
3155
Gene S 226 aa
833
Region P : 832 aa
1374
Region X : 154 aa
1821
1800
DR2
DR1
5'
1814
1818
1836
1901
Pre-C
1934
AAA~
AAA~
Gene C : 183 aa
2450
2307
3.5 kb RNA
2848
Pre-S1 : 128 aa
3172
55aa
pre-S2
156
– strand
+ strand
400
800
1200
2000
2400
2800
3182/1
Eco RI
Bgl II
Bgl II
Bgl II

form is the 42 nm Dane particle that represent the complete virus and its presence indicates active viral replication. It consists of an outer coat of envelope proteins embeded in a lipid bilayer and a nucleocapsid structure. Treatment of the Dane particle with nonionic detergents (e.g. NP40) results in removal of the outer viral coat and liberation of the nucleocapsid (Almeida et al, 1971). The nucleocapsid consists of the core proteins (HBcAg) (Almeida et al, 1971) and contains a circular DNA molecule, a DNA polymerase activity (Robinson:1975), a protein kinase activity (Albin et al, 1980; Gerlich et al, 1982), a DNA-linked protein (Gerlich et al, 1980). During active viral replication, an additional antigen, HBeAg (Magnius et al, 1972), related to the core protein is detected in the serum. The HBV genome (Galibert et al, 1979; Valenzuela et al, 1980) (fig. 1) is a small circular partially double stranded DNA, the long strand has a fixed length of about 3200 b.p. while the short strand has a variable length (Delius et al, 1983). The 5'ends of both strands overlap each other, thereby maintaining the circular structure of the genome. Analysis of the DNA sequence of the long strand indicates the presence of four major conserved Open Reading Frames (ORFs), S,C,P and X (Tiollais et al, 1981). Two ORFs, S (Peterson et al, 1977) and C (Pasek et al, 1979) have been assigned to identified gene products and functions; the hepatitis B virus envelope proteins and the hepatitis B virus core proteins respectively. These proteins have been characterized in infected individuals and expressed in animal cells in tissue culture. The other two ORFs, P and X are thought to correspond to a viral DNA polymerase with reverse transcriptase activity (Toh et al, 1983) and a protein X (Moriarty et al, 1985) with unknown function respectively.

2.The hepatitis B virus envelope proteins:

As mentioned above, HBV envelope proteins constitute the envelope of empty HBsAg particles and HBV particles. These two populations of particles are present in the plasma of infected individuals and can be biophysically separated by ultracentrifugation based on their densities. On cesium chloride gradients, HBsAg particles have a density of about 1.20 g/ml while HBV particles, having a dense core structure, are heavier and have a density of about 1.3 g/ml. By the electron microscope, the HBsAg particles appear as small spherical empty particles of about 22 nm in diameter while the HBV appear as a 42 nm double layered sphere with a dense core structure.

ORF S which codes for the envelope proteins is divided into three regions, preS1, preS2 and S all in a continuous reading frame with each region starting with an ATG codon. Thus, three differenproteins sharing the same C-terminus but different at the N-terminus can be translated depending on which ATG is utilized for translation initiation. This organization of the ORF S is highly conserved in all HBV isolates as well as in other hepadna viruses (Tiollais et al, 1985).

Electrophoretic and biochemical analysis of the envelope proteins (Stibbe et al, 1982; Heermann et al, 1984) and comparison of tryptic digests (Stibbe et al, 1983) with the sequence of the ORF S recently led to defining the envelope protein composition. The envelope of the complete virus contains three proteins called the major (S),the middle (M) and the large (L) proteins.

The S protein is 226 amino acids (aa) long and is encoded by the S region. It exists in two forms, glycosylated [GP27] and non-glycosylated

[GP24]. GP27 posseses a complex N-linked glycan at aa number 146. HBsAg is a conformational antigen carried on S protein . Treatment of HBsAg particles with detergents in absence of reducing agents (Mishiro et al, 1980) resulted in partial solubilization of the particles and release of dimers of two S protein molecules linked by disulfide bridge(s) probably representing the structural unit of the HBV envelope proteins that bears full HBsAg antigenicity. Treatment of the HBV envelope proteins with reducing agents results in a drastic decrease in HBsAg antigenicity (Mishiro et al, 1980).

The M protein is 281 aa long and is encoded by the preS2 and the S region. It is a glycoprotein present in two forms, GP33 and GP36 according to the extend of glycosylation. GP33 contains one high-mannose glycan at aa 4 of the M protein. GP36 contains two glycans, one high-mannose glycan at aa 4 and one complex-type glycan at aa 201 of the M protei The 55 aa encoded by the preS2 region are in general hydrophilic and carry another dominant epitope located at the surface of HBsAg particles (Neurath et al, 1984; Neurath et al, 1985). The epitope is a linear one and is not destroyed by denaturation (Machida et al, 1983). Studies have shown that the the M protein as an immunogen can induce an immune response stronger than that of the S protein and can change an HBsAg-non-responder animal into a responder (Millich et al, 1985). These studies indicate, therefore, that inclusion of the M protein into the HBV vaccine can provide better protection and may help those who do not benefit from the currently available vaccine which is made of the S protein only.

The L protein (HBV subtype ayw) is 389 aa long and is encoded by the preS1, preS2 and S regions and is present in a glycosylated [GP42] and non-glycosylated [GP39] forms. GP42, again, carries one complex-type glycan at aa 309 of the L protein. An antibody immune response to the preS1 has been shown to specifically precipitate HBV particles and to correlate with better prognosis of HBV infection (Alberti et al, 1978; Alberti et al, 1984; Klinkert et al, 1986). The preS1 has also been demonstrated to be responsible for direct binding of HBV to hepatocytes (Neurath et al, 1985; Neurath et al, 1986). Studies on the composition of the HBV and HBsAg particles indicate great variability in their envelope proteins (Heermann et al, 1984). During active viral replication, the M protein can represent more than 10% of the total envelope proteins in both the 22 nm and the complete 42 nm viral particles. In the absence of viral replication, the M protein is present in lower aunts. The L envelope protein can be found mostly during active viral replication and is preferentially associated with the complete virus particle, accounting for about 10% of its envelope proteins (Heermann et al, 1984).

A major HBV mRNA transcript of about 2.1 kilobase (kb) can be identified in cells infected with HBV or carrying HBV DNA (Cattaneo et al, 1983 and 1984; Standring et al, 1984). Transcription of the 2.1 kb mRNA is initiated from an SV40-late promoter-like sequences (Cattaneo et al, 1983 and 1984) that are present within the preS1 region upstream from the preS2 region. This mRNA which is responsible for expression of the M and S proteins (Heermann et al, 1984) has been found to have multiple start sites on either side of the ATG initiation codon of the M protein (Standring et al, 1984). As a result, some of the mRNA will not contain this AUG and can not encode for the M protein. Factors which influence the initiation of mRNA transcription on either side of the M protein ATG will regulate the expression ratio of the M to the S protein. A mechanism for preferential translation initiation at an internal ATG codon has been suggested to play a role in the regulation of expression of the M and S protein and to further explain for the predominance of the S over the M protein (Stibbe et al, 1983). The existance of such mechanism is supported by few examples of eukaryotic proteins (Liu et al, 1984). A scanning model has been proposed for ribosomal selection of initiator codons whereby not only proximity to the 5' terminus of the mRNA but also presence of so called consensus sequences flanking an ATG codon will determine its selection for initiation of translation (Kozak et al, 1983, 1984 and 1986). Indeed such consensus sequences were characterized to flank the S rather than the M protein ATG codon (Stibbe et al, 1983). A promoter similar to the typical eukaryotic TATAA promoter and located just upstream from the preS1 region (Rall et al, 1983) is probably responsible for transcription of the entire S ORF into about 2.5 kb HBV mRNA (Pourcel et al, 1982; Gough et al, 1983). Transcription of the entire S ORF would alow expression of the L protein.

Expression of HBsAg was achieved in E.Coli but in low quantities as a fusion protein (Charnay et al, 1980; Edman et al, 1981; Fujisawa et al, 1983). Much more higher levels of expression have been achieved in transformed mammalian cell lines and yeast cells utilizing different transfection and expression strategies (Christman et al, 1982; Smith et al, 1983; Davis et al, 1985; Valenzuela et al, 1982 and 1985). In these systems, HBsAg particles are secreted (except in the yeast) as 22 nm particles and consist of the S protein and resemble the natural HBsAg particles in its structure and biochemical and immunological properties. The particles band at a density of 1.2 g/ml in CsCl by isopycnic sedimentation and appear as empty spheres by electron microscopy of negatively stained preparations. It is evident now that the S proteins are capable of self assembly into particles and that the preS sequences are not required for expression,assembly or secretion of S. Expression of HBsAg particles made of both S and M proteins in different cell culture systems has been reported in several studies (Mitchel et al, 1984). The secreted particles were similar to the natural HBsAg particles biophysically and morphologically.

Recently, there have been reports on expression of L proteins in different cell culture systems (Cheng et al, 1986; Ou et al, 1987; Mclachlan et al, 1987), in transgenic mice (Chisari e al, 1986), and by injecting Xenopus oocytes with specific mRNA (Persing et al, 1986; Standring et al, 1986). These reports indicate that the expressed L protein is not secreted but strongly retarded at the cell membranes at an early secretory stage probably at the level of the endoplasmic reticulum. Several studies have clearly indicated that the expression of of the L protein inhibited to great extend the assembly and secretion of other HBV envelope proteins as HBsAg particles. In search for the reason of this behaviour of the L protein, the protein was found to be co-translationally modified at its N-terminus by cleavage of the first methionine and linkage of a myristic acid to the glycine residue at aa position number 2 (Persing et al, 1987). There are several examples of other proteins known to be modified similarily like the pp60 v-src (Schulz et al, 1985). Interestingly, all these proteins share the feature of having a glycine residue at aa position 2 and the association of the protein in each case with the cell membranes seems to be an important aspect of its function. The presence of a glycine residue at aa position 2 is noticed to be preserved in the L envelope proteins of all Hepadna viruses (Persing et al, 1987). How does this peculiar structure and behaviour of the L envelope proteins relate to their interaction with other HBV envelope proteins and what function does it serve is still not established. But it is suggested that the L proteins may play a key role in the formation and secretion of the HBV.

3. The hepatitis B virus core proteins:

The HBcAg particles isolated from the hepatocytes of HBV infected individuals or released fro HBV particles by disrupting their envelopes were first immunologically characterized by Almeida et al (Almeida et al, 1971). The particle is about 27 nm in diameter, carries a dominant antigenic epitope HBcAg, and can be physically separated from other HBV particles based on their higher density of about 1.35 g/ml in cesium chloride density gradients (Barker et al, 1974). The major polypeptide component that represent the structural unit of the particle has been chemically identified (Budkowska et al, 1979; Takahashi et al, 1979) and found to be encoded by ORF C on the genome of HBV (Pasek et al, 1979). this polypeptide has been determined to be 183 aa long with a calculated molecular weight of 21.1 kd (Tiollais et al, 1981). The C-terminus of this core protein is rich in arginine residues and has a sequence similar to those present on DNA binding proteins (Nakano et al, 1976).

Although HBcAg, in the form of HBcAg particles is found in serum only in association with HBV particles, another antigenic protein moeity present free in the serum and related to HBcAg is HBeAg (Magnius et al, 1972). The presence of HBeAg in the serum correlates with HBcAg as a marker for HBV infectivity and may indicate a poor disease prognosis. The HBeAg is present in the serum in two relatively high and low molecular weight forms (Takahashi et al, 1978). These forms represent different aggregation levels of the protein and association with IgG molecules. Chemical characterization of HBeAg reveals the presence of a polypeptide with a molecular weight of about 15.5 to 17 kd (Tedder and bull, 1979; Takahashi et al, 1980).

Several studies have indicated the structural relationship between HBcAg particles and HBeAg by demonstrating that disruption of HBcAg particles by detergents and reducing agents results in the release or formation of HBeAg (Takahashi et al, 1979; Neurath and Strick, 1979; Budkowska et al, 1979). These results were further confirmed when similar treatment of recombinant HBcAg particles resulted also in conversion of the HBcAg particles into HBeAg (Mackay et al, 1981). It was clear from these studies that for the formation of HBcAg particles, the core protein molecules are assembled together in such a way as to exihibit the HBcAg epitopes on the surface of the particle while the HBeAg epitopes are hidden in a cryptic manner. Although the molecular weight of the HBeAg polypeptides released from HBcAg particles was detected to be about 19 kd, the addition of proteolytic enzymes such as pronase resulted in the formation of HBeAg with a polypeptide molecular weight of about 15.5 kd similar to that of the serum derived HBeAg polypeptides (Takahashi et al, 1980).

The structural relationship between HBcAg and HBeAg proteins was further defined in a series of studies showing that the two major HBcAg epitopes and the two major HBeAg epitopes (as defined by monoclonal antibodies) are carried in one copy, each, on the 19 kd polypeptide while conversion of the 19 kd polypeptide into the 15.5 kd polypeptide results in loss of one of the two HBcAg epitopes (Takahashi et al, 1981 and 1983). The previous studies also showed that although HBeAg proteins were made from these polypeptides, yet the HBcAg epitopes present on the individual polypeptides were masked and only HBeAg epitopes were exhibited on the native protein. Chemical analysis and carboxy-terminal end amino acid sequencing of the 15.5 kd polypeptide in comparison with the aa sequence of the complete core polypeptide indicated that it represents a part of the core polypeptide that has been truncated of 34 aa residue from its C-teminus. The calculated molecular weight of this truncated polypeptide is 16.8 kd rather than 15.5 kd.

On the ORF C which codes for the core proteins (Pasek et al, 1979), there are two in-frame ATGs, one at position 1901 on the HBV map and another at position 1814 further upstream. This organization of the HBV genome is also preserved in all hepadna viruses. Both ATGs are candidates for traslation initiation of the core protein. The sequence between the two ATG's is termed pre-core (Tiollais et al, 1985).

Two major HBV transcripts can be identified in cells infected with the virus, a 2.1 and a 3.5 kilobase (kb) long mRNA's (Cattaneo et al, 1983 and 1984). The 3.5 mRNA is the only candidate for encoding for the core proteins and also for serving as an HBV pregenomic RNA. Several studies have shown the presence of several functional mRNA transcriptional initiation sites both upstream and downstream from the precore ATG codon (Rall et al, 1983; Roosinck et al, 1986). Mapping of the 5' end of the 3.5 mRNA shows several starting sites downstream or upstream from the ATG codon of the pre-core region (Zelent et al, 1987). Similar to the envelope proteins, two core proteins identical along their 183 C-terminus aa but one has 29 extra hydrophilic aa at its N-terminus can be expressed. It is not known which protein is being initially expressed in cells infected with HBV, and how this expression is regulated; transcriptionally, translationally or post-translationally. It is observed, however, that while both intracellular core particles and secreted HBeAg are expressed in cells with active HBV replication, only HBeAg is expressed and secreted in cells carrying the HBV genome without active virus replication (Zelent et al, 1987).

This raised questions about how would expression of the core proteins with or without the precore sequences relate to the expression of core particles versus HBeAg.

Several studies have been conducted to express HBV core proteins under control of heterologous promoters in the yeast (Miyanohara et al, 1986) and different mammalian cell lines (Roosinck et al, 1986; Ou et et al, 1986; Mclachlan et al, 1987). Expression of the core proteins without the precore sequences resulted in formation of the 27 nm core-like particles which sedimented in CsCl at 1.3 to 1.35 g/ml similar to naturally occuring naked core particles. Interestingly, the expression of the pre-core/core sequences in the yeast cells led to formation of unstable particles which were easily dissociated into HBeAg. In the mammalian cell lines, pre-core sequences acting like signal peptide, directed the association of the core proteins with the cell membranes and led to the secretion of HBeAg rather than formation of core particles. In this way, the precore sequences acted like a signal peptide by targeting the core proteins to the secretory pathway. These studies seem to suggest different pathways for the formation of core particles as compared to expression and secretion of HBeAg. It is still not known, however, whether both pathways are essential in the assembly and release of HBV particles.

4.The baculovirus expression system

The Autographa californica Nuclear polyhedrosis virus (AcNPV) (reviewed by Kelly, 1982) is a large (about 130 Kb) double stranded DNA virus that belongs to the baculovirus family and infects several species of insects and is capable of infecting spodoptera fugipedra cells in culture (Carstens et al, 1979). During the course of infection, this baculovirus demonstrates a biphasic replicatiocycle (Tjia et al, 1979; Granados, 1980) and produces two different forms of infectious particles. Early between 10 and 24 hours post infection, extracellular virus is produced by budding of nucleocapsids through the cytoplasmic membrane. By 15 to 18 hours post infection a late gene called the polyhedrin gene (Van der Beek et al, 1980; Smith et al, 1983a) is turned on and produces large amounts of polyhedrin, a 29 KD protein, that accumulate in the nuclei of infected cells in the form of occlusion bodies with the baculoviruses embeded in them. The polyhedrin gene continues to be expressed as late as 4 days post infection or until the infected cells lyse. In infected spodoptera cell cultures, polyhedrin accumulates to very high levels accounting for about 50% of the total cellular proteins (Smith et al, 1983a). Viral occlusions are important for the natural life cycle of the virus by providing the means for lateral transmission of the virus. When infected larvae die, millions of viral occlusions are left in the decomposing tissue. The polyhedrin protects the embeded virus from inactivation by environmental factors that would otherwise inactivate the virus. When larvae feed on contaminated plants, the ingested viral occlusions are dissolved in the insect gut releasing the virus which invades and infects the cells of the gut lumen. Secondary infection spreads to other cells by extracellular virus. Once cells are infected, the viral DNA is released into the nucleus, cellular chromatin is degraded and the virus directs the production of viral proteins. Viral mutants in which the polyhedrin gene is inactivated by deletions or insertions are capable of infecting insect cells and produce extracellular virs without occlusion bodies (Smith et al, 1983b).

Several properties of AcNPV make this virus a very useful expression vector for cloned eukaryotic genes :1) The potential of the viral genome to carry large pieces of additional foreign DNA (Smith et al, 1983c). 2) Neither the virus nor the recombinant viral vectors are pathogenic to vertebrates (Carbonell et al, 1985). 3) the polyhedrin gene provides a non-essential region of the AcNPV in which to insert foreign DNA. 4) A very strong polyhedrin promoter which directs transcription late in infection after extracellular virus is produced and after most host and viral genes are turned off (Smith et al, 1982). 5) The absence of the occlusion bodies in recombinant viruses provides a visual marker to select for these viruses (Smith et al, 1983c).

The capability of the baculovirus and the insect cell expression system to faithfully express foreign eukaryotic proteins in a properly processed and biologically active forms is seen in an increasing number of studies. The human interferon (Smith et al, 1983c) and interleukin-2 (Smith et al, 1985) are among several examples of recombinant proteins that have been successfuly expressed utilizing the baculovirus expression system. Recombinant interferon is properly glycosylated, its signal peptide cleaved and the protein secreted. Recombinant interleukin-2 is also secreted after cleavage of its signal peptide. The influenza virus haemagglutinin (Kuroda et al, 1986) has been also expressed in insect cells using the baculovirus expression system and shown to be fully processed and in a biologically active form. The insect cells have been shown to also be capable of supporting studies on constitution of simple biological structures when their components are expressed in these cells using the baculovirus expression system. Co-expression of the three influenza polymerase proteins in insect cells resulted in their assembly into their native biologically active complex (Detjen et al, 1987; Angelo et al, 1987).

Thus, besides the ability of expressing large amounts of recombinant proteins which facilitates their study and production for commercial use, this system provides a unique tool to examine at the molecular level the process of assembly of structures like viruses by co-expressing their components in the insect cells.

MATERIALS AND METHODS

1.Plasmids and viruses:

Cloning of HBV DNA (subtype ayw) (fig. 1) into E.Coli and construction of plasmid pTHBV-1 (fig. 2) which consists of two copies of HBV DNA arranged in a head-to-tail tandem and inserted into the EcoRI site of pBR322 have both been described by our laboratory (Christman et al, 1982). Plasmid pTHBV-1 was used as a source for different HBV DNA fragments.

The AcNPV baculovirus is a generous gift of Dr. Max D. Summers of Department of Entomology, Texas A&M University, Texas. Virus stocks are grown by infecting cultured S. Frugiperda insect cells. Extracellular virus is collected and stored at 4^{o} C.

Plasmid pAc610 is described below in section 3.

2.Cells and culture conditions:

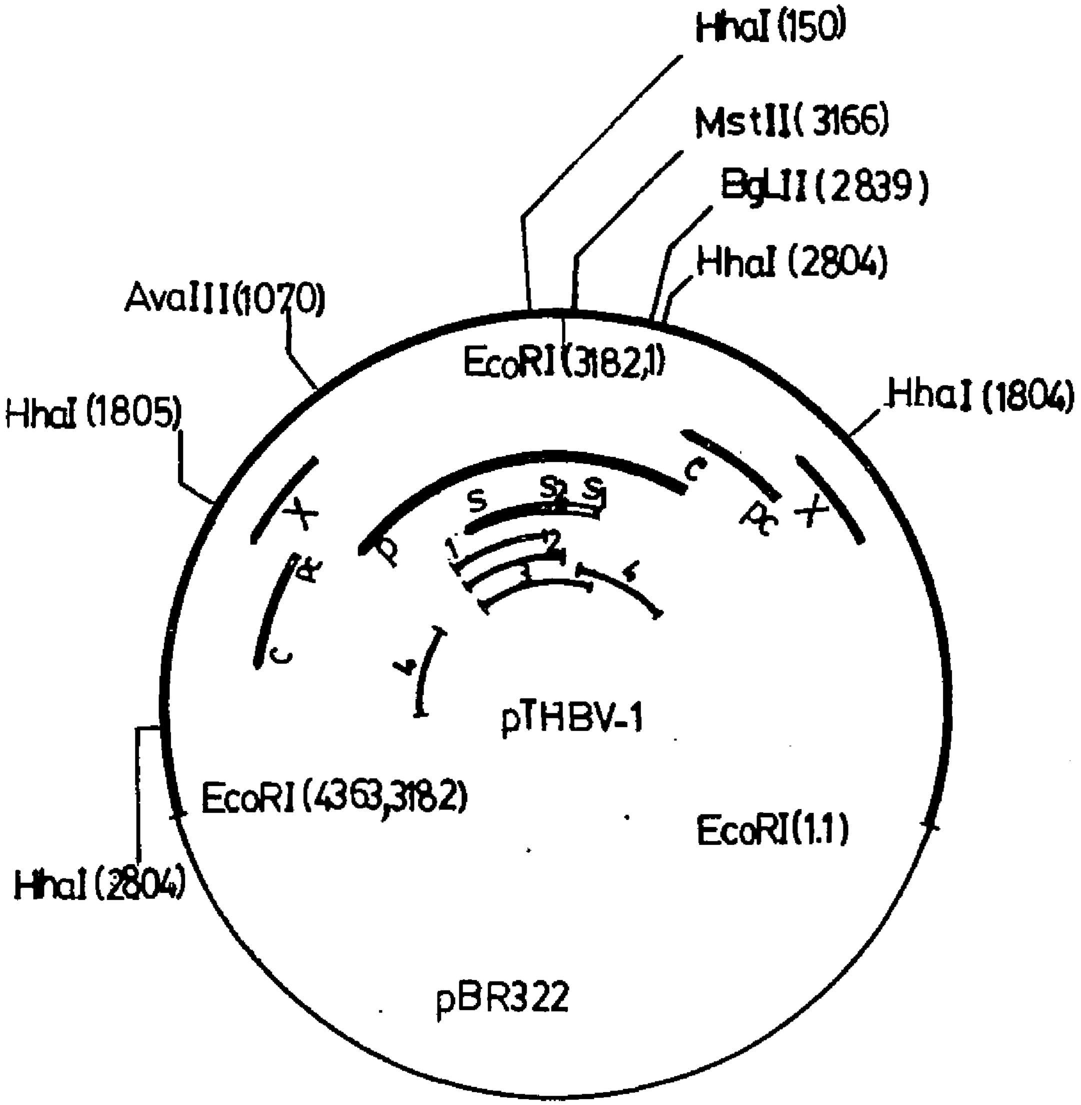
Sf9 spodoptera frugiperda insect cell line (fig. 8) has been purchased from the American Type Culture Collection and is maintained in our laboratory under standard conditions in Grace's culture medium modified with lactalbumin hydrosylate and yeastolate and supplemented with 10% heat inactivated fetal bovine serum (Hink, 1970) . The cells are grown at 27^{o} C, have a doubling time of about 24 hours and are passed 1:5 every third day by resuspending cell monolayers in fresh medium by rocking the culture flask to dislodge the cells from the culture surface.

3.Cloning of HBV genes into baculovirus transfer vectors:

The large size of the AcNPV and the lack of unique restriction sites into which foreign genes can be easily inserted in a site specific manner make it necessary to rely on random in vivo recombination between the

Figure 2. Map of pTHBV-1 indicating the positions of cleavage sites for restriction enzymes used in preparation of different HBV DNA fragments used for construction of different baculovirus transfer plasmids.

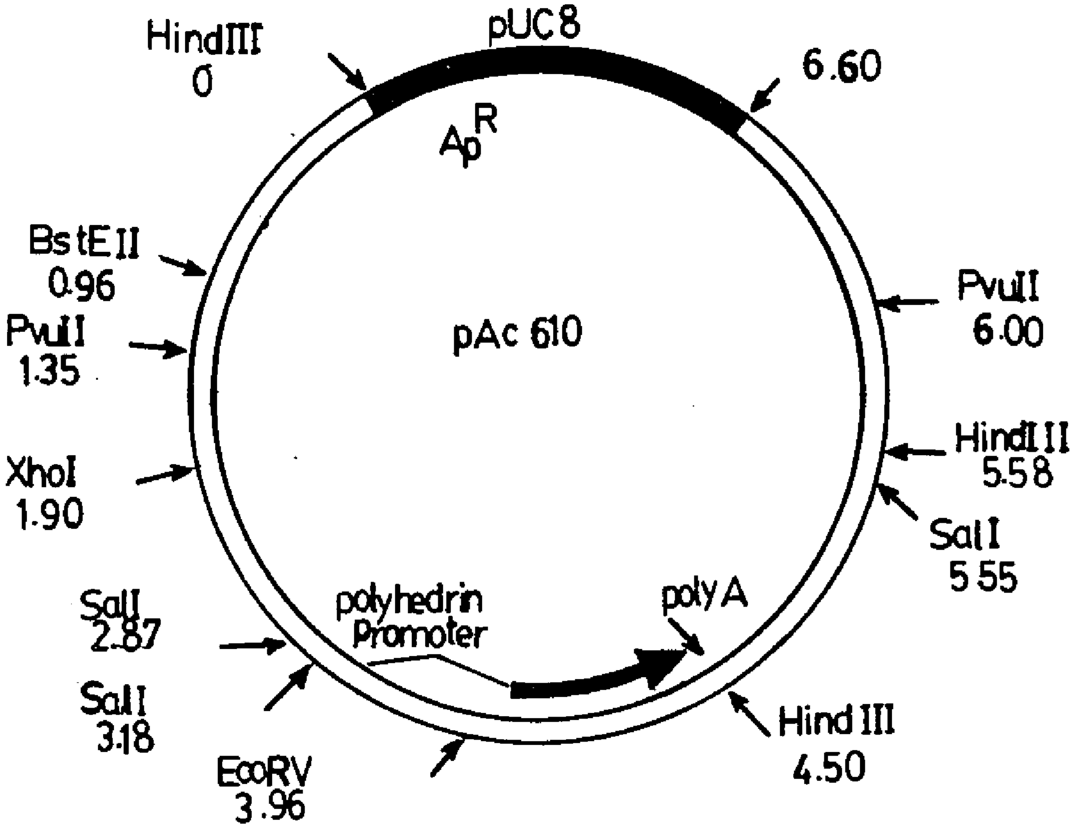
Fragment 1 represents the HBV DNA coding sequences for the S envelope protein. Fragment 2 represents the HBV DNA coding sequences for the M envelope protein. Fragment 3 represents the HBV DNA coding sequences for the L envelope protein. Fragment 4 represents the HBV DNA coding sequences for the precore-core protein. The HBV sequences on the pTHBV-1 are indicated with a heavier line and the open reading frames are indicated by symbols S, C, P and X.

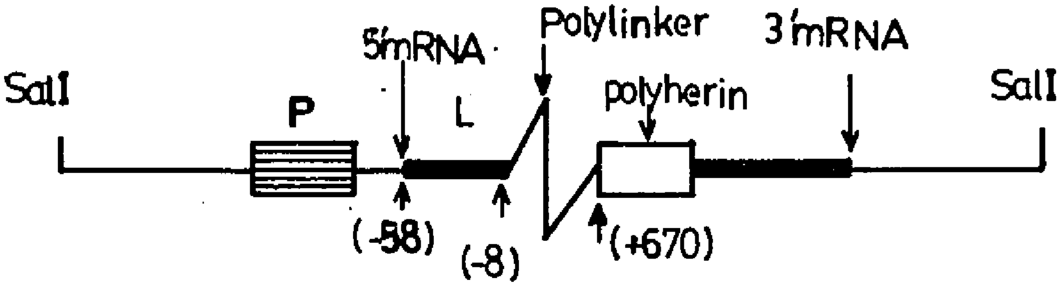

HhaI (150)
MstII (3166)
BglII (2839)
HhaI (2804)
AvaIII (1070)
EcoRI (3182,1)
HhaI (1804)
HhaI (1805)
X
Pc
p
C
S
S2
S1
C
Pc
X
1
2
3
4
4
pTHBV-1
EcoRI (4363,3182)
EcoRI (1.1)
HhaI (2804)
pBR322

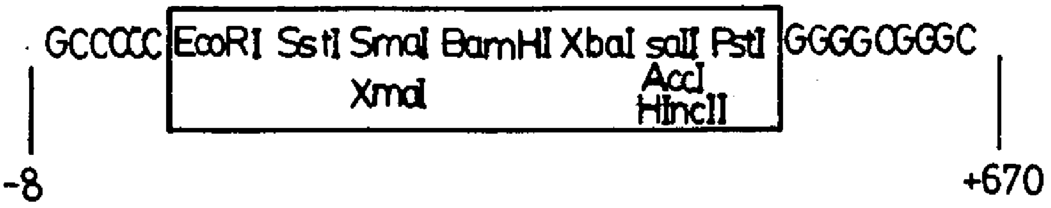
baculovirus and foreign genes for the generation of recombinant viruses. In order to ensure that foreign genes recombine only within the right contest of the polyhedrin gene which has been mapped and sequenced (Smith et al, 1983a), several chimeric plasmid vectors (transfer vectors) have been constructed (Smith et al, 1983c and 1985). Each of these has both a truncated inactive polyhedrin gene with a cloning site near the intact polyhedrin promoter and flanking viral DNA linked to the E.coli plasmid pUC8. The use of these transfer vectors achieves two goals. First, it allows convenient insertion and cloning of foreign genes adjacent to the polyhedrin promoter. Second, recombination between the transfer vector carrying the foreign gene and the wild-type virus mostly if not always occurs in the polyhedrin gene region and this generates recombinant viruses that lack the intact polyhedrin gene. In order to express mature recombinant proteins instead of fusion proteins, we selected transfer vector pAc610 (fig. 3) (Smith et al, 1985). Plasmid pAc610 has in addition to pBR322 origin of replication and the B-lactamase gene for selection in E.coli, a truncated polyhedrin gene consisting the promoter region, the RNA transcription leader sequences, a polylinker region (Messing, 1983) with unique cloning sites for EcoRI, SacI, SmaI, BamHI, XbaI and PstI and the polyhedrin 3'mRNA region including the polyadenylation signal. The polyhedrin protein encoding sequences including the translation initiating ATG have been removed from pAc610 and the vector is used to insert foreign genes in such a way that the first ATG is that of the foreign protein.

The HBV DNA sequences coding for the three envelope proteins S, M and L and for the precore-core protein were cloned into pAc610 to generate

Figure 3. AcNPV transfer vectors pAc610. The circular restriction map represents the transfer plasmid pAc610. The thick arrow marks the location of the sequences coding for the truncated polyhedrin mRNA; numbers at restriction sites indicate distance in kilobase pairs from the Hind III end of the pUC8 backbone. ApR is ampicillin resistance gene. The upper linear map is a detailed description of the 2370 bp SalI fragment containing the truncated polyhedrin gene. The polyhedrin gene sequences from nucleotide -8 to nucleotide +670 (where the first nucleotide of the translation initiation codon of the polyhedrin is assigned position +1) have been deleted from this plasmid. The hatched box represents the polyhedrin promoter (P). The open box represents the undeleted polyhedrin coding sequences. The thick line marks the begining and end of the polyhedrin mRNA where L is the leader sequences. The gap in the polyhedrin gene has been filled with a polylinker region. The lower linear map is a detailed restriction map of the polylinker region including parts of the flanking polyhedrin mRNA sequences.


HindIII
0
pUC8
6.60
Ap^R
BstEII
0.96
PvuII
1.35
XhoI
1.90
SalI
2.87
SalI
3.18
EcoRV
3.96
pAc 610
polyhedrin
Promoter
poly A
Hind III
4.50
SalI
5.55
HindIII
5.58
PvuII
6.00


SalI
P
5'mRNA
L
Polylinker
polyherin
3'mRNA
SalI
(-58)
(-8)
(+670)


GCCCCC
EcoRI
SstI
SmaI
XmaI
BamHI
XbaI
salI
AccI
HincII
PstI
GGGGCGGGC
-8
+670

four HBV baculovirus hybrid plasmids pAcS, pAcS2S, pAcS1S2S and pAcPCC respectively.

For construction of pAcS (fig. 4), pTHBV-1 was restricted by HhaI and AvaIII which cleave 7 bp upstream and 235 bp downstream, respectively, from the initiation and termination codons of the S protein and generate a 920 bp long DNA fragment. The HhaI end was treated with klenow polymerase (Maniatis et al, 1982) and the fragment was inserted between the SmaI and PstI sites in the polylinker region of pAc610 so that the blunted HhaI end is ligated (Zimmerman et al, 1985) to the blunt SmaI end and the AvaIII end and PstI end are ligated together by virtue of their having identical [sticky] ends.

For construction of pAcS2S (fig. 5), pTHBV-1 was restricted by MstII and AvaIII which cleave 8 bp upstream and 235 bp downstream,respectively, from the initiation and the termination codons of the M protein and generate a 1086 bp long DNA fragment. The MstII end was treated with klenow polymerase and the fragment was inserted between the SmaI and PstI sites in the polylinker region of pAc610 so that the blunted MstII end is ligated to the blunt SmaI end and the AvaIII end and PstI end are ligated together by virtue of their having identical "sticky" ends.

For construction of pAcS1S2S (fig. 6), pTHBV-1 was restricted by BglII and AvaIII which cleave 11 bp upstream and 235 bp downstream, respectively, from the initiation and the termination codons of the L protein and generate a 1413 bp long DNA fragment. This fragment was inserted between the BamHI and PstI sites in the polylinker region of pAc610 so that the BglII and BamHI ends and the AvaIII and PstI ends are ligated together by virtue of their having identical "sticky" ends.

Figure 4. Diagram of pAc-S, containing a fusion between the HBV S protein coding DNA sequences (shaded area) and the AcNPV polyhedrin coding DNA sequences (hatched area). For this construction, the HBV S protein coding DNA sequences were inserted in the polylinker region of pAc610. The fused sequences are included within the RNA transcript (large arrow) initiated by the polyhedrin promoter (P). Also included are baculovirus sequences flanking the polyhedrin gene (double lines) and pUC8 sequences including the origin of replication and a gene for ampicillin resistance (solid line).

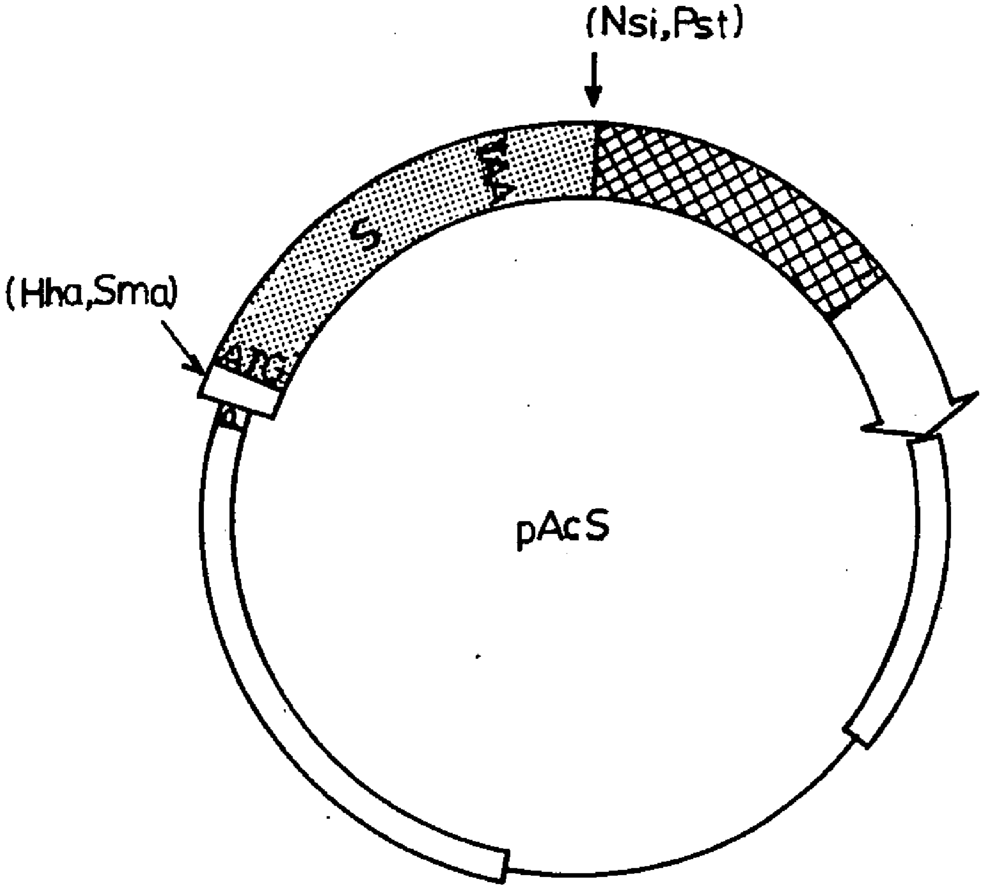

(Nsi,Pst)
TAA
S
(Hha,Sma)
ATG
pAcS

Figure 5. Diagram of pAc-S2S, containing a fusion between the HBV M protein coding DNA sequences (shaded area) and the AcNPV polyhedrin coding DNA sequences (hatched area). For this construction, the HBV M protein coding DNA sequences were inserted in the polylinker region of pAc610. The fused sequences are included within the RNA transcript (large arrow) initiated by the polyhedrin promoter (P). Also included are baculovirus sequences flanking the polyhedrin gene (double lines) and pUC8 sequences including the origin of replication and a gene for ampicillin resistance (solid line).

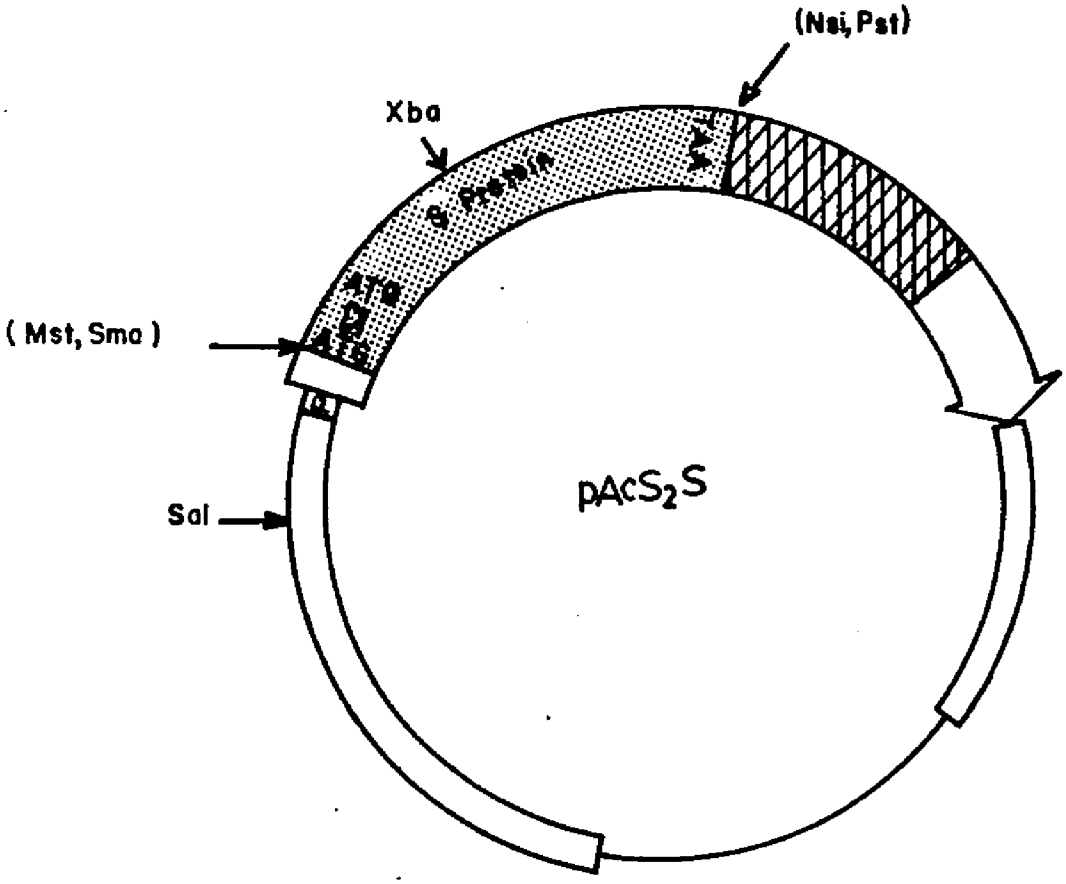

(Nsi, Pst)
Xba
S Protein
ATG
ATG
(Mst, Sma)
Sal
$pAcS_2S$

Figure 6. Diagram of pAc-S1S2S, containing a fusion between the HBV L protein coding DNA sequences (shaded area) and the AcNPV polyhedrin coding DNA sequences (hatched area). For this construction, the HBV L protein coding DNA sequences were inserted in the polylinker region of pAc610. The fused sequences are included within the RNA transcript (large arrow) initiated by the polyhedrin promoter (P). Also included are baculovirus sequences flanking the polyhedrin gene (double lines) and pUC8 sequences including the origin of replication and a gene for ampicillin resistance (solid line).

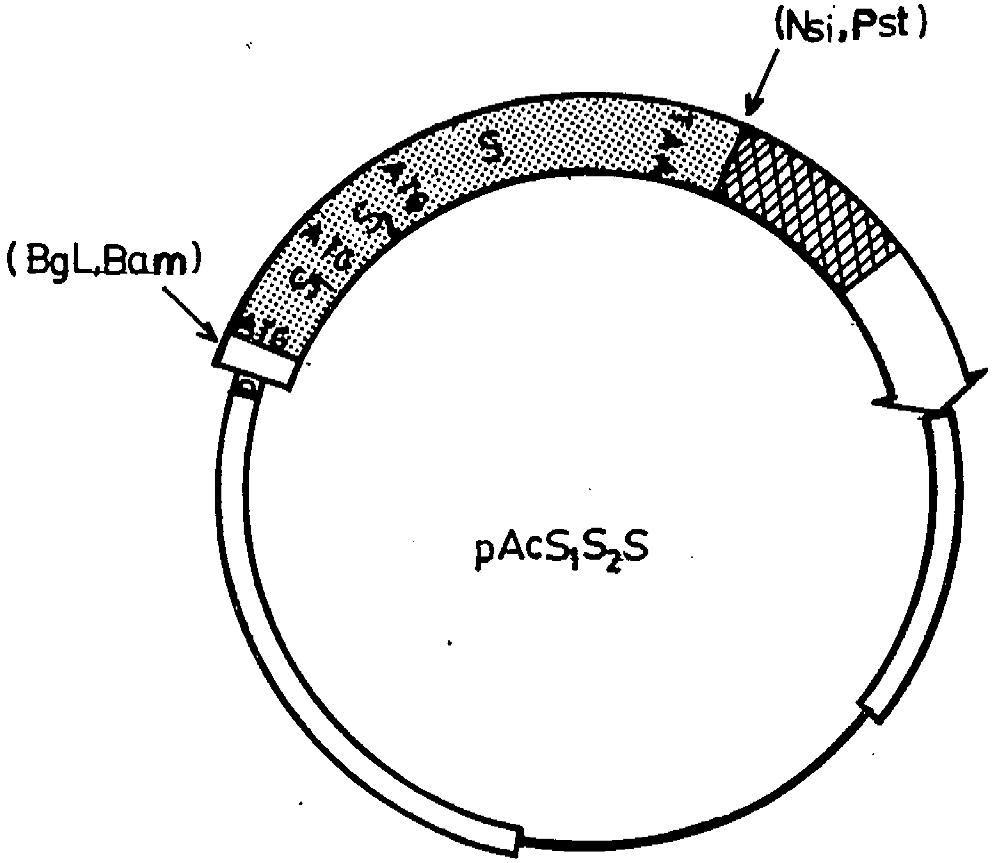
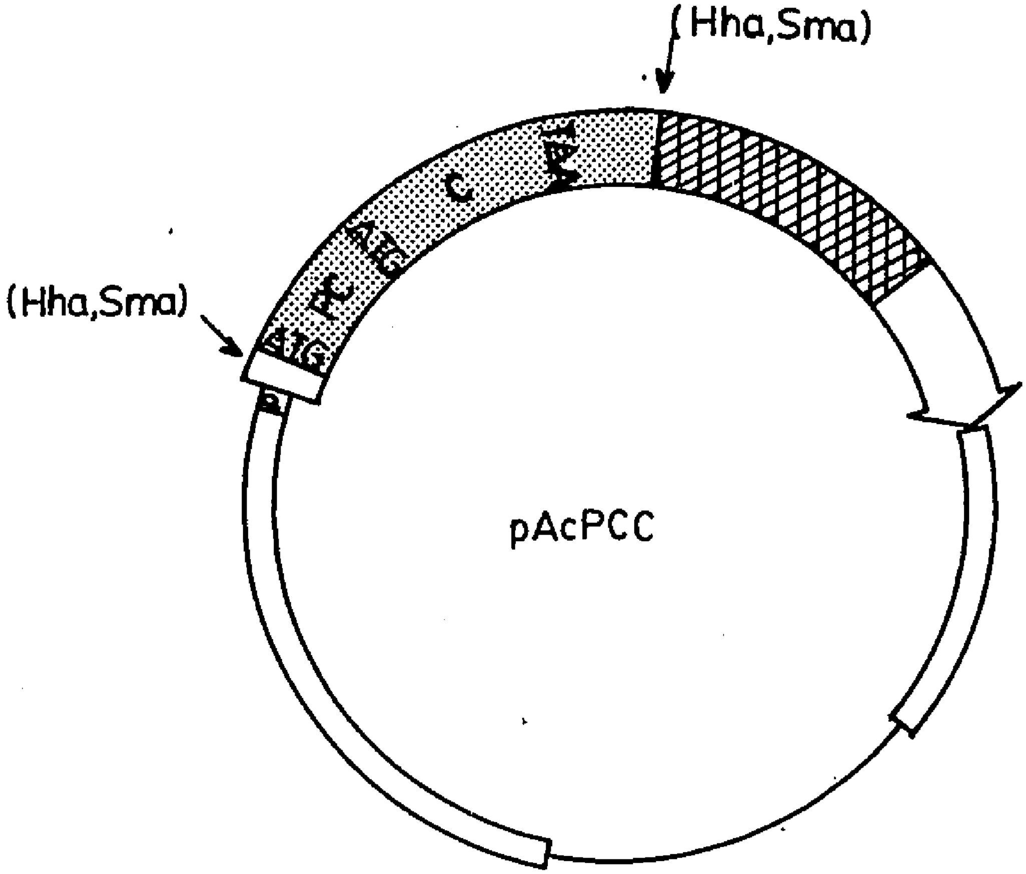

(Nsi, Pst)
(BgL, Bam)
ATG
ATG
S2
ATG
S1
pAcS$_1$S$_2$S

Figure 7. Diagram of pAc-PCC, containing a fusion between the HBV precore-core protein coding DNA sequences (shaded area) and the AcNPV polyhedrin coding DNA sequences (hatched area). For this construction, the HBV precore-core protein coding DNA sequences were inserted in the polylinker region of pAc610. The fused sequences are included within the RNA transcript (large arrow) initiated by the polyhedrin promoter (P). Also included are baculovirus sequences flanking the polyhedrin gene (double lines) and pUC8 sequences including the origin of replication and a gene for ampicillin resistance (solid line).


(Hha,Sma)
(Hha,Sma)
ATG
K
ATG
C
A
p
pAcPCC

For costruction of pAcPCC (fig. 7), pTHBV-1 was restricted by HhaI which cleaves 11 bp upstream and 350 bp downstream, respectively, from the initiation and the termination codons of the precore-core protein and generate a 999 bp long DNA fragment. This fragment was treated with klenow polymerase and inserted into the SmaI site of pAc610 by blunt end ligation.

Standard methods (Maniatis et al, 1982) and conditions recommended by the materials manufacturers were used for restriction of DNA, isolation of pure DNA fragments and ligation. After transformation of E.coli, ampicillin resistant clones carrying different plasmids were selected, their plasmids harvested by standard methods and the presence and orientation of the HBV DNA insert were confirmed by restriction analysis.

4.Isolation of baculovirus DNA:

The baculovirus was grown in the insect cells as described above. 48 hours after infection, the DNA of the extracellular baculovirus was isolated from the cell culture medium by the following method:

100 ml of infected cell culture medium was clarified by centrifugation at 10,000 x G for 10 minutes, polyethylene glycol 8000 was added to 2% and the medium was incubated on ice for 2 hours. The virus was pelleted by centrifugation at 10,000 x G for 10 minutes and resuspended in 0.1 M tris-HCl, pH 7.5, 10 mM EDTA. Sodium dodecyl sulfate was added to 1%, proteinase K was added to 0.5 mg per ml, and the suspension incubated at 50^{o} C for one hour. The suspension was extracted twice with phenol, once with ether, and the DNA precipitated by the addition of 2 volumes of ethanol. The wild-type viral DNA isolated by this method was used for the cotransfection experiments as described below.

5.Cotransfection of insect cells with plasmid and baculovirus DNA:

The DNA of wild-type baculovirus along with each of the plasmids pAcS, pAcS2S, pAcS1S2S, or pAcPCC DNA was used to cotransfect cultured insect cells (Smith et al, 1983b) using the following method: Half confluent cells were seeded at about 1 to 2 ($3x10^{6}$ cells/25 ml flask) in 2 ml fresh medium. 1 ug of purified wild-type viral DNA was mixed with 2 ug of CsCl-purified plasmid DNA in 950 ul of HEBS/CT solution (0.137 M NaCl, 0.006 M glucose, 0.005 M KCl, 0.0007 M Na2HPO4, 0.02 M HEPES, pH 7.1)/ (15 ug calf thymus DNA/ml). CaCl2 was added to the mixture and the mixture incubated at room temperature for 30 minutes. The mixture was then added to the cells and incubated at 27^{o}C for 4 hours. The DNA containing medium was replaced with fresh cell culture medium and the cells were incubated for 4 to 6 days and examined daily for signs of viral infection.

6.Nick translation of HBV DNA:

For the preparation of nick translated probes (Rigby et al, 1977), HBV DNA was isolated from pTHBV-1 after cleaving the plasmid with EcoRI, separating it from pBR322 by electrophoresis in 1% agarose gel, electro-eluting the HBV DNA, and precipitating it twice with ethanol. 250 ng of HBV DNA were nick translated at 14^{o}C for 3 hours in 25 ul of solution containing : 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.1 mM DTT, 50 ug/ml bovine serum 32 albumin, 60 uM dATP and dGTP, 10 uM dTTP, 6 uM dCTP, 250 uCi P-alpha-dCTP (specific activity 3000 Ci/mmole), 50 pg of DNase from bovine pancreas, and 12 U. of E.coli DNA polymerase I. After the incubation, the reaction mixture was made up to 20 mM with EDTA, diluted to 0.1 ml with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and passed on a sephadex G-50 (medium) column to separate the probe from unincorporated radioactivity.

7.DNA dot blot hybridization for selection of recombinant baculovirus:

Ten fold dilutions of a mixture of wild-type and recombinant baculoviruses (medium from the cotransfection experiments) were used to infect insect cells seeded in 96-well cell culture plates at $5x10^5$ cells/ml. After standing at room temperature for one hour, the infection medium was replaced with fresh medium and the cells were incubated for one week at 27^oC. Infection was assessed by appearance of polyhedrin occlusion bodies in several wells of the plates. When infection is apparent, 10 ul medium samples from each well were removed and treated with 0.4 M NaOH to release and denature the viral DNA. The samples were then dot blotted (Kafatos et al:1979) on positively charged nylon membranes (BioRad's Zeta-probe membranes). The DNA binds covalently in a denatured form to the membranes. The membranes were then washed with 2 x SSC (20 x SSC: 3 M NaCl, 0.3 M trisodium citrate) for 10 minutes and air dryed. Dry membranes were prehybridized overnight at 42^o C in 50% formamide, 4 x SSPE (20 x SSPE: 3.6 M NaCl, 0.2 M Na2HPO4, 0.02 M EDTA), 10 x Denhardt's (100 x Denhardt's: 2% BSA, 2% polyvinylpyrrolidone, 2% ficoll), 0.5 mg/ml denatured salmon DNA, and 1% SDS. This prehybridization buffer was then replaced with a fresh that has in addition to the above, 10% dextran sulfate and a nick translated ^{32}P-HBV DNA. Hybridization was carried out at 42^oC for 24 hours and membranes were washed 15 minutes each with: 1) 2 x SSC, 0.1% SDS, 2) 0.5 x SSC, 0.1% SDS, 3) 0.1 x SSC, 0.1% SDS, all at room temperature and finally at 50^o C with 0.1 x SSC, 1% SDS. The membranes were air-dryed and autoradiographed to identify clones that have HBV DNA-recombinant baculoviruses. Positive clones were evaluated for presence or absence of polyhedrin occlusion bodies by the microscope. Several cycles of dilution cloning were repeated to obtain recombinant baculovirus clones pure of any wild-type virus.

8.Infection of insect cells with recombinant baculovirus:

Stocks of recombinant and wild-type viruses were prepared by infecting insect cells using the method described in the next paragraph. 5 days after infection, cell culture medium was collected, clarified by centrifugation at 1,000 G for 5 minutes, and stored at 4^{o}C. Baculovirus can be stored at this temperature for over two years without any loss in virus titer. Freezing can be used for long term storage although it results in substanial decrease of virus titer. It is important to keep an early passage virus stock for use to avoid any build up of defective viruses.

Cell cultures near confluency were passed at about 1 to 3 into fresh culture flasks (about 3×10^{6} cells/25 cm^{2} flask). Freshly seeded cells were allowed to attach to the surface for one hour, the medium was removed and 2 ml of virus stock inoculum was added per a 25 cm^{2} flask. After incubation period of one hour at 27^{o}C, the inoculum was removed and fresh medium (5 ml/ 25 cm^{2} flask) was added. Infected cells were incubated for different periods up to 6 days when all cells are lysed and examined daily for infecion and expression of different proteins as described below. Generally, infected cells were incubated for 3 days at which time different cell fractions including the extracellular medium were harvested for protein analysis. At this stage of cell infection (3 days), cells were found to be still viale but they start to lyse from this point on. Cell viability was easily evaluated by microscopic examination and confirmed by trypan blue uptake. This is checked by adding 0.1 ml of trypan blue (0.4% in PBS pH 7.2) to 1 ml of cell suspension and examining the cells under the microscope at a low magnification.

9.Isolation of RNA from cells infected with baculovirus:

Total cellular RNA was isolated from insect cells infected for 48 hours with a recombinant or wild-type virus. When working with RNA, all solutions, tubes...etc. were treated with 1% diethylpyrocarbonate (DEPC) over night and autoclaved thereafter. RNA was isolated by the method developed by Chirgwin et al,1974. Cells were lysed by addition of 2 ml of 4 M guanidinium thiocyanate, 0.5% sarcosyl NL-30, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol and 0.1% antifoam A. The cell lysate was transfered on ice to a 30 ml Corex centrifuge tube and mixed with 0.025 volume of 1 M HOAc and 1 volume of ethanol by continuous vortexing and nucleic acids were precipitated at -20^{o} C overnight. The precipitate was collected by centrifugation at -10^{o} C at 6,000 G for 10 minutes and resuspended, with vigorous shaking, in 0.5 original volume of cell lysate of guanidine hydrochloride, 0.025 M HOAc, pH 7.0, 5 mM DTT. Nucleic acids were again precipitated by the addition of 0.025 volume of 1 M HOAc and 0.5 volume of ethanol and repeating the same steps twice. The final RNA pellet was washed with 75% ethanol, vacuum dryed, and dissolved in DEPC treated dH2O. For storage, RNA solution was mixed with 0.1 volume of 5 M KOAc, pH 5.0 and 2 volumes of 95% ethanol and placed at -20^{o} C.

10.Preparation and 5'end radiolabeling of plasmid DNA probes:

A DNA probe used for S1 nuclease analysis of RNA from cells infected with a recombinant virus was prepared (Maxam and Gilbert, 1980) using the corresponding recombinant HBV-pAc610 plasmid. A DNA fragment that spans the junction between the polyhedrin promoter and the inserted HBV DNA and extends in both directions upstream and downstream was radiolabeled at its 5'ends by kinating the dephosphorylated 5'extensions as follows: The phosphatase reaction was carried out for one hour at 37^{o} C in 200 ul of 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 U. calf alkaline intestinal phosphatase/ 10 pmoles of DNA ends. The reaction was stoped by the addition of EDTA to 50 mM and heating at 60^{o} C for 30 minutes. The dephosphorylated DNA was precipitated with ethanol and the kination reaction of the protruding 5'ends was carried out at 37^{o} C for one hour using 1 U. of phage T4 polynucleotide kinase per 10 pmoles of 5'ends in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 5 mM DTT, 1 mM spermidine and ^{32}P-gamma-ATP (specific activity 5,000 Ci/mmole) at concentration of 2 pmole per 1 pmole of 5'ends of the DNA in a volume of 50 ul. In order to obtain a probe radiolabeled on one strand only, labeled DNA was restricted with another endonuclease and purified by preparative gel electrophoresis.

11.S1 nuclease and polyacrylamide/urea gel electrophoresis:

For S1 nuclease analysis (Berk and Sharp, 1977), 25 ug of RNA, radiolabeled probe, and 100 ug of carrier E.coli tRNA were resuspended in 30 ul of hybridization buffer (80% deionized formamide, 0.4 M NaCl, 40 mM piperazine-N,N'-bis[2-ethane-sulfonic acid] {PIPES}, pH 6.8, 0.5 mM EDTA), denatured for 10 minutes at 85 C and hybridized at 52^{o} C for three hours. The samples were then diluted with 320 ul of ice-cold S1 buffer (50 mM NaOAc, pH 4.6, 0.28 M NaCL, 4.5 mM ZnCl2, pH 5.0, 20 ug/ml denatured salmon sperm DNA). Digestion was carried out for 2 hours at 20° C with 12.000 U. of S1 nuclease. At the end of the reaction S1 nuclease was deactivated by adding 6 ul of 2 M Tris-base and 8 ul of 0.5 M EDTA. The nucleic acids were precipitated after addition of 0.25 volume of 10 M NH4OAc and 2 volumes of ethanol by storing overnight at -20° C. The nucleic acid pellet was collected by centrifugation at 10,000 G for 5 minutes, vacuum dryed, and resuspended in 10 ul of 2 mM Tris-HCl, pH 7.5, 0.05 mM EDTA. Before loading onto the 7 M urea/polyacrylamide gels described below, samples were mixed with 2 volumes of loading buffer (88% deionized formamide, 10 mM EDTA, 0.2% BpB and XCFF), denatured at 90° C for 3 minutes and quick chilled on ice.

For preparation of polyacrylamide gels for nucleic acids electrophoresis, 40% polyacrylamide stock solutions (38 g acrylamide and 2 g bisacrylamide) were used. To prepare 8% acrylamide gel, 10 ml of acrylamide stock solution were mixed with 5 ml of 10 x TBE (0.89 M Tris-base, 0.89 M Boric Acid, 25 mM EDTA), 1.6 ml ammonium persulfate, and 33.4 ml of 37.5% glycerol. Polymerization was catalyzed by the addition of 50 ul of TEMED. gels were formed 1.5 mm thick and run in 1 x TBE at a constant 200 voltage after loading the samples. The 7 M urea/ 8% polyacrylamide gels used for the S1 analysis were prepared by adding 30 g of urea to 12 ml of acrylamide stock solution, 16.9 ml ml of dH2O and the dissolved material was stirred with 0.5 g of AG501-X8(D) resin and filtered through a 0.45 um filter membrane. To 43.4 ml of this solution, 5 ml of 10 x TBE, 1.6 ml ammonium persulfate, and 50 ul of TEMED were added. These gels (1.5 mm thick) were run at 300 volts to keep the gel surface temperature at about 50° C. After the nucleic acids electrophoresis, the gels were rnsed in dH2O, dryed and autoradiographed

12.Preparation of extracellular, cytoplasmic and membrane cell fractions:

Different cell fractions were collected 3 days days after infection with baculoviruses. Cells were gently resuspended in their medium by pipetting and transfered to 15 ml tubes on ice. Phenyl Methane Sulfonyl Fluoride (PMSF) and Leupeptin (Proteolytic enzyme inhibitors) were added to a final concentration of 0.2 mM of PMSF and 2 ug/ml of leupeptin and the cell suspension was centrifuged at 1,000 x G for 5 minutes. The supernatant was collected as the extracellular fraction and stored frozen until further experiments. The cell pellet was resuspended in hypotonic lysis buffer, 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.2 mM PMSF and 2 ug/ml Leupeptin to a final volume equal to the supernatant, incubated on ice for 10 minutes with vortexing at maximum speed for one minute and checked for cell lysis under the microscope. The lysed cells were centrifuged at 1,000 x G for 5 minutes and the supernatant was collected as the soluble cytoplasmic fraction. The pellet, representing the insoluble membrane/nuclei fraction was then resuspended in 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, 2 ug/ml Leupeptin and 0.5% NP 40 in a volume equal to 0.1 of the cytpolasmic fraction, incubated for 10 minutes on ice with vortexing. The solubilization of the membranes and the presence of a nuclei preparation free of cellular membranes was checked by the microscope and the mixture was centrifuged at 1,000 G for 5 minutes. The supernatant, representing the membrane fraction was collected and stored frozen.

13.Radio-immunoassay for HBV antigens:

HBV envelope proteins were measured using a commercially available solid-phase radio-immunoassay kits (HBsAg kit, Connaught labs). The test utilizes an anti-HBs antibody that recognizes a common epitope(s), HBsAg, present on the S protein and thus shared by all envelope proteins. The test results are expressed as radioactive counts per minutes (cpm) and a sample is considered positive if it has cpm of 2.1 or more times higher than the cpm of a known negative control. A standard positive control with a known amount of HBsAg (20 ng/ ml) was used to calculate the amount of HBsAg present in a given sample. For this purpose, a positive sample was appropriately diluted to give cpm near to the cpm of the standard positive control. The amount of HBsAg was calculated using the following formula: (sample cpm - negative control cpm) X dilution factor X 20 ng / (positive control cpm - negative control cpm) X 1 ml.

HBV core proteins were detected using the commercially -available radio-immunoassay kit Abbott-HBe (Abbott). The test utilizes a human anti-HBe serum which always contains, in addition, anti-HBc antibody and hence it measures total HBeAg and HBcAg present in a given sample. The test results are expressed as radioactive counts per minutes (cpm) and a sample is considered positive if it has cpm of 2.1 or more times higher than the cpm of a known negative control. A single batch of HBeAg positive control, arbitrarily defined as having one unit per ml, was used as a standard to measure the amount in units of HBeAg and HBcAg in a given sample presence of HBeAg and HBcAg. For this purpose, a positive sample was appropraitely diluted to give cpm near to the cpm of the standard positive control and the amount of HBeAg and HBcAg was calculated using the following formula: (sample cpm - negative control cpm) X dilution factor X 1 U. / (positive control cpm - negative control cpm) X 1 ml.

14.Cesium Chloride isopycnic ultracentrifugation of HBV proteins:

Cesium Chloride was added at 1.38 gm per 4.5 ml of different cell fractions containing HBV proteins and the mixed solution was centrifuged at 235,000 G for 60 hours at 4 ^{o}C. Fractions were collected, the density of each fraction was measured and fractions were assayed for HBV proteins.

15.Electron microscopy of HBV-related particles:

Positive Cesium Chloride gradient fractions were dialyzed against 0.9% NaCl. Formvar/Carbon coated Copper grids were floated on a drop of the sample for 10 minutes, drained of excess fluid, floated again on filtered 1% aquaous Urinyl Acetate for one minute, drained of excess fluid and allowed to air dry. The grids were then examined under the electron microscope for the presence of negatively stained particles. In some cases, the samples were first incubated with antibody against a relative HBV protein for one hour at room temperature, centrifuged in eppendorf tubes at 16,000 G for 30 minutes and the pellet was resuspended in 0.9% NaCl and used for electron microscopy study.

16.Immunological probes for HBV proteins:

Goat anti-HBs antiserum (DAKO Co.) or rabbit anti-HBs antiserum (CalBiochem.) raised against HBsAg of ad/ay subtype isolated from serum of hepatitis patients was used for for building of Sepharose affinity columns for separation of HBV envelope proteins (see below). Rabbit anti-HBc serum (from DAKO Co.) raised against purified recombinant HBcAg from E.coli was used for immunoprecipitation of HBV core proteins. This antiserum was found to contain anti-HBc and anti-HBe antibodies both in high titers as determined by the CORAB test (Abbott) for the anti-HBc antibody and the ABBOTT-HBe test (Abbott) for the anti-HBe antibody respectively (data not shown). Therefore, this antibody was suitable for immunoprecipitation of HBeAg as well as HBcAg. For immunostaining of HBV envelope proteins on western blots, several other antibodies were used: 1) Anti-S, a rabbit hyperimmune serum raised against a synthetic peptide corresponding to aa (amino acid) 135 to 155 on the S protein (Neurath et al, 1982). Thus, this antibody can recognize an epitope(s) carried on S, M and L envelope proteins. 2) Anti-preS2, a rabbit hyperimmune serum raised against synthetic peptide corresponding to aa 1 to 25 on the M protein (Neurath et al, 1985). Monoclonal-Anti-gly-preS2, a monoclonal antibody Q19/10 against the glycosylated pre-S2 region of HBV envelope protein. The epitope for this antibody is present on both native and denatured M proteins that are gylcosylated on the preS2 region (Heermann et al, 1984). 4) Monoclonal-Anti-preS1, a monoclonal antibody MA18/7 against the preS1 region of HBV envelope protein. The epitope for this antibody is present on both native and denatured L protein (Heermann et al, 1984). For immunostaining of HBV core proteins on western blots, the rabbit anti-HBc serum was used and found to detect different forms of HBV core proteins in a denatured form.

17.Immunoprecipitation of HBV proteins:

The IgG fraction was purified from anti-HBs goat anti-serum on a protein A affinity column successfully following a method described by Delacroix and Vaerman, 1979 (data not shown) and used to build an anti-HBs affinity column as follows: CNBr-activated Sepharose 4B dry beads (Sigma) were hydrated and washed with 1 mM HCl (200 ml/gm beads) on sintered glass filter (medium). The IgG fraction dissolved in 0.1 M NaHCO3, pH 8.3, 0.5 M NaCl coupling buffer was mixed with the gel suspension at 5 mg protein per one mL gel for 2 hours at room temperature with continuous mixing. After the protein bound to the gel, the gel was mixed with 0.2 M glycine, pH 8.0 for 2 hours at room temperature to block remaining active sites on the beads. The gel was then washed with the coupling buffer followed by 0.1 M NaAc buffer, pH 4, 0.5 M NaCl and again with the coupling buffer. The gel was finally stored at 4 ^{o}C in in 10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA and 0.05% Na3N. For immunoprecipitation of HBV envelope proteins for SDS/polyacrylamide gel electrophoresis (SDS/PAGE), 5 to 10 ul of gel were incubated with 1 ml of extracellular or cytoplasmic fractions containing HBV envelope proteins with continuous mixing at room temperature for one hour. The gel was then washed 3 times with PBS (10 mM phosphate buffer, pH 7.2, 0.15 M NaCl) and immuno-immobilized proteins were solubilized with 2 x SDS/PAGE sample loading buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.05% Bromophenol Blue dye).

The IgG fraction of the rabbit anti-HBs antiserum was also purified on a protein A affinity column and covalently linked to sepharose similar to linkage of the IgG fraction of the goat anti-HBs antiserum.

For preparation of anti-HBc affinity gel, rabbit anti-HBc antiserum was incubated with Protein A affinity gel at 1 ul (14 ug IgG) of serum per 2 ul of protein A gel in PBS. The anti-HBc/protein A affinity gel was then used to immunoprecipitate HBV core proteins by incubating 2 ul of this gel with 1 ml of extracellular or cytoplasmic fractions containing HBV core proteins. The gel was then washed three times with PBS and immunoimmobilized proteins were solubilized as described for the HBV envelope proteins.

18.SDS/polyacrylamide gel electrophoresis:

For the electrophoresis of proteins, 0.5 mm. thick gels were prepared essentially as described by Laemmli, 1970. A 12.5% polyacrylamide gel was prepared using a 40% polyacrylamide stock solution (1 g bisacrylamide per 37.5 g acrylamide) and contained 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.1% ammonium persulfate, and 0.1% TEMED. After the polymerization of the lower gel, the upper stacking gel (3% polyacrylamide, 0.125 M Tris-HCl, pH 6.6, 0.1% SDS, 0.1% ammonium persulfate, and 0.1% TEMED) was casted and allowed to polymerize. Protein samples were boiled for 5 minutes and rapidly cooled on ice immediately before loading on the gel. The gel was run in 50 mM Tris-HCl, pH 8.3, 0.384 M glycine, 0.1% SDS buffer at a costant current of 20 mA. Prestained protein molecular weight markers were run on each gel and used to follow the migration of proteins on the gels.

19.Western blotting and immunostaining of HBV proteins:

Immediately after protein electrophoresis, the gel was soaked for 10 minute in the transfer buffer (25 mM Tris-HCL, pH 8.3, 0.192 M glycine, and 20% methanol) and protein bands were electrophoretically transferred to nitrocellulose membranes (Towbin et al, 1979) in trasfer buffer at constant voltage of 60 V. for 3 hours. Efficiency of transfer was checked by the transfer of the prestained protein molecular weight markers to the membrane. These markers were also used to determine the molecular weight of different immunostained protein bands later. For immunostaining, the nitrocellulose membranes were rinsed once in TBST (10 mM Tris-HCl, pH

8.0, 150 mM NaCl, and 0.05% Tween 20) and incubated in 1% BSA (bovine serum albumin) in TBST for 30 minutes at room temperature to block remaining protein binding sites on the membrane. The membranes were then incubated with a relevant primary antibody diluted in TBST for 1 hour at room temperature, washed three times 10 minutes each with TBST, incubated with an alkaline phosphatase conjugated secondary antibody (anti-rabbit IgG or anti-mouse IgG) diluted in TBST for 30 minutes at room temperature, washed three times 10 minutes each with TBST, and finally incubated in the alkaline phosphatase substrate color developing solution (0.2 mg nitro blue tetrazolium and 0.1 mg 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl2).

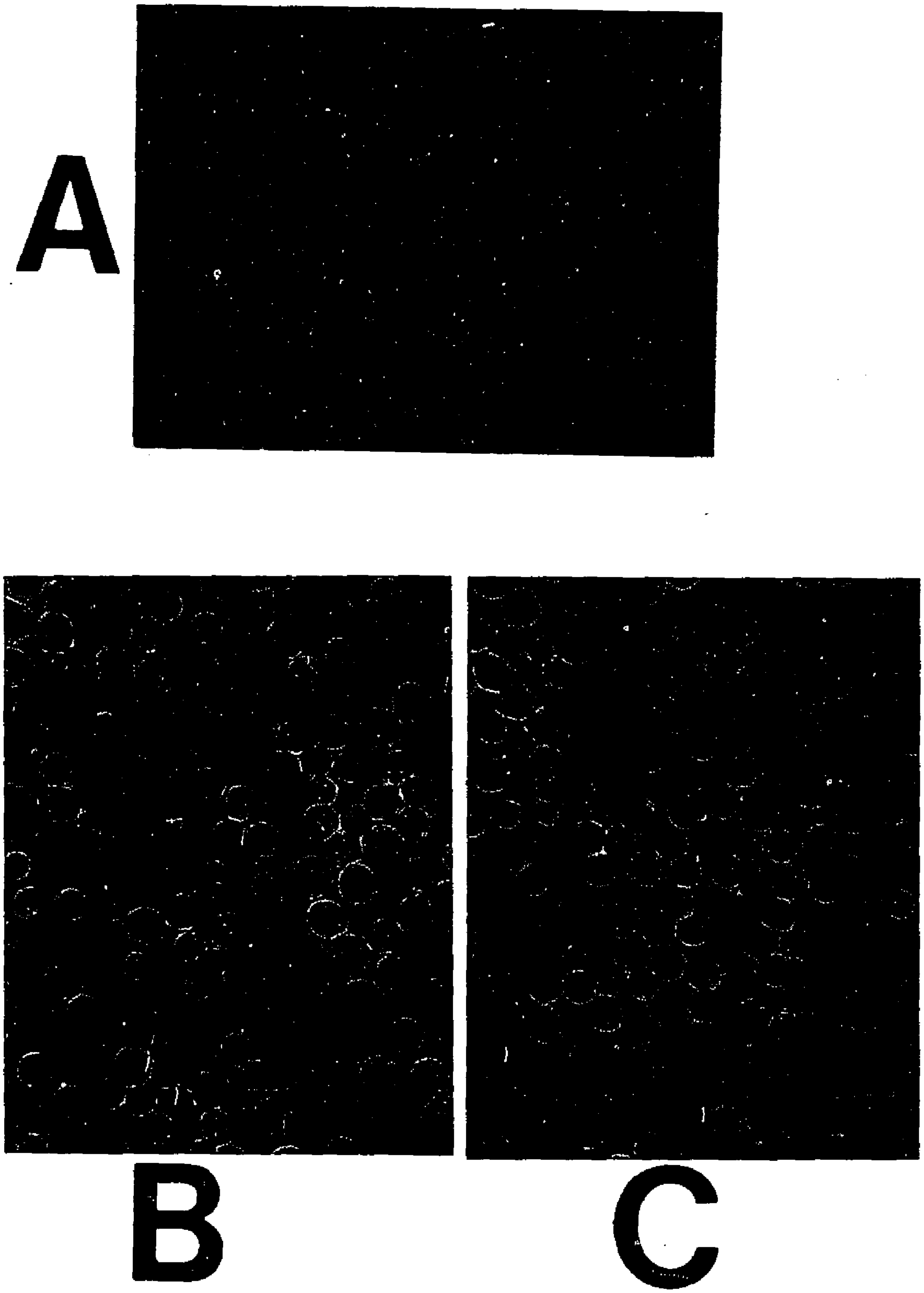
RESULTS

1.Production of recombinant baculoviruses:

The baculovirus is a lytic DNA virus. About ten hours after the insect cells are infected with the wild-type virus, virus starts to be released from the cells by budding until it reachs a maximum level by about 36 hours after infection. At about 15 to 18 hours after infection, the polyhedrin gene (Van der Beek et al, 1980; Smith et al, 1983a) of the baculovirus is turned on and starts to produce large amounts of polyhedrin, a 29 kd protein, that accumulates in the nuclei of the infected cells forming large occlusion bodies that contain embedded baculoviruses. Infected cells containing these occlusion bodies can be easily characterized under the light microscope (fig. 8, B) from other infected cells laking these occlusion bodies (fig. 8, C). Viral mutants in which the polyhedrin gene is inactivated by deletions or insertions are capable of infecting insect cells (Smith et al, 1983b) (fig. 8, C) and are released as extracellular viruses without formation of occlusion bodies. We have successfully utilized the occlusion body marker in combination with a standard DNA dot blot hybridization technique (Kafatos et al, 1979) to develope a system for selection and purification of recombinant baculoviruses by dilution cloning.

After cloning different HBV DNA coding sequences into the transfer plasmids, each of the hybrid plasmids: pAcS, pAcS2S, pAcS1S2S, and pAcPCC (figs. 4,5,6 and 7) was used along with the wild-type baculoviral DNA to transfect insect cells according to the method outlined before. Under these standard conditions, it is estimated (Summers et al, 1986) that

Figure 8. Sf9 spodoptera frugiperda insect cells under the light microscope. A: uninfected cells 48 hours after seeding are almost confluent. B: cells 48 hours after seeding and infection with wild-type baculovirus. The cells stopped dividing, became larger than uninfected cells and full of polyhedrin inclussion bodies. C: cells 48 hours after seeding and infection with recombinant baculovirus. These cells also stopped dividing, became larger than uninfected cells but do not have polyhedrin inclussion bodies and can be easily differentiated from those with polyhedrin. Cell culture conditions and infection were done as described in Methods.


A
B
C

recombination between the viral DNA and the transfer plasmid at the polyhedrin gene region results in the formation of recombinant viruses that lack the intact polyhedrin gene at a rate of about 0.1% to 5% of the total viruses produced which remain mostly of the wild type (Summers et al, 1986).

In all four co-transfection experiments, signs of viral infection of the insect cells and the formation of occlusion bodies were obvious by day three of the experiment. Six days after the co-transfection, the cell culture medium was collected and ten-fold serial dilutions were used to infect insect cells in 96-well culture plates at one ul per well as described in the methods section. At 1:1,000 dilution, about 6 days of cell culture incubation were required for appearance of signs of viral infection in most of the wells. Longer time was required with the 1: 10,000 dilution while some wells lacked any signs of viral infection. At this stage the DNA dot blot hybridization was done and showed the presence of recombinant baculoviruses with HBV DNA sequences in few wells. All the wells with recombinant baculoviruses still contained wild-type viruses and were run through other cycles of dilution cloning. Two cycles were enough to obtain pure recombinant baculovirus clones in the case of AcNPV-S, AcNPV-S2S, and AcNPV-PCC. In case of AcNPV-S1S2S we were unable to identify the wells with recombinant baculoviruses during the cloning except when the DNA dot blot hybridization was done much later after the cell infection (about 12 days) when most cells were lysed. Also several cycles of cloning were required to purify AcNPV-S1S2S. The reason(s) for the difficulty in purifying AcNPV-S1S2S as compared to the other recombinant baculoviruses is not understood, but may suggest that the putative expression of the HBV L envelope protein may have enterfered with or delayed the release of the extracellular baculovirus until the cells were lysed, a situation which gave in the same time some selective growth advantage to the wild-type over the recombinant baculoviruses.

2.Expression and secretion of HBV proteins:

Infection of insect cells with different recombinant baculoviruses resulted in expression of considerably high amounts of each of the HBV envelope or core proteins. Table I and II summarize the kinetics of expression and secretion of each of these proteins.

Infection of insect cells with AcNPV-S which has the DNA coding sequences for the HBV S envelope protein (table I,A) resulted in early synthesis of HBsAg which increased to five fold (total amount) by day 2, 15 fold by day 3, 30 fold by day 4, and finally to 45 fold by day 5/6 when the cells were lysed. At this stage a total of about 2.7 ug of HBsAg/ml were made by these cells. The expressed HBsAg was secreted into the medium in quantities proportional to the total amount of expressed HBsAg until the cells started to lyse by day 4 and the soluble cytoplasmic (presumably post-membrane pre-secretory) HBsAg started to leak into the medium.

Infection of the insect cells with AcNPV-S2S which has the DNA coding sequences for the HBV M envelope protein resulted in expression and secretion of HBsAg (table I,B) with kinetics generally similar to that in case of AcNPV-S. Although total amount of HBsAg (up to 3.7 ug/ml cell culture by day 5/6) expressed using AcNPV-S2S was consistantly higher than when using AcNPV-S throught the cell culture period, the secretion

TABLE I. Kinetics of expression and secretion of HBsAg in insect cells infected with recombinant baculoviruses.

Days in culture	% viable cells	Amount of HBsAg in ug/ ml of cell culture				Ratio total/ medium	Ratio medium/ cytoplasm
		medium	cytoplasm	membrane	total		
A:							
<u>AcNPV-S</u>							
1	>95%	0.015	0.013	0.040	0.070	4.6	1.0
2	>95%	0.067	0.040	0.226	0.333	4.9	1.7
3	90%	0.200	0.105	0.661	0.966	4.8	1.9
4	70%	0.468	0.200	1.198	1.866	4.0	2.3
5	40%	0.800	0.265	1.534	2.599	3.2	3.0
6	<30%	0.933	0.200	1.533	2.666	2.8	4.6
B:							
<u>AcNPV-S2S</u>							
1	>95%	0.013	0.013	0.067	0.093	7.0	1.0
2	>95%	0.066	0.067	0.334	0.467	7.0	1.0
3	90%	0.200	0.200	1.000	1.400	7.0	1.0
4	70%	0.465	0.334	1.668	2.467	5.3	1.4
5	40%	0.865	0.468	2.340	3.673	4.2	1.8
6	<30%	1.000	0.335	2.340	3.675	3.7	3.0

(continued next page).

TABLE I. Kinetics of expression and secretion of HBsAg in insect cells infected with recombinant baculoviruses (continued).

Days in culture	% viable cells	Amount of HBsAg in ug/ ml of cell culture				Ratio total/ medium	Ratio medium/ cytoplasm
		medium	cytoplasm	membrane	total		
C:							
AcNPV-S1S2S							
1	>95%	-	-	0.020	0.020	-	-
2	>95%	-	-	0.107	0.107	-	-
3	90%	-	-	0.320	0.320	-	-
4	70%	-	-	0.560	0.560	-	-
5	40%	-	-	0.827	0.827	-	-
6	<30%	-	-	0.827	0.827	-	-

Table I: Insect cells were infected with different recombinant baculoviruses. At indicated time points, cell fractions were prepared and assayed for HBsAg using HBsAg radio-immunoassay kits (Connaught) as described in the Methods. Values for each fraction represent the amount of HBsAg in each fraction per one ml of cell culture suspension. These values were calculated according to the formula presented in the Methods and using a standard HBsAg positive control with known amount of HBsAg (20 ng/ml). The data represent the average values of duplicate determinations on samples from three different experiments.

of HBsAg in the former case (in presence of pre-S2 sequences) was relatively retarded.

Infection of the insect cells with AcNPV-S1S2S which has the DNA coding sequences for the HBV L envelope proteins resulted in expression of HBsAg in a quite different way (table I,C). Although total HBsAg increased over the incubation period (up to 0.8 ug HBsAg/ml cell culture by day 5/6), it remained totally associated with the cell membranes even after the cells were lysed and no HBsAg was secreted.

Infection of the insect cells with AcNPV-PCC which has the DNA coding sequences for the HBV precore-core proteins resulted in expression and efficient secretion of HBeAg (table II). HBeAg was secreted much faster than HBsAg into the medium. The test for HBeAg measured both HBeAg and HBcAg. This is because virually all anti-HBe sera contain anti-HBc antibodies. Several immunoassay methods have been designed to measure HBcAg or to differentiate between HBeAg and HBcAg (Roosinck et al, 1986; Ou et al, 1986; Mclachlan et al, 1987). These assays depend on the use of monospecific or monoclonal anti-HBcAg antibodies. Anti-HBc sera used in this study (Abbott anti-HBc and anti-HBc raised in rabbits against pure recombinant HBV core particles, DAKO) were evaluated for presence of anti-HBeAg activity by Abbott-HBe assay and shown to be positive (data not shown). A test for HBcAg based on the percentage inhibition of radiolabeled anti-HBc binding to immobilized HBcAg in the CORAB radioimmunoassay (Abbott) was used to evaluate for the expression of HBcAg in the insect cells infected with AcNPV-PCC and found to be slightly positive (Price et al, 1983). But we were not able to draw any conclusion about the percentage of HBcAg to HBeAg from this test because of the low

TABLE II. Kinetics of expression and secretion of HBV core proteins in insect cells infected with recombinant baculoviruses.

Days in culture	% viable cells	Amount of HBV core proteins in U./ ml cell culture				Ratio total/ medium	Ratio medium/ cytoplasm
		medium	cytoplasm	membrane	total		
AcNPV-PCC							
1	>95%	0.324	0.046	0.370	0.740	2.3	7.0
2	>95%	1.620	0.231	1.852	3.703	2.3	7.0
3	90%	5.000	0.556	5.556	11.112	2.2	8.9
4	70%	11.112	0.740	8.334	20.186	1.8	15.0
5	40%	17.780	0.740	12.962	31.482	1.8	24.0
6	<30%	17.780	0.740	12.962	31.482	1.8	24.0

Table II: Insect cells were infected with recombinant baculoviruses. At indicated time points, cell fractions were prepared and assayed for HBeAg and HBcAg using Abbott-HBe radio-immunoassay kits (Abbott) as described in the Methods. Values for each fraction represent the amount in units of HBeAg and HBcAg in each fraction per one ml of cell culture suspension. These values were calculated according to the formula presented in the Methods and using a standard HBeAg positive control with arbitrarily defined amount of HBeAg (one unit/ml). The data represent the average values of duplicate determinations on samples from three different experiments.

sensitivity of the test.

3.Physicochemical and morphological characterization of HBV proteins:

The density of HBV envelope proteins secreted by insect cells infected with either AcNPV-S or AcNPV-S2S was determined by cesium chloride isopycnic ultracentrifugation (Fig. 9). The secreted HBV envelope proteins banded in the gradient at a mean density of about 1.22 g/ml in proximity to the density of the HBsAg particles found in the serum of HBV infected individuals. There was no noticeable difference in the density between HBV envelope proteins secreted by cells infected with AcNPV-S or AcNPV-S2S. The particulate form and the morphology of this HBV envelope proteins was further characterized by electron microscopy and immunoelectron microscopy of negatively stained HBV envelope proteins (Fig. 10). The abundant presence of negatively stained empty spherical particles with an avarage size of 22 nm is seen in figure (10, A). To confirm that these particles were made of HBV envelope proteins samples were first immunoprecipitated with anti-HBs antibody before processing for electron microscopy (Fig. 10, B). The density of the HBV precore-core proteins expressed by insect cells infected with AcNPV-PCC was also determined on a cesium chloride gradient. These proteins banded at a mean density of about 1.35 g/ml along with most of the serum proteins (fig. 9), in agreement with the density of HBV core proteins or HBeAg found in the serum of HBV infected individuals. To further determine whether precore-core proteins were assembled into core particles, both secreted and cytoplasmic fractions containing HBV precore-core proteins were examined by electron and immunoelectron microscopy but no core particles were detected (data not shown). The presence of the baculovirus in some

Figure 9. CsCl isopycnic centrifugation of HBV envelope and core proteins secreted in the medium of insect cells three days after infection with AcNPV-S (or AcNPV-S2S) or AcNPV-PCC. CsCl was added to the clarified medium to a density of 1.25 g/ml, and the solution was centrifuged for 60 hours at 235,000 x G, fractions were collected, and the densities of the fractions were measured (open circles). AcNPV-S (or AcNPV-S2S) medium fractions were assayed for HBsAg (open squares) and AcNPV-PCC medium fractions were assayed for HBeAg/HBcAg (closed circles) as described in the Methods part. Results with AcNPV-S and AcNPV-S2S were identical.

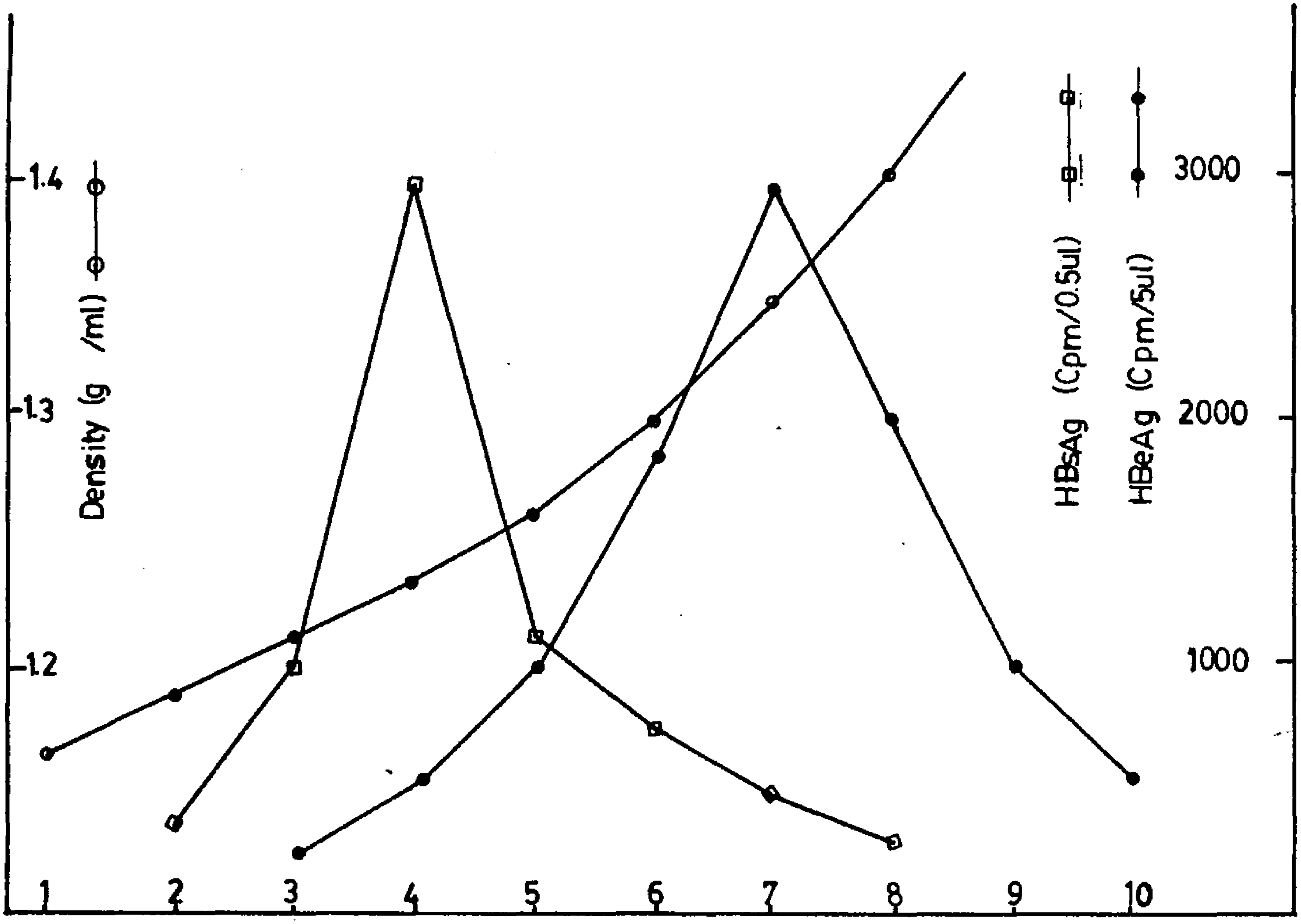
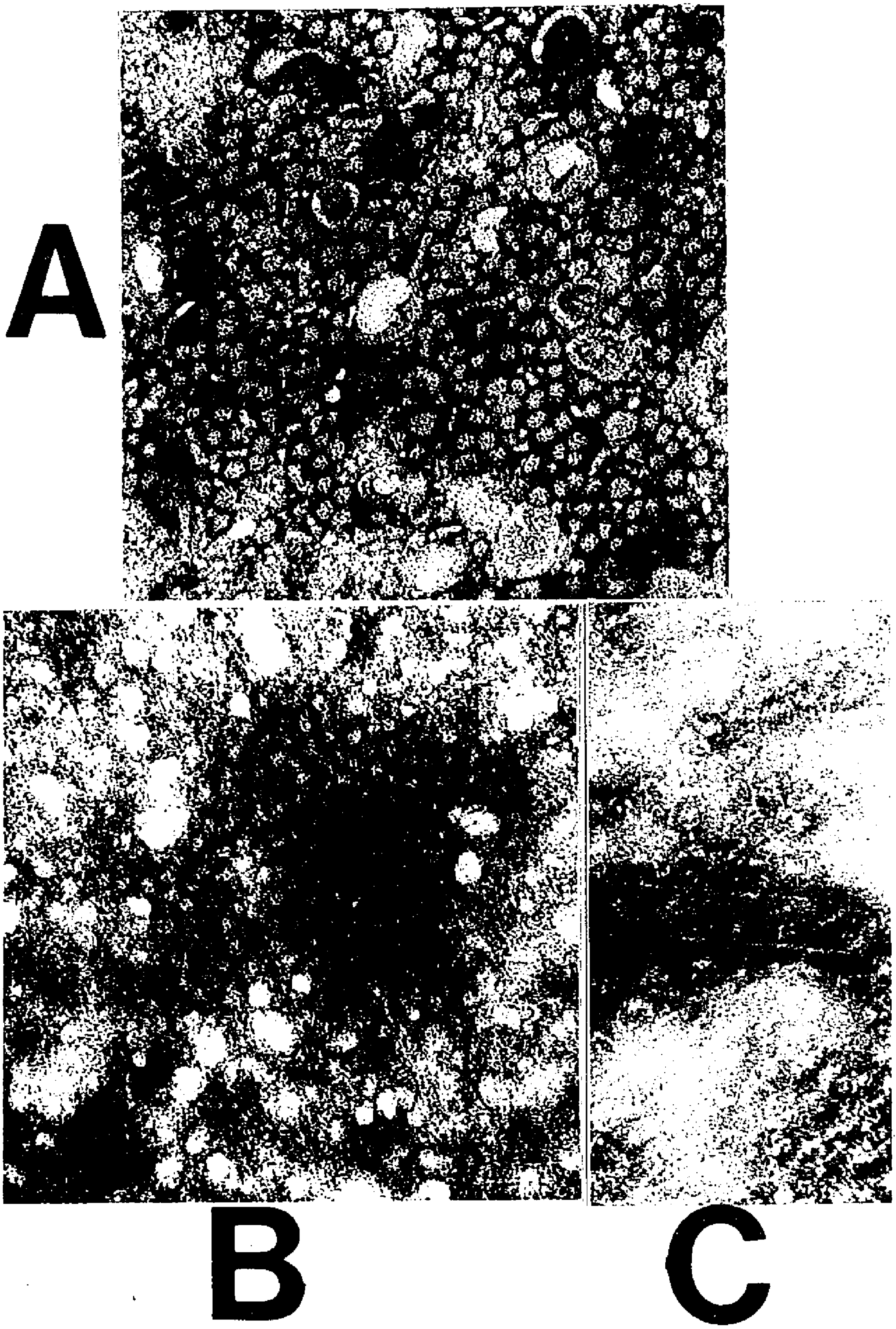

1.4
1.3
1.2
Density (g /ml)
3000
2000
1000
HBsAg (Cpm/0.5ul)
HBeAg (Cpm/5ul)
1
2
3
4
5
6
7
8
9
10

Figure 10. Electron micrographs of negatively stained HBsAg particles produced by insect cells infected with AcNPV-S (or AcNPV-S2S). HBsAg positive CsCl fractions (from previous experiment) were dialyzed against 0.9% NaCl. The samples were processed for electron microscopy as described in the Methods part. A: Negatively stained HBsAg particles. B: Same sample as in A but was first incubated with goat anti-HBs serum (DAKO Lab.) and then processed for electron microscopy. C: The baculovirus (large cylinder-like structure) was detected in some samples.


A
B
C

of the electron-micrographs (fig. 10, C) prompted us to determine whether any of the HBV proteins expressed in the insect cells is associated with the baculovirus. Polyethylene glycol (PEG) has been commonly used to precipitate and concentrate macromolecules like viruses based on their sizes. Baculoviral DNA was isolated from extracellular baculoviruses using a 2% PEG precipitation step of the virus from the cell culture medium (Methods). Table III indicates that while the baculovirus was present in the 2% PEG pellet and not in the 10% PEG pellet, the HBV proteins did not precipitate with 2% PEG and remained to precipitate only with 10% PEG. This clearly indicates that the HBV proteins did not associate to any detectable degree with the baculoviruses.

4.Immunochemical characterization of the HBV proteins:

The HBV proteins expressed in the insect cells were further characterized by immunoprecipitation and SDS-polyacrylamide gel electrophoresis followed by western blotting of electrophoresed polypeptides to nitrocellulose membranes and immunostaining utilizing an array of polyclonal and monoclonal antibodies.

Western blotting and immunostaining with rabbit anti-S antiserum (Neurath et al, 1982) of electrophoresed HBV envelope proteins expressed in insect cells infected with AcNPV-S indicated the presence of a 24 kd polypeptide. This polypeptide, corresponding to the unglycosylated HBV S envelope protein was present in the medium, cytoplasm, and membrane fractions of the insect cells (fig. 11: lanes 3, 4 and 6) but not in the insect cells infected with wild-type AcNPV (lanes 1, 2 and 5). No 27 kd polypeptide that corresponds to the glycosylated S protein was detected by this antibody. This is probably because the anti-S antibody does not

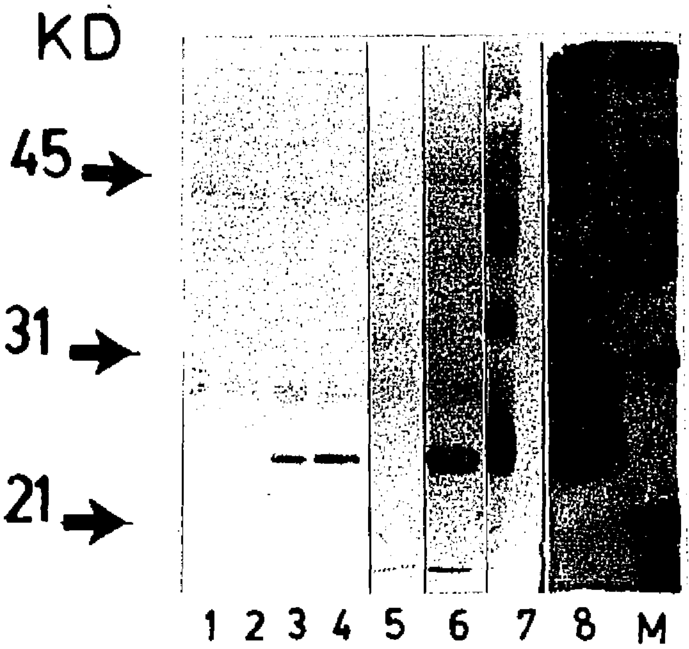
TABLE III. Polyethylene glycol fractionated precipitation of HBV proteins and extracellular baculoviruses from insect cell culture medium.

Medium from cells infected with	Percentage of HBsAg or HBeAg/HBcAg in......			Presence of extracellular baculoviruses in.......	
	2% PEG pellet	10% PEG pellet	Supernatant	2% PEG pellet	10% PEG pellet
1)AcNPV-S	<5%	>90%	<5%	+	-
2)AcNPV-S2S	<5%	>90%	<5%	+	-
3)AcNPV-PCC	<5%	70%	25%	+	-

Table III. Three days old medium from insect cells infected with with each of the indicated recombinant baculoviruses was collected. Polyethylene glycol (PEG) 8000 was added to 2% and the medium was incubated on ice for 2 hours. The medium was centrifuged at 10,000 xG for 10 minutes. The pellet was collected and PEG was added to the supernatant to 10%, incubated again for 2 hours on ice, centrifuged at 10,000 xG for 10 minutes and the pellet was separated from the supernatant. Each fraction was assayed for HBsAg (1 and 2) or HBeAg/HBcAg (3). The presence of extracellular virus in the 2% or the 10% pellets was assessed by the presence (+) or absence (-) of viral DNA isolated according to the protocol outlined in the Methods.

Figure 11. Immunochemical characterization of HBV envelope proteins produced by insect cells infected with AcNPV-S. Three days after infection, medium, cytoplasmic, and membrane fractions were collected and prepared for electrophoresis as described in Methods. The samples were run on a 12.5% SDS-polyacrylamide gel along with pre-stained standard molecular weight protein markers, electrophoretically transfered to nitrocellulose membranes, and immunostained with rabbit anti-S antiserum all under conditions described in Methods. The pre-stained markers were used to follow the electrophoresis, to determine efficiency of protein transfer to nitrocellulose membranes and to directly calculate the molecular weight of the immunostained bands on the nitrocellulose membranes.

Lanes 1 to 7 contain cell fractions equal to 0.1 ml cell culture per lane immunostained with rabbit anti-S antiserum (1:500). Wild-type virus infected cell medium (lane 1), cytoplasm (lane 2), and membrane (lane 5). AcNPV-S infected cell medium (lane 3), cytoplasm (lane 4), and membrane (lane 6). Lane 7 represents 1 ug of HBsAg purified from infectious human plasma. Lanes 8 and M are silver staining of SDS-polyacrylamide gel containing 1 ug of purified HBsAg (lane 8) (as in lane 7) and markers (lane M). Silver staining was done using a BioRad kit as instructed by the manufacturer (BioRad). HBV envelope proteins did not stain with Coomassie blue.

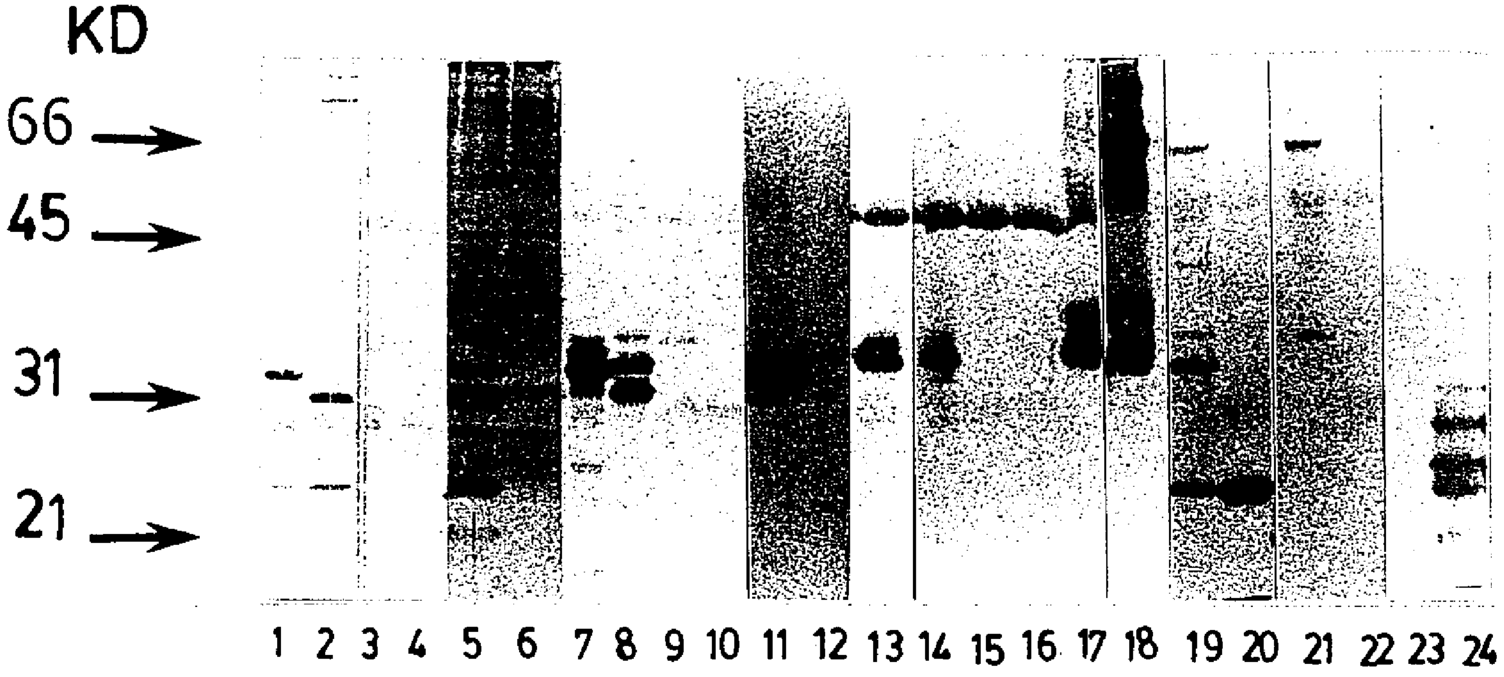

KD
45
31
21
1
2
3
4
5
6
7
8
M

recognize glycosylated forms of the S protein. This reasoning is supported by the fact that this antibody was raised against a synthetic peptide corresponding to the glycosylation site on the 27 kd S protein (Neurath et al, 1982). Glycosylation may mask the antigenic epitope on the polypeptide as shown in fig. 11, lanes 7 and 8. Lane 8 is a silver staining of HBsAg purified from infectious human plasma electrophoresed on an SDS-polyacrylamide gel. While both the 24 and 27 kd S proteins can be seen on the gel, only the 24 kd S protein was detected by the anti-S antibody (lane 7). Therefore, with this immunological probe, no conclusion can be made about the presence of the 27 kd S protein. The anti-HBsAg antiserum could not be used because it does not react with denatured or reduced HBV envelope proteins (Mishiro et al, 1980) (data not shown).

Western blotting and immunostaining of electrophoresed HBV envelope proteins expressed in insect cells infected with AcNPV-S2S (fig. 12) indicated the presence of polypeptides corresponding to the M protein and the S protein. Immunostaining with anti-S antibody demonstrated the presence of a 33 kd and a 24 kd polypeptides in the medium (lane 1) while the cytoplasm contained a 31 kd and a 24 kd polypeptides (lane 2). All three polypeptides; the 33 kd, 31 kd, and 24 kd were present in the membrane fraction (lane 5). Reflected by the intensity of various bands, it can be estimated that the 24 kd polypeptide represented about 20 % of the total amount of the envelope proteins. Immunostaining with anti-preS2 (Neurath et al, 1985) resulted in detection of both The 33 kd and the 31 kd polypeptides in the medium (lane 7), cytoplasm (lane 8) and membrane (lane 11) fractions. The intensity of the bands was much higher

Figure 12. Immunochemical characterization of HBV envelope proteins produced by insect cells infected with AcNPV-S2S. Three days after infection, medium, cytoplasmic, and membrane fractions were collected and prepared for electrophoresis as described in Methods. The samples were run on a 12.5% SDS-polyacrylamide gel along with pre-stained standard molecular weight protein markers, electrophoretically transfered to nitrocellulose membranes, and immunostained with different antibodies all under conditions described in Methods. The pre-stained markers were used to follow the electrophoresis, to determine efficiency of protein transfer to nitrocellulose membranes and to directly calculate the molecular weight of the immunostained bands on the nitrocellulose membranes.

All lanes contain cell fractions equal to 0.1 ml cell culture per lane except lanes 17 and 18 which contain 1 ug of HBsAg purified from infectious human plasma. Lanes 19 to 24 represent cell fractions prepared in the absence of proteolytic enzyme inhibitors. Lanes 1 to 6 and 19 to 24 are immunostained with rabbit anti-S antiserum. Lanes 7 to 12 and 18 are immunostainedwith rabbit anti-preS2 antiserum. Lanes 13 to 17 are immunostained with monoclonal anti-glycosylated preS2 antibody. AcNPV-S2S infected cell medium (lanes 1, 7, 13, and 19), cytoplasm (lanes 2, 8, and 20), and membranes (lanes 5, 11, 14, and 24). Wild-type virus infected cell medium (lanes 3, 9, 15 and 21), cytoplasm (lanes 4, 10, and 22), and membranes (lanes 6, 12, 16, and 23).


KD
66
45
31
21
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

than when stained with anti-S antibody probably because of a higher affinity of anti-S2 antibody. The glycosylation of the M proteins was analyzed using a monoclonal antibody that recognizes only the glycosylated forms of the M protein (monoclonal-anti-gly-preS2). Only the 33 kd polypeptide was detected with this antibody in the medium (lane 13) and the membrane (lane 14). Lanes 17 and 18 represent HBsAg particles purified from infectious human plasma, electrophoresed, western blotted and immunostained with anti-preS2 and monoclonal-anti-gly-preS2 respectively.

When the different cell fractions were prepared in the absence of some proteolytic enzyme inhibitors, the ratio of M to S proteins shifted toward the S protein. As indicated in figure 12 there was an equal amounts of the 33 kd and the 24 kd polypeptides the medium (lane 19), only the 24 kd was present in the cytoplasm (lane 20) and the 33 and 31 kd polypeptides in the membrane were converted into several smaller polypeptides toward the 24 kd polypeptide (lane 24). This clearly demonstrates the high sensitivity of the M proteins to cleavage with proteolytic enzymes. The mixed expression of the M and S proteins in insect cells infected with AcNPV-S2S could be due to one or more of the following posibilities: multiple mRNA transcription initiation sites upstream and downstream of the M protein ATG codon, (Cattaneo et al, 1983 and 1984; Standring et al, 1984) translation initiation at both the M and S proteins ATG codons (Stibbe et al, 1983), or cleavage of the M protein into the S protein could have accounted for the mixed presence of the M and S envelope proteins (Itoh et al, 1986a).

Western blotting and immunostaining of electrophoresed HBV envelope proteins expressed in insect cells infected with AcNPV-S1S2S (fig. 13) confirmed the absence of any envelope proteins in the medium (lane 1) or the cytoplasm (lane 3) and the presence of 39 kd and 42 kd L proteins in association with the cell membranes (lane 5) using monoclonal anti-preS1 antibody (Heermann et al, 1984). It can be noticed that there is a smear of very faint bands polypeptides of lower molecular weights. These may represent different stages of growth of the L protein. The accumulation of the L protein at the membrane may have resulted in slowing down of this process. The presence of the 39 kd and 42 kd polypeptides in the membrane fraction is confirmed by their immunostaining with anti-preS2 (lane 7). A polypeptide band of about 25 kd in the membrane was immunostained with both anti-preS2 and anti-preS1 (lanes 5 and 7) and probably represent a cleaved L protein. The 39 kd polypeptide band was also immunostained with anti-S antibody but only after overloading of the gel with the sample as can be noticed from the high background in both the sample and its negative control (lanes 9 and 10). The difficulty was probably bacause of the low affinity of the anti-S antibody as indicated previously. It is not unexpected that the 42 kd polypeptide did not stain with anti-S since this polypeptide is probably the glycosylated form of the 39 kd polypeptide.

Electrophoresis and western blotting of HBV core proteins expressed in insect cells infected with AcNPV-PCC (fig. 14) and their immunostaining with rabbit anti-HBc antiserum, which also contained anti-HBe antibodies, showed the presence of two major 26 kd, and 17 kd polypeptides as well as other minor 22 kd and 19 kd polypeptides in the medium (lanes 1 and 3). The intense band at about 50 kd was also present

Figure 13. Immunochemical characterization of HBV envelope proteins produced by insect cells infected with AcNPV-S1S2S. Three days after infection, medium, cytoplasmic, and membrane fractions were collected and prepared for electrophoresis as described in Methods. The samples were run on a 12.5% SDS-polyacrylamide gel along with pre-stained standard molecular weight protein markers, electrophoretically transfered to nitrocellulose membranes, and immunostained with different antibodies all under conditions described in Methods. The pre-stained markers were used to follow the electrophoresis, to determine efficiency of protein transfer to nitrocellulose membranes and to directly calculate the molecular weight of the immunostained bands on the nitrocellulose membranes.

Cell fractions equal to: 0.1 ml cell culture per lane immunostained with monoclonal anti-preS1 antibody (lanes 1 to 6), 0.2 ml cell culture per lane immunostained with rabbit anti-preS2 antiserum (lanes 7 and 8), and 1 ml cell culture per lane immunostained with rabbit anti-S antiserum (lanes 9 and 10). AcNPV-S1S2S infected medium (lane 1), cytoplasm (lane 3), and membrane (lanes 5, 7, and 9). Wild-type virus infected cell medium (lane 2), cytoplasm (lane 4), and membrane (lanes 6, 8, and 10).

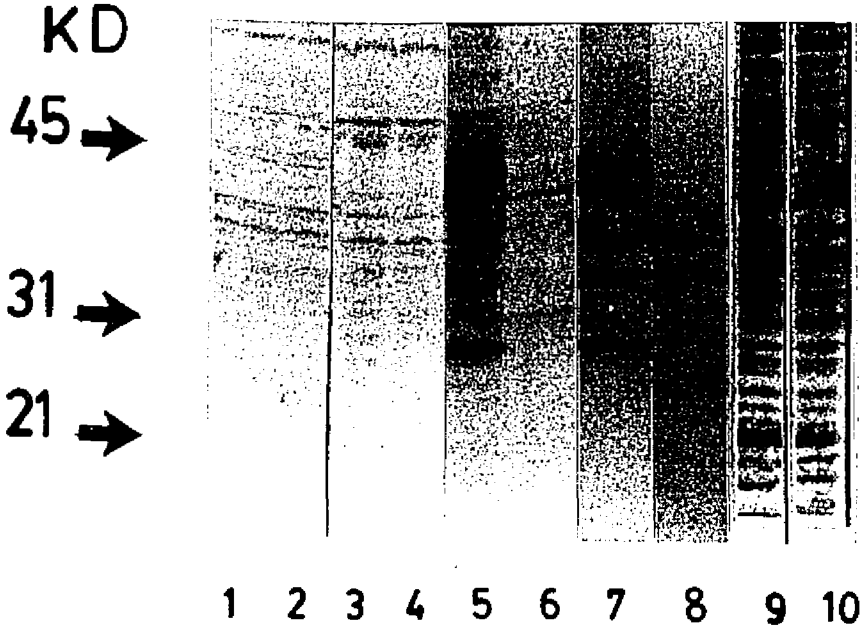
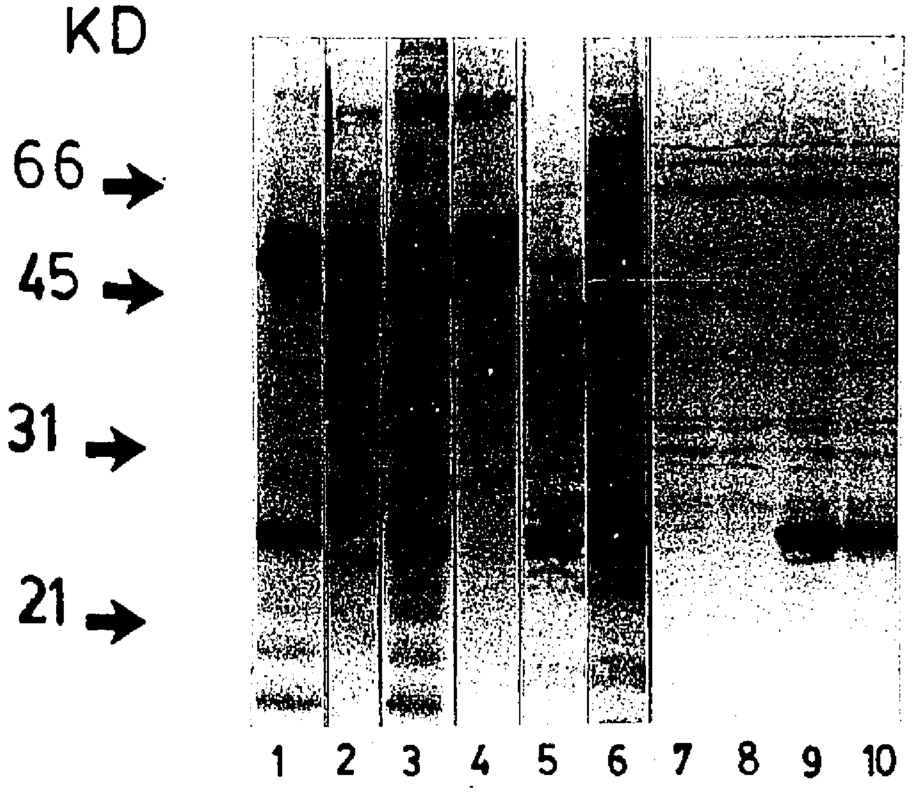

KD
45
31
21
1
2
3
4
5
6
7
8
9
10

Figure 14. Immunochemical characterization of HBV core proteins produced by insect cells infected with AcNPV-PCC. Three days after infection, medium, and cell lysate fractions were collected and prepared for electrophoresis as described in Methods. The samples were run on a 12.5% SDS-polyacrylamide gel along with pre-stained standard molecular weight protein markers, electrophoretically transfered to nitrocellulose membranes, and immunostained with rabbit anti-HBc/HBe antiserum all under conditions described in Methods. The pre-stained markers were used to follow the electrophoresis, to determine efficiency of protein transfer to nitrocellulose membranes and to directly calculate the molecular weight of the immunostained bands on the nitrocellulose membranes.

All lanes were immunostained with rabbit anti-HBc/HBe antiserum.

Samples in lanes 3, 4 and 6 were prepared in the absence of proteolytic enzyme inhibitors. Samples in lanes 1 to 4 containing cell fractions equal to 0.1 ml cell culture per lane were prepared by immunoprecipitation with rabbit anti-HBc/HBe antiserum. Samples in lanes 7 to 10 containing cell fractions equal to 0.3 ml cell culture per lane were prepared by immunoprecipitation ith rabbit (lanes 7 and 9) or goat (lanes 8 and 10) IgG containing anti-HBc antibodies immobilized on sepharose beads. Samples in lanes 5 and 6 containing cell fractions equal to 0.3 ml cell culture per lane were not immunoprecipitated prior to analysis.

AcNPV-PCC infected cell medium (lanes 1, 3, 9 and 10) and total cell lysate (lanes 5 and 6). Wild-type virus infected cell medium (lanes 2, 4, 7 and 8).


KD
66
45
31
21
1
2
3
4
5
6
7
8
9
10

in the negative control (lanes 2, 4) and is the result of binding of the enzyme conjugated anti- rabbit IgG to the heavy chain of the rabbit IgG molecules that were used in the immunoprecipitation of the core proteins. Immunostaining of the cellular proteins (cytoplasmic and membrane fractions) demonstrated the presence of only the 26 kd polypeptide (lanes 5 and 6) which appears to be the primary product that is expressed and secreted. The smaller polypeptides present in the medium may be processed products of the original protein. The presence (lanes 1 and 5) or absence (lanes 3 and 6) of proteolytic enzyme inhibitors did not change the polypeptide pattern. The calculated molecular weight of the precore-core protein is about 25 kd and the 26 kd polypeptide may represent that protein. Also the 17 kd polypeptide may correspond to the HBeAg whose polypeptide molecular weight has been determined to range from 15.5 to 19 kd (calculated M.W. of HBeAg polypeptides is 16.8 kd; Takahashi et al, 1983).

Two anti-HBs antisera, one rabbit and one goat antiserum, were found to contain anti-HBc but not anti-HBe antibodies using the CORAB and Abbott-HBe tests from Abbott. These anti-HBs antisera were commercially developed against HBsAg obtained from infectious human plasma that may have contained HBeAg or HBcAg. When these antisera were used to immunoprecipitate core proteins from the medium of AcNPV-PCC infected insect cells, only the 26 kd polypeptide but not the other smaller polypeptides was immunoprecipitated as detected by immunostaining with the rabbit anti-HBc/HBe antibodies (lanes 9 and 10).

5.Characterization of HBV-specific RNA transcripts and determination transcription initiation sites:

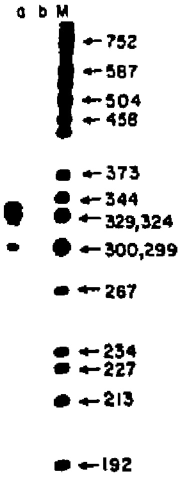
As mentioned above, one of the posibilities for the mixed expression of both the M and S envelope proteins upon infection of the insect cells with AcNPV-S2S was multiple mRNA transcription initiation sites upstream and downstream of the M protein ATG codon. To verify this posibility, S1 nuclease analysis of RNA from cells infected with AcNPV-S2S was performed to define the 5'end(s) of the HBV-specific transcripts. The 5'end radio-labeled (*) SalI-XbaI* DNA fragment from plasmid pAc-S2S which spans sequences from SalI (nucleotide 3180) site on pAc610 to the XbaI (nucleotide 249*) site on HBV DNA (fig. 15) and which includes the polyhedrin promoter/mRNA leader sequences, the polylinker region, and all the inserted preS2 and early S sequences was used as a probe. This probe was hybridized to RNA from cells inected with AcNPV-S2S or with AcNPV (as a control) and the hybrids were subjected to S1 nuclease digestion (fig. 15, lanes a and b). Two major and one minor digestion products were observed when AcNPV-S2S RNA was used. The sizes of these products indicated very clearly that the sites of initiation of transcription began with the polyhedrin mRNA leader sequences upstream of the inserted HBV DNA sequences.

6.Effect of co-expression of different HBV proteins in insect cells on their secretion and co-assembly into virus-related particles:

In a series of experiments, insect cells were infected with different combinations of recombinant baculoviruses to induce co-expression of different HBV proteins in these cells. Tables IV and V summarize the results of the effect of co-expression on the production and secretion of the HBV proteins.

It was noticed above that the expression of preS2 sequences has a

Figure 15. Characterization of the 5' ends of the HBV-specific RNA transcripts isolated from insect cells infected with AcNPV-S2S. Two days after infection, total cellular RNA was isolated, hyridized to a 5' end labeled (*) probe corresponding to a SalI-XbaI* DNA fragment (1129 nt) on pAcS2S which includes the polyhedrin promoter/mRNA leader sequences, all the preS2 region sequences and part of the S region sequences, digested with S1 nuclease and digestion-resistant hybrids were denatured and analyzed on a urea 6% polyacrylamide gel. Autoradiograph of DNA probe fragments protected with RNA from cells infected with AcNPV-S2S (lane a) or wild-type virus (lane b).


a b M
752
587
504
458
373
344
329,324
300,299
267
234
227
213
192

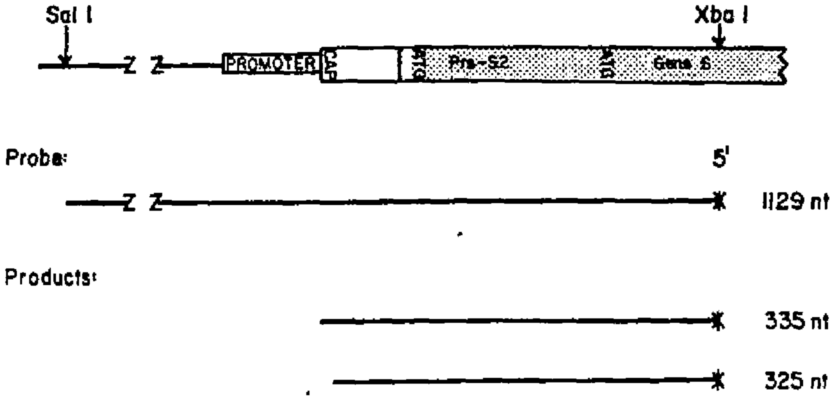

Sal I
Xba I
PROMOTER
CAP
ATG
Pre-S2
ATG
Gene S
Probe:
5'
1129 nt
Products:
335 nt
325 nt

TABLE IV. Kinetics of expression and secretion of HBsAg in insect cells <u>co-infected</u> with different recombinant baculoviruses.

Co-infection with AcNPV-.....	Amount of HBsAg in ug/ ml of cell culture (3 day)				Ratio total/ medium	Ratio medium/ cytoplasm
	medium	cytoplasm	membrane	total		
S.	0.200	0.105	0.661	0.966	4.8	1.9
S2S.	0.200	0.200	1.000	1.400	7.0	1.0
S1S2S.	-	-	0.320	0.320	-	-
S, S2S.	0.200	0.200	0.865	1.265	6.4	1.0
S, S1S2S.	0.027	0.015	0.627	0.669	24.8	1.8
S2S, S1S2S.	0.032	0.035	0.867	0.934	29.2	0.9
S, S2S, S1S2S.	0.040	0.040	0.853	0.933	23.3	1.0
S, PCC.	0.107	0.048	0.352	0.507	4.7	2.2
S2S, PCC.	0.107	0.093	0.533	0.733	6.8	1.1
S1S2S, PCC.	-	-	0.160	0.160	-	-
S, S2S, PCC.	0.127	0.106	0.560	0.793	6.2	1.2
S, S1S2S, PCC.	0.014	0.008	0.306	0.328	23.4	1.7
S2S, S1S2S, PCC.	0.022	0.020	0.560	0.602	27.4	1.1
S, S2S, S1S2S, PCC.	0.035	0.030	0.665	0.730	21.0	1.2

Table IV: Insect cells were co-infected with equal volumes of different recombinant baculoviruses stocks. Three days after co-infection, cell fractions were prepared and assayed for HBsAg using HBsAg radioimmunoassay kits (Connaught) as described in the Methods. Values for each fraction represent the amount of HBsAg in each fraction per one ml of cell culture suspension. These values were calculated according to the formula presented in the Methods and using a standard HBsAg positive control with known amount of HBsAg (20 ng/ml). The data represent the average values of duplicate determinations on samples from three different experiments.

TABLE V. Kinetics of expression and secretion of HBV core proteins in insect cells <u>co-infected</u> with different recombinant baculoviruses.

Co-infection with AcNPV-.....	Amount of HBV core proteins in U./ml of cell culture (three days)				Ratio total/ medium	Ratio medium/ cytoplasm
	medium	cytoplasm	membrane	total		
PCC.	5.000	0.556	5.556	11.112	2.2	8.9
S, PCC.	2.778	0.314	3.240	6.332	2.3	8.9
S2S, PCC.	2.780	0.319	3.242	6.341	2.3	8.8
S1S2S, PCC.	2.773	0.316	3.245	6.334	2.3	8.8
S, S2S, PCC.	2.037	0.232	2.223	4.492	2.2	8.8
S, S1S2S, PCC.	2.034	0.230	2.219	4.483	2.2	8.8
S2S, S1S2S, PCC.	2.035	0.235	2.227	4.497	2.2	8.7
S, S2S, S1S2S, PCC.	1.574	0.175	1.841	3.590	2.3	8.9

Table V: Insect cells were co-infected with equal volumes of recombinant baculoviruses stocks. Three days after co-infection, cell fractions were prepared and assayed for HBeAg and HBcAg using Abbott-HBe radioimmunoassay kits (Abbott) as described in the Methods. Values for each fraction represent the amount in units of HBeAg and HBcAg in each fraction per one ml of cell culture suspension. These values were calculated according to the formula presented in the Methods and using a standard HBeAg positive control with arbitrarily defined amount of HBeAg (one unit/ml). The data represent the average values of duplicate determinations on samples from three different experiments.

slight retardation effect on the secretion of HBV envelope proteins as compared to when only the S proteins are expressed. When both M and S proteins are co-expressed in insect cells, the kinetics of secretion approximated that of the M protein alone (table IV). When the L envelope protein was co-expressed with the M, S or both proteins, a dramatic decrease in the amount of secreted envelope proteins and accumulation of these proteins at the cell membrane level occured. The co-expression of HBV core proteins did not have any effect on the expression or secretion of the HBV envelope proteins as shown in table IV. Vice versa, the co-expression of ar all of the HBV envelope proteins did not affect the kinetics of expression or secretion of the HBV core proteins (table V).

In order to study whether the secreted HBV envelope proteins that resulted from different co-expression experiments were made of a mixture of these envelope proteins that were assembled together, aliquots of the secreted HBV envelope proteins were assayed for HBsAg with or without prior immunoprecipitation with anti-HBs, anti-preS2, or anti-preS1 antibodies. The results in table VI indicate very clearly that when different HBV envelope proteins were co-expressed in insect cells, not only were they all secreted, although to varying degrees, but they were also associated or assembled with each other. Although the L envelope protein was not detected in the medium of cells when expressed alone and inhibited the secretion of other envelope proteins, the little secreted envelope proteins in the last case contained some L protein. The preS1, preS2 and HBsAg antigenic epitopes were all present on the surface of the assembled and secreted HBV envelope proteins. It is also shown in table VI that co-expression of HBV core proteins did not result in any

TABLE VI. Co-assembly of different HBV envelope proteins and their association with HBV core proteins.

Co-infection with AcNPV-......	Percentage of HBsAg present in the medium after immuno-precipitation with...........					% of HBV core proteins in medium ar immunoprecipitation with...	
	-	anti-HBs	anti-preS2	anti-preS1	anti-HBc/e	-	anti-HBc/e
S.	100%	05%	97%	96%	97%	-	-
S2S.	100%	05%	04%	95%	96%	-	-
S, S2S.	100%	04%	06%	95%	95%	-	-
S, S1S2S.	100%	06%	07%	04%	97%	-	-
S2S, S1S2S.	100%	05%	04%	06%	96%	-	-
S, S2S, S1S2S.	100%	04%	03%	06%	96%	-	-
PCC	-	-	-	-	-	100%	03%
S, PCC.	100%	-	-	-	96%	100%	04%
S2S, PCC.	100%	-	-	-	95%	100%	04%
S, S2S, PCC.	100%	-	-	-	95%	100%	05%
S, S1S2S, PCC.	100%	-	-	-	93%	100%	04%
S2S, S1S2S, PCC.	100%	-	-	-	91%	100%	06%
S, S2S, S1S2S, PCC.	100%	-	-	-	90%	100%	05%

Table VI: Insect cells were co-infected with equal volumes of different recombinant baculovirus stocks. Three days after infection, cell culture medium was collected and assayed for HBsAg and HBeAg/HBcAg as described before. Immunoprecipitation was done as described in the Methods part on 1 ml aliqouts of the medium before the assays for HBV proteins. HBsAg or HBeAg/HBcAg values without prior immunoprecipitation were calculated as formulated before and were considered 100%.

significant association between the envelope and core proteins. But this did not exclude the possibility that core proteins may be present inside the assembled envelope protein particles with no HBeAg/HBcAg epitopes available on the surface.

To test for the last possibility, the secreted HBV envelope protein particles were immunoprecipitated to separate them from the co-expressed, but unassociated, core proteins present in the medium. But direct immunoprecipitation of the envelope proteins with anti-HBs antiserum was not possible because of the presence of anti-HBc antibodies in the anti-HBs antiserum as shown above. Therefore, the unassociated core proteins were first removed from the samples by immunoprecipitation with rabbit anti-HBc antibodies. This removal was monitored using the HBeAg/HBcAg radio-immunoassay. Then, the envelope proteins were immunoprecipitated with anti-HBs antibodies, run on a gel, western blotted, and immunostained with rabbit anti-HBc antibodies. Lanes 1 and 2 in figure (16) show the absence of any detectable core proteins associated with the envelope proteins when immunoprecipitated with goat or rabbit anti-HBs. Lanes 3 and 4 of the same figure represent positive controls and show the presence of the 26 kd core protein before its separation from the unassociated envelope proteins.

The secretion of the L envelope protein upon its co-expression with other envelope proteins was also characterized by gel electrophoresis, western blotting, and immunostaining with monoclonal anti-preS1 antibody. Figure (17) demonstrates the presence of 39 kd and 42 kd polypeptides in the medium of cells expressing the L protein along with the S protein (lane 3), the M protein (lane 4), or both the S and M proteins (lane 7).

Figure 16. Immunochemical characterization of HBV core proteins association with secreted HBV envelope protein particles upon their co-expression by insect cells co-infected with AcNPV-PCC, AcNPV-S, AcNPV-S2S, and AcNPV-S1S2S. Three days after co-infection, secreted HBV envelope proteins were immunoprecipitated with anti-HBs antiserum and prepared for electrophoresis as described in Methods. The samples were run on a 12.5% SDS-polyacrylamide gel along with pre-stained standard molecular weight protein markers, electrophoretically transfered to nitrocellulose membranes, and immunostained with rabbit anti-HBc/HBe antiserum all under conditions described in Methods. The pre-stained markers were used to follow the electrophoresis, to determine efficiency of protein transfer to nitrocellulose membranes and to directly calculate the molecular weight of the immunostained bands on the nitrocellulose membranes.

Immunoprecipitation with rabbit anti-HBs (lane 1) or prior to removal of free secreted core proteins (lane 3). Immunoprecipitation with goat anti-HBs (lane 2) or prior to removal of free secreted core proteins (lane 4).

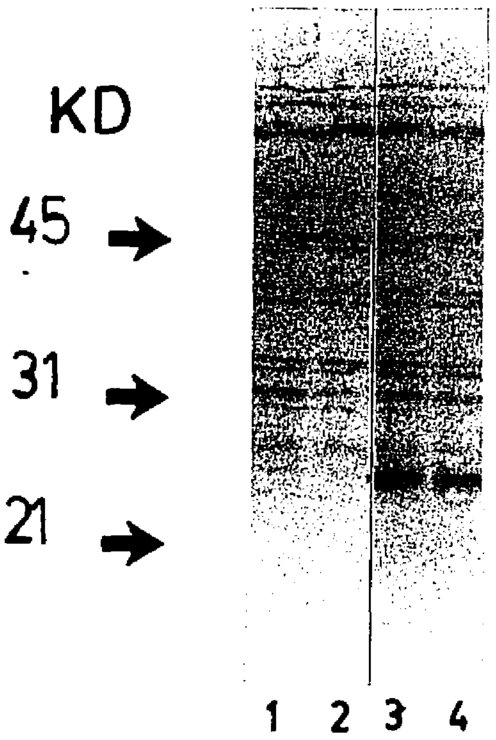
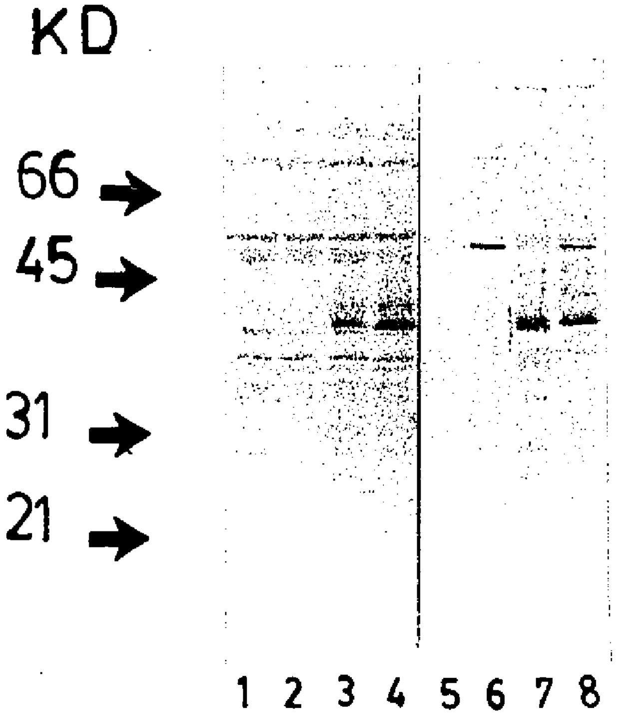

KD
45
31
21
1
2
3
4

Figure 17. Immunochemical characterization of HBV L envelope proteins secreted by insect cells infected with AcNPV-S1S2S (lanes 1 and 5), AcNPV-S2S (lane 2), AcNPV-S1S2S/ AcNPV-S2S (lane 3), AcNPV-S1S2S/ AcNPV-S (lane 4), or AcNPV-S1S2S/ AcNPV-S2S/ AcNPV-S (lane 7). The soluble cytoplasmic fraction from cells infected with AcNPV-S1S2S (lane 6) or from cells infected with AcNPV-S1S2S/ AcNPV-S2S/ AcNPV-S (lane 8) was also analysed for presence of HBV L protein. Three days after co-infection, secreted HBV envelope proteins were immunoprecipitated with anti-HBs antiserum and prepared for electrophoresis as described in Methods. The samples were run on a 12.5% SDS-polyacrylamide gel along with pre-stained standard molecular weight protein markers, electrophoretically transfered to nitrocellulose membranes, and immunostained with monoclonal anti-preS1 antibodies all under conditions described in Methods. The pre-stained markers were used to follow the electrophoresis, to determine efficiency of protein transfer to nitrocellulose membranes and to directly calculate the molecular weight of the immunostained bands on the nitrocellulose membranes.


KD
66
45
31
21
1
2
3
4
5
6
7
8

The 39 kd and 42 kd L polypeptides were also present in the cytoplasm of the same cells that were co-expressing all envelope proteins (lane 8). But it can be noticed that the amount of L proteins secreted was very little; probably presenting not more than 5% of total secreted envelope proteins.

The insect cell culture media from different co-infection experiments were further analyzed by cesium chloride isopycnic ultracentrifugation. There were no detectable difference in the mean density (1.22 g/ml) of the secreted envelope proteins regardless of their composition of different envelope proteins (fig. 9). The co-expression of HBV core proteins did not affect the density of the secreted envelope proteins, another indication of the absence of association between the HBV core and envelope proteins which if was present may have increased the density to that of the DNA-free Dane particles.

When the secreted HBV envelope proteins made of all three envelope proteins were examined by the electron microscopy, they were also shown to be in the form of empty spherical particles (fig. 18) but with a size larger (about 40 nm) than that of the HBV envelope protein particles that do not contain the L protein (about 20 to 25 nm).

Figure 18. Electron micrographs of negatively stained HBV envelope protein particles secreted by insect cells co-infected with AcNPV-S, AcNPV-S2S, and AcNPV-S1S2S. HBsAg positive CsCl fractions (from previous experiment) were dialyzed against 0.9% NaCl. The samples were processed for electron microscopy as described in the Methods part.

DISCUSSION

Studies on the expression and properties of individual hepatitis B virus proteins have been conducted extensively with a hope to better understand the pathobiology of the virus and provide new approaches to the diagnosis, treatment, and protection against this virus. These studies were critical particularly in the light that the virus could not be used to infect cultured cells in order to study its life cycle. Until now only two of the four encoded HBV proteins, the envelope and the core proteins have been characterized in great detail; thanks to the advances in molecular biology and recombinant DNA technology. Many of the properties of these proteins are now known, yet some aspects of their processing or interaction with each other particularly in the process of the virus assembly are not clear. The HBV assembly itself at the molecular level is still an unknown process. Several systems have been exploited to study the expression of HBV envelope or core proteins including prokaryotic cells, yeast cells, and different mammalian cells. The strategies followed in each system were as diverse as the systems themselves. Yet, the HBV envelope and core proteins proved to generally behave in a similar manner indicating that their properties and fate are determined by their own structure more than by the environment surrounding them. The baculovirus and insect cells system provides a new eukaryotic expression system not only to study properties of each of the HBV proteins but also to study under controled conditions the interaction of each of these proteins with the other proteins particularly in relation to the process of their assembly into virus-related particles.

This kind of approach may allow us in the future to understand the molecular events and factors involved in the production of HBV. The relatively high level of expression of different HBV proteins may also be utilized in the production of HBV-specific diagnostic or preventive reagents. One has to regard, however, that it might not be valid to extrapolate results on the behaviour of different HBV proteins in insect cells to that in the human cells. But the fact that the results of this work come in general agreement with other works provides an argumental ground for these results.

When different recombinant baculoviruses were generated, they were capable of infecting insect cells and inducing production of different HBV proteins in these cells. However, there were significant differences in the levels of production of these proteins. When different transfer plasmids were used in the generation of recombinant baculoviruses, different amounts of proteins were produced (Summers et al, 1986). It is not known whether the presence of extra DNA sequences or sequences from a polylinker region flanking inserted genes has an effect on the gene expression. Therefore, the strategy has been to insert the coding sequences of genes into the baculovirus with as few flanking sequences as possible. However, the structure or properties of the expressed protein may also contribute to the level of production of that protein. Expression of HBV L protein has been noticed to be generally low in comparable to other HBV envelope proteins in their natural host cells (Heermann et al, 1984) as well as in other heterologous expression systems (Cheng et al, 1986; Ou et al, 1987; Mclachlan et al, 1987) although its gene is put under the control of a strong promoter and the

ATG initiation codon of the protein is flanked with typical consensus sequences (Stibbe et al, 1983; Heermann et al, 1984). The reason for that is not known but the accumulation of the L protein at the cell membrane may down regulate its production.

All S, M and L proteins seem to be made in association with the cell membranes but only the S and M proteins are secreted to outside. Although both S and M proteins are secreted, yet the secretion of the M proteins is slightly retarded compared to that of the S protein. It has been shown that the HBV envelope protein is initially synthesized as a transmembrane polypeptide which is later transported to the endoplasmic reticulum lumen in a particulate form prior to its secretion (Eble et al, 1986).It also has been suggested that the preS1 and, to a much less degree, the preS2 inhibits the secretion of HBV envelope proteins (Standring et al, 1986). Our results clearly come in agreement with these studies. Secretion of HBV core proteins upon expression of the complete precore-core protein confirm the suggession that precore sequences allows the secretion of these proteins (Roosinck et al, 1986; Ou et al, 1986; Mclachlan et al, 1987) but does not prove it as an exclusive prerequisite for secretion. Comparison of the secretion efficiency of HBV core to envelope proteins demonstrates a significant difference with the core proteins being much more efficiently secreted. It has been noticed in other studies that the HBV envelope proteins are slowly secreted when generally compared with other secreted proteins (Eble et al, 1986) and this may be an intrinsic feature of these proteins. Immunochemical analysis of the HBV envelope proteins expressed in insect cells infected with AcNPV-S indicated the presence of the S protein with a molecular weight of 24 kd as the naturally existing protein. Although we did not have an antibody capable of detecting the glycosylated form of this protein, its presence can not be excluded based on the fact that the insect cells were able to support glycosylation of different foreign proteins and were particularly able, as shown in this work, to support glycosylation of the M and L envelope proteins.

Infection of the insect cells with AcNPV-S2S resulted in expression of the M envelope protein which was found secreted in the medium as a glycosylated 33 kd polypeptide and as both 33 kd glycosylated and 31 kd non-glycosylated polypeptides inside the cells. The glycosylation of the 33 kd M polypeptide was confirmed when it reacted with a monoclonal antibody that recognize only the naturally glycosylated form of this protein. M proteins larger than 33 kd were also detected particularly in the medium and may represent the the 36 kd M protein. It is noticed that previous studies describing expression of the M proteins showed the presence of the 33 kd or 32 kd polypeptides but not the 36 kd polypeptide (Mitchel et al, 1984; Mclachlan et al, 1987).

About 20% of the HBV envelope proteins produced by insect cells infected with AcNPV-S2S was S protein which was present in all cell fractions. This has been a consistent finding in all studies describing the expression of the M protein regardless the system used except that there has been a great variance in the percentage of M to S proteins from one study to another.

Transcriptional regulation mechanisms by which mRNA's may initiate upstream or downstream of the ATG translation initiation codon for the M protein and thus may determine translation of either the M or the S protein might play a role in the differential expression of these proteins in naturally HBV infected individuals (Cuttaneo et al, 1984; Standring et al, 1984; Rall et al, 1983; Heermann et al, 1984). The SV40 late promoter-like sequences that are located within the preS1 region and are probably responsible for the transcription of the M and S protein mRNA (Cattaneo et al, 1984) have been deleted from our AcNPV-S2S construct and all mRNA should initiated via the polyhedrin promoter. Yet it was important to determine whether multiple transcription initiation sites of the mRNA still play a role in this heterologous system. S1 nuclease analysis showed clearly that all HBV specific mRNA in insect cells infected with AcNPV-S2S initiated at the polyhedrin mRNA leader sequences upstream from the M protein first ATG codon. Presumably, shorter mRNA needed for the synthesis of the S protein were not made because the HBV mRNA start sites within the preS2 region could not be utilized by the baculovirus transcriptional apparatus.

Thus although transcriptional regulation mechanisms may have played a role in the expression of the M and S proteins in other systems, it is excluded in our system which leaves us with two other possible mechanisms. The first is a preferential translation initiation at an internal ATG codon. This mechanism has been suggested to play a role in the regulation of expression of some eukaryotic proteins (Liu et al, 1984). A scanning model has been proposed for ribosomal selection of initiator codons whereby not only proximity to the 5' terminus of the mRNA but also presence of so called consensus sequences flanking an ATG codon will determine its selection for initiation of translation (Kozak et al, 1983, 1984 and 1986). Indeed such consensus sequences were characterized to flank the S rather than the M protein ATG codon (Stibbe et al, 1983) and thus translational regulation mechanisms are suggested to contribute to the predominant expression of the S protein over the M protein not only in naturally HBV infected individuals but also in cultured cells expressing these proteins.

The other mechanism responsible for the mixed expression of the S and the M proteins could be a post-translational protein cleavage mechanism. It has long reported that the preS2 sequences are very sensitive to cleavage with proteolytic enzymes (Machida et al, 1983; Heermann et al, 1984). Complete conversion of the M protein expressed in the yeast cells into the S protein was reported to be affected by the extraction method and the presence of proteolytic enzyme inhibitors (Itoh et al, 1986a). Moreover, site specific mutation of the cleavage sit in the M protein into another non-sensitive site resulted in expression of only the M protein and prevented its conversion into S protein (Itoh et al, 1986b). When insect cell fractions from the AcNPV-S2S infection were prepared in the absence of proteolytic enzyme inhibitors, namely leupeptin which inhibits enzymes like trypsin, or in the presence of PMSF only, part of the M proteins in the medium, all of the M proteins in the cytoplasm, and most of the M protein in the membrane were converted into S protein. Polypeptides with sizes intermediate between the M and S proteins were also detected in the membrane fraction. The sizes of these polypeptides come in agreement with cleavage at sites that were mapped on the M protein as trypsin sensitive sites (Stibbe et al, 1983). These findings highly suggest that protein cleavage plays an important role in processing of the M protein into the S protein. This can be only confirmed by amino acid sequence analysis of the N-terminus of the S protein produced in conjunction with the M protein.

HBV L envelope proteins corresponding to the natural 39 kd and its glycosylated form 42 kd proteins were made in insect cells infected with AcNPV-S1S2S and were essentially associated with the cell membranes. This supports the suggession that properties of the HBV proteins are determined mainly by their own structure and that the insect cell system is capable of supporting studies on these proteins. The apparent glycosylation of the L protein indicates that the protein enter the early secretory pathway but is blocked at this stage. Myristilation of the L protein has been suggested to be a contributing factor to the secretory behaviour of the protein (Persing et al, 1987). The insect cell system has been shown to support sevaral processes of ptein modification from higher species. Our results suggest, although do not indicate, that the insect cells may also be capable of supporting one more kind of protein processing and that is the addition of myristic acid to proteins. Neither the M or the S proteins were detected indicating that internal mRNA translation initiation codons are not utilized in this case. The 25 kd polypeptide detected with both anti-preS1 and anti-preS2 antibodies but not with anti-S antibody is probably a cleaved L protein or a growing peptide.

The structural relationship between HBcAg particles and HBeAg proteins is well documented although not precisely defined. Although HBeAg is a marker for HBV replication in infected individuals, the expression and secretion of HBeAg has always dominated expression of HBcAg particles in cells carrying HBV DNA and expressing HBV proteins but not supporting viral replication (Christman et al, 1982; Zelent et al, 1987). The characterization of a precore coding DNA sequences has stimulated several studies on its effect on the expression of HBV core proteins (Roosinck et al, 1986; Ou et al, 1986; Mclachlan et al, 1987). These studies suggest that in the presence of precore sequences, core proteins are mainly expressed as HBeAg which is effeciently secreted to outside of the cells while in the absence of these precore sequences, the core proteins are essentially expressed as intracellular HBcAg particles. In the insect cells, the inclusion of precore sequences, also resulted in expression and efficient secretion of HBV core proteins. It seems that in contrast to the effect of the preS1 sequences on the secretion of the envelope proteins, the precore sequences act as a signal peptide which direct the core protein to the cell membranes in its way to be secreted.

Based on morphological criteria, no HBcAg particle formation was detected by electron microscopy. We did not, however, have an immunoassay test to evaluate how much HBeAg versus HBcAg was expressed by the insect cells. It has been shown that HBcAg as well as HBeAg epitopes are carried on the core proteins but the HBeAg epitopes become cryptically masked in the core particle structure. A series of elegant studies demonstrated that HBcAg epitopes are also present on the HBeAg proteins but may be cryptically masked on some forms of the protein in turn (Takahashi et al, 1981 and 1983). Carefull evaluation of different immunoassay designed to measure HBcAg (Roosinck et al, 1986; Ou et al, 1986; Mclachlan et al, 1987) indicates that the core particle structure rather than presence of HBcAg epitopes is the critical factor in some but not all these HBcAg immunoassays. Therefore, it seems that although the conventional usage of

HBcAg and HBeAg has its practical advantages, yet these designations can not be used to make physicochemical distinctions of the core proteins.

Immunochemical analysis of the HBV core proteins expressed in insect cells demonstrated that a 26 kd polypeptide corresponding to the complete precore-core protein is present inside the cells. The presence of two major polypeptide bands of 26 kd and 17 kd as well as two minor bands of 22 kd and 19 kd indicate first that the precore sequences, if acting as a signal peptide, are not cleaved in the process of secretion of the core proteins and second that further cleavage takes place probably in the process of expression of HBeAg. Cleavage of the precore sequences would result in conversion of the 26 kd polypeptide into a core polypeptide whose calculated molecular weight is 21.1 kd (Pasek et al, 1979), and disruption of of the core particle proteins has been shown to result in its conversion into a 19 kd and/or a 15.5/16.5 (Takahashi et al, 1980) HBeAg. It has been shown that the 15.5 kd HBeAg polypeptide (calculated molecular weight 16.8 kd) is the result of the removal of about 34 amino acids from the C-terminus of the complete core polypeptide (Takahashi et al, 1983). Although the sizes of the HBV core or HBeAg proteins present in the medium of the insect cell cultures are in general agreement with the previous sequence of events, yet there is no direct evidence that they correlate with each other. Little information is available from other studies to support or contradict our results. Only one study (Mclachlan et al, 1987) has shown the secretion in the medium of one major polypeptide corresponding to our 17 kd band and two minor ones corresponding to our 22 kd and 19 kd bands. In the same study, although expression of the complete precore-core protein was presumed, it was not detected neither in the medium or inside the cells. Therefore, it not clear whether the secretion of the 26 kd polypeptide observed in the insect cells is an authentic behaviour of the precore-core proteins, an artifact of the insect cells, or due to some leakage of the cellular proteins. But it will be interesting to study possible expression and secretion of the precore sequences and possible immune response during the natural course of HBV infection.

Immunoprecipitation of the secreted core proteins with antiserum containing anti-HBc but not anti-HBe antibodies resulted in immunoprecipitation of only the 26 kd polypeptide indicating that cleavage of the 26 kd into the 22 kd, 19 kd and 17 kd polypeptides results in masking or losing of the HBcAg epitopes on the cleaved polypeptides. HBcAg and probably HBeAg epitopes are expressed on the 26 kd polypeptide. Processing or cleavage of the secreted core proteins occured and was not affected by presence or absence of proteolytic enzyme inhibitors during the preparation of the samples. This indicates that such cleavage is an authentic property of the proteins rather than an artifact of the system (although this can not be completely excluded). It is not clear, however, whether this cleavage is a self-cleavage process as has been recently suggested (Miller, 1987).

Co-expression of the HBV L envelope protein along with other M or S envelope proteins resulted in dramatic inhibition of assembly and secretion of HBsAg particles which comes in support of other recent studies and further indicates the appropriateness of the insect cell system to study properties and interactions between different HBV proteins. The precise nature and site of action of the inhibitory influence of the L proteins are not clear. But it seems from recent studies that the addition of myristic acid to the L protein (Persing et al, 1987) could be important in anchoring the protein in a transmembrane configuration (thereby preventing its spontaneous secretion) and in orienting the preS1 domains to allow interactions with other HBV viral components.

Immunoprecipitation of the little amount of HBV envelope proteins secreted as a result of their co-expression, with anti-preS1, anti-preS2 or anti-HBs provides an experimental proof for the ability of the L protein to specifically recognize and interact with other HBV envelope proteins (probably via identical domains present on these proteins) leading first to significant inhibition of their assembly and secretion as HBsAg particles and second to some degree of co-assembly of all envelope proteins expressed into some particulate structure with the antigenic epitopes of the preS1, preS2 and HBsAg all expressed on the surface of the particles similar to the HBV particles. It is suggested that the fact that the L protein inhibits the formation of HBsAg particles and is preferentially associated with the HBV rather than HBsAg particles, may reflect a specific role in the assembly and morphogenesis of other structures like filamentous particles or the HBV particles. The electron microscopy studies showed that the unusual HBsAg particles made of all three envelope proteins upon their co-expression were larger than the common 22 nm particles approximating in size the 42 nm HBV. This suggests that the presence of the L protein plays a role in determining the size of the HBV particles. Previous studies, although suggested formation of particulate structures of envelope proteins larger than the

HBsAg particles in the presence of the L protein (Ou et al, 1987; Mclachlan et al, 1987), yet did not detect or characterize these structures. Our results, therefore, remain to be reproduced in other systems to allow drawing any conclusions. The presence of L proteins in the particle structure did not, however, affect the density of these particles.

The surface proteins of most enveloped animal viruses do not undergo spontaneous budding but the initiation of budding is triggered by the interaction with nucleocapsid components. It has been suggested that expression of precore-core and or core proteins may play a role in assembly and or secretion of HBV by interaction with the envelope proteins (Ou et al, 1986). The baculovirus-insect cell system allowed us to conduct for the first time a series of experiments to study the interaction between different HBV envelope and core proteins.

Co-expression of the HBV precore-core proteins along with different envelope proteins did not affect the kinetics of expression or secretion of either group. Detailed immunoassay, immunoprecipitation, physiochemical, morphological and more important immunochemical analysis of the assembled and secreted particles produced in these experiments indicated, however, that there was no interaction or association between the HBV envelope and core proteins. There could be two explanations for this lack of interaction. First, the insect cell system may be different from the natural environment required for this interaction. The second explanation which is more likely (based on the demonstration of the suitability of the insect cell system to support authentic expression and behaviour of different HBV proteins), is that other factors, like expression of core proteins without the precore core sequences, presence of HBV DNA, or other HBV components, may be essential for the formation of core-containing particles or HBV particles. Further experiments utilizing the insect cell expression system can allow us to test many of these factors and delineate the molecular requirements and events associated with the assembly and secretion of the HBV, an essential but yet unknown aspect of the pathobiology of the HBV.

REFRENCES

Alberti, A., S. Diana, G. Sculard, and A. Eddlestone. 1978. Detection of a new antibody system reacting with Dane particles in hepatitis B virus infection. British Medical Journal 2:1056.

Alberti, A., P. Pontisso, E. Schiavon, and G. Realdi. 1984. An antibody which precipitates Dane particles in acute hepatitis type B: relation to receptor sites which bind polymerized human serum albumin on virus particles. Hepatology 4:220.

Albin, C., and W. Robinson. 1980. Protein kinase activity in hepatitis B virus. J Virol 34:297.

Almeida, J., D. Rubenstein, and E. Scott. 1971. New antigen-antibody system in Australia-antigen-positive hepatitis. Lancet 2:1225.

Angelo, C., G. Smith, M. Summers, and R. Krug. 1987. Two of the three influenza viral proteins expressed by using baculovirus vectors form a complex in insect cells. J Virol 61:361.

Barker, L., J. Almeida, J. Hoofnagle, R. Gerety, D. Jackson, and P. Mcgrath. 1974. Hepatitis B core antigen: immunology and electron microscopy. J Virol 14:1552.

Berk, A., and P. Sharp. 1977. Sizing and mapping of early adenovirus mRNA by gel electrophoresis of S1 endonuclease-digested hybrids. Cell 12:721.

Budkowska, A., B. Kalinowska, and A. Nowoslawski. 1979. Identification of two HBeAg subspecificities revealed by chemical treatment and enzymatic digesion of liver derived HBcAg. J Immunol 123:1415.

Carbonell, L., M. Klowden, and L. Miller. 1985. Baculovirus-mediated expression of bacterial genes in dipteran and mammalian cells. J Virol 56:153.

Carstens, E., S. Tjia, and W. Doerfler. 1979. Infection of Spodoptera frugiperda cells with Autographa californica polyhedrosis virus. I. Synthesis of intracellular proteins after virus infection. Virology 99:386.

Cattaneo, R., H. Will, N. Henanandez, and H. Schaller. 1983. Signals regulating hepatitis B surface antigen transcription. Nature 305:338.

Cattaneo, R., H. Will, and H. Schaller. 1984. Hepatitis B virus transcription in the infected liver. EMBO J 3:2191.

Charnay, P., M. Gervais, A. Louise, F. Galibert, and P. Tiollais. 1980.

Biosynthesis of hepatitis B virus surface antigen in Escherichia coli. Nature 286:893.

Cheng, K., G. Smith, and B. Moss. 1986. Hepatitis B virus large surface protein is not secreted but is immunogenic when selectively expressed by recombinant vaccinia virus. J Virol 60:337.

Chirgwin, J., A. Przybyla, R. MacDonald, and W. Rutter. 1974. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294.

Chisari, F., P. Filippi, A. McLachlan, D. Milich, M. Riggs, S. Lee, R. Palmiter, C. Pinkert, and R. Brinster. 1986. Expression of hepatitis B virus large envelope polypeptide inhibits hepatitis B surface antigen secretion in transgenic mice. J Virol 60:880.

Christman, J.K., M. Gerber, P. Price, C. Flordellis, J. Edelman, and G. Acs. 1982. Amplification of expression of hepatitis B surface antigen in 3T3 cells cotransfected with a dominant-acting gene and cloned viral DNA. PNAS 79:1815.

Dane, D. C. Cameron, and M. Briggs. 1970. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. lancet 1:695.

Davis, A., B. Kostek, B. Mason, C. Hsiao, J. Morin, S. Dheer, and P. Hung. 1985. Expression of hepatitis B surface antigen with a recombinant adenovirus. PNAS 82:7560.

Delacroix, D., and J. Vaerman. 1979. Simple purification of goat IgG1 and IgG2 subclasses by chromatography on protein A-sepharose at various pH. Mol Immunol 16:837.

Delius, H., N. Gough, C. Cameron, et al. 1983. Structure of the hepatitis B virus genome. J Virol 47:337.

Detjen, B., C. Angelo, M. Katze, and R. Krug. 1987. The three influenza virus polymerase (P) protein not associated with viral nucleocapsids in the infected cell are in the form of a complex. J Virol 61:16.

Eble, B., V. Lingappa, and D. Ganem. 1986. Hepatitis B surface antigen: an unusal secreted protein initially synthesized as a transmembrane polypeptide. Mol Cell Biol 6:1454.

Edman, J., R. Hallewell, P. Valenzuela, H. Goodman, and W. Rutter. 1981. Synthesis of hepatitis B surface and core antigens in E.coli. Nature 291:503.

Fujisawa, Y., Y. Ito, R. Sasada, Y. Ono, K. Igarashi, R. Marumoto, M. Kikuchi, and Y. Sugino. 1983. Direct expression of hepatitis B surface antigen gene in E.coli. Nucl Acids Res 11:3581.

Galibert, F., E. Mandart, F. Fitoussi, P. Tiollais, and P. Charnay. 1979. Nucleotide sequence of the hepatitis B virus genome (subtype awy) cloned in E.coli. Nature 281:646.

Gerlich, W., and W. Robinson. 1980. Hepatitis B virus contains protein attached to the 5' terminus of its complete DNA strand. Cell 21:801.

Gerlich, W., U. Goldmann, R. Muller, W. Stibbe, and W. Wolff. 1982. Specificity and localization of the hepatitis B virus associated protein kinase. J Virol 42:761.

Gough, N. 1983. Core and e antigen synthesis in rodent cells transformed with hepatitis B virus DNA is associated with greater than genome length viral messenger RNAs. J Mol Biol 165:683.

Granados, R. 1980. INfectivity and mode of action of baculoviruses. Biotech Bioeng 22:1377.

Heermann, K., U. Goldmann, W. Schwartz, T. Seyffarth, H. Baumgarten, and W. Gerlich. 1984. Large surface proteins of hepatitis B virus containing the pre-S sequence. J Virol 52:396.

Hink, W.F. 1970. Established insect cell line from the cabbage looper, Trichoplusia ni. Nature 226:466.

Itoh, Y., T. Hayakawa, and Y. Fujisawa. 1986a. Expression of hepatitis B virus surface antigen P31 gene in yeast. Biochem Biophys Res Commun 138:268.

Itoh, Y., and Y. Fujisawa. 1986b. Synthesis in yeast of hepatitis B surface antigen modified P31 particles by gene modification. Biochem Biophys Res Commun 141:942.

Kafatos, F.C., C.W. Jones, and A. Efstratiadis. 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucl Acids Res 7:1541.

Kelly, D. 1982. Baculovirus replication. J Gen Virol 63:1.

Klinkert, M., L. Theilmann, E. Pfaff, and H. Schaller. 1986. Pre-S1 antigens and antibodies early in the course of acute hepatitis B virus infection. J Virol 58:522.

Kozak, M. 1983. Comparison of initiation of protein synthesis in procaryotes, eucaryotes and organelles. Microbiol Rev 47:1.

Kozak, M. 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucl Acids Res 12: 857.

Kozak, M. 1986. Bifunctional messenger RNAs in eukaryotes. Cell 47:481.

Kuroda, K., C. Hauser, R. Rott, H. Klenk, and W. Doerfler. 1986. Expression of the influenza virus hemagglutinin in insect cells by a baculovirus vector. EMBO J
5:1359.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680.

Laub, O., L. Rall, M. Truett, et al. 1983. Synthesis of hepatitis Bsurface antigen in mammalian cells: expression of the entire gene and the coding region. J Virol 48:271.

Liu, C., C. Simonsen, and A. Levinson. 1984. Initiation of translation at internal AUG codons in mammalian cells. Nature 309:82.

Mackay, P., S. Lee, and K. Murray. 1981. The conversion of hepatitis B core antigen synthesized in E.coli into e antigen. J Med Virol 8:237.

Magnius, L., and J. Espmark. 1972. New specifities in Australia antigen positive sera distinct from Le Bouvier determinants. J Immunol 109:1017.

Machida, A., S. Kishimoto, H. Ohnuma, H. Miyamoto, K. Baba, K. Oda, T. Nakamura, Y. Miyakawa, and M. Mayumi. 1983. A hepatitis B surface antigen polypeptide (P31) with the receptor for polymerized human as well as chimpanzee albumin. Gastroenterology 85:268.

Maniatis, T., E. Fritsch, and J. Sambrook. 1982. Manual of Molecular cloning, Cold Spring Harbor Press, Cold Spring Harbor,N.Y.

Marion, P., S. Oshirol, D. Reghery, G. Scullard, and W. Robinson. 1980. A virus in beechey ground squirrels which is related to hepatitis B virus in humans. PNAS 77:2941.

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.

Maxam, A., and W. Gilbert. 1980. Sequencing end-labeled DNA with base specific chemical cleavages. Methods in Enzymology 65:499.

Mclachlan, A., D. Milich, A. Raney, M. Riggs, J. Hughes, J. Sorge, and F. Chisari. 1987. Expression of hepatitis B virus surface and core antigens: Influence of pre-S and precore sequences. J Virol 61:683.

Messing, J. 1983. New M13 vectors for cloning. Methods in Enzymology 101:20.

Michel, M., P. Pontisso, Sobczak, Y. Malpiece, R. Streeck, and P. Tiollais. 1984. Synthesis in animal cells of hepatitis B surface antigen particles carrying a receptor for polymerized human serum albumin. PNAS 81:7708.

Miyanohara, A., T. Imamura, M. Araki, K. Sugawara, N. Ohtomo, and K. Matsubara. 1986. Expression of hepatitis B virus core antigen gene in Saccharomyces cerevisiae: synthesis of two polypeptides translated from different initiation codons. J Virol 59:176.

Miller, R. 1987. Proteolytic self-cleavage of hepatitis B virus core protein may generate serum e antigen. Science 236:722.

Millich D., G. Thornton, A.R. Neurath, S. Kent, M. Michel, P. Tiollais, and F. Chisari. 1985. Enhanced immunogenicity of the pre-S region of hepatitis B surface antigen. Science 228:1195.

Mishiro, S., et al. 1980. A 49,000 dalton polypeptide bearing all antigenic determinants and full immunogenicity of 22 nm hepatitis B surface antigen particles. J Immunol 124:1589.

Moriarty, A.M., H. Alexander, R.A. Lerner, and G.B. Thorton. 1985. Antibodies to peptides detect new hepatitis B antigen: serological correlation with hepatocellular carcinoma. Science 227:429.

Nakano, M., T. Tobita, and T. Ando. 1976. Int J Pept Protein Res 8:565.

Neurath, A.R., and N. Strick. 1979. J Gen Virol 42:645.

Neurath, A.R., S. Kent, and N. Strick. 1982. Specificity of antibodies elicited by a synthetic peptide having a sequence in common with a fragment of a virus protein, the hepatitis B surface antigen. PNAS 79:7871.

Neurath, A.R., S. Kent, and N. Strick. 1984. Location and chemical synthesis of a pre-S gene coded immunodominant epitope of hepatits B virus. Science 224:392.

Neurath, A.R., S. Kent, N. Strick, P. Taylor, and C. Stevens. 1985. Hepatitis B virus contains pre-S gene-encoded domains. Nature 315:154.

Neurath, A.R., S. Kent, N. Strick, and K. Parkeer. 1986. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell 46:429.

Ou, J., and W. Rutter. 1985. Hybrid hepatitis B virus-host transcripts in human hepatoma cell. PNAS 82:83.

Ou, H., O. Laub, and W. Rutter. 1986. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. PNAS 83:1578.

Ou, J-H., and W.J. Rutter. 1987. Rgulation of secretion of the hepatitis B virus major surface antigen by the pre-S1 protein. J Virol 61:782.

Pasek, M., T. Goto, W. Gilbert, B. Zink, H. Schaller, P. Mackay, G.

Leadbetter, and K. Murray. 1979. Hepatitis B virus genes and their expression in E.coli. Nature 282:575.

Persing, D., H. Varmus, and D. Ganem. 1986. Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. Science 234:1388.

Persing, D., H. Varmus, and D. Ganem. 1987. The pre-S1 protein of hepatitis B virus is acylated at its amino terminus with myristic acid. J Virol 61:1672.

Peterson, D., I. Roberts, and G. Vyas. 1977. Partial amino acid sequence of two major component polypeptides of hepatitis B surface antigen. PNAS 74:1530.

Pourcel, C., et al. 1982. Transcription of the hepatitis B surface antigen gene in mouse cells transformed with cloned viral DNA. J Virol 42:100.

Price, P., S. Ostrove, C. Flordellis, M. Sells, S. Thung, M. Gerber, J. Christman, and G. Acs. 1983. Characterization of RNA transcripts and virally coded proteins synthesized in mouse fibrobasts transfected with hepatitis B DNA. Bioscience Reports 3:1017.

Rall, L., D. Standring, O. Laub, et al. 1983. Transcription of hepatitis B virus by RNA polymerase II. Mol Cell Biol 3:1766.

Rigby, P., M. Dieckman, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113:237.

Robinson, W. 1975. DNA and DNA polymerase in the core of Dane particle of hepatitis B. Am J Med Sci 270:151.

Robinson, W., and L. Lutwick. 1976. The virus of hepatitis, type B. New Engl J Med 295:1168 and :1232.

Robinson, W.S., P. Marion, and M. Feitelson. 1982. The hepadna virus group: hepatitis B and related viruses. In W. Szmuness, J. Alter and J. Maynard (ed.), Viral Hepatitis: 1981 International Symposium. Franklin Institute Press, Philadelphia.

Roossinck, M., S. Jameel, S. Loukin, and A. Siddiqui. 1986. Expression of hepatitis B core region in mammalian cells. Mol Cell Biol 6:1393.

Schulz, A., L. Henderson, S. Oroszlan, E. Garber, and H. Hanafusa. 1985. Amino terminal myristylation of the protein kinase pp60 src, a retroviral transforming protein. Science 227:427.

Smith, G., J. Vlak, and M. Summers. 1982. In vitro translation of Autographa californica nuclear polyhedrosis virus early and late mRNAs. J

Virol 44:199.

Smith, G., M. Mackaett, and B. Moss. 1983. Infectious vaccinia virus recombinants that express hepatitis B virus surface antigen. Nature 302:490.

Smith, G., J. Vlak, and M. Summers. 1983a. Physical analysis of Autographa californica nuclear polyhedrosis virus transcripts for polyhedrin and 10.000 molecular weight protein. J Virol 45:215.

Smith, G., M. Fraser, and M. Summers. 1983b. Molecular engineering of Autographa californica nuclear polyhedrosis virus genome: deletion mutations within the polyhedrin gene. J Virol 46:584.

Smith, G., M. Summers, and M. Fraser. 1983c. Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol Cell Biol 3:2156.

Smith, G., G. Ju, B. Ericson, J. Moschera, H. Lahm, R. Chizzonite, and M. Summers. 1985. Modification and secretion of human interleukin 2 produced in insect cells by a baculovirus expression vector. PNAS 82:8404.

Standring, D., W. Rutter, H. Varmus, and D. Ganem. 1984. Transcription of the hepatitis B surface antigen gene in cultured murine cells initiates within pre-surface region. J Virol 50:563.

Standring, D., J-H. Ou, and W.J. Rutter. 1986. Assembly of viral particles in Xenopus oocytes: presurface antigens regulate secretion of the hepatitis B viral surface envelope particle. PNAS 83:9338.

Stibbe, W., and W. Gerlich. 1982. Variable protein composition of hepatitis B surface antigen from different donors. Virology 123:436.

Stibbe, W., and W. Gerlich. 1983. Structural relationship between minor and major proteins of hepatitis B surface antigen. J Virol 46:626.

Summers, J., J. Smolec, and R. Snyder. 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. PNAS 75:4533.

Summers, J., and W. Mason. 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermeiate. Cell 29:403.

Summers, M., and G. Smith. 1986. Manual of methods for baculovirus vectors and Insect cell culture procedures.

Takahashi, K., M. Imai, Y. Miyakawa, S. Iwakiri, and M. Mayumi. 1978. Duality of hepatitis B e antigen in serum of persons infected with hepatitis B virus. PNAS 75:1952.

Takahashi, K., Y. Akahane, T. Gotanda, T. Miashiro, M. Imai, Y. Miyakawa, and M. Mayumi. 1979. Demonstration of hepatitis B e antigen in the core of Dane particles. J Immunol 122:275.

Takahashi, K., M. Imai, T. Gotanada, T. Sano, A. Oinuma, S. Mishiro, Y. Miyakawa, and M. Mayumi. 1980. Hepatitis B e antigen polypeptides isolated from sera of individuals infected with hepatitis B virus: Comparison with HBeAg polypeptide derived from Dane particles. J Gen Virol 50:49.

Takahashi, K., M. Imai, M. Nomura, A. Oinuma, A. Machida, G. Funatsu, Y. Miyakawa, and M. Mayumi. 1981. Demonstration of the immunogenicity of hepatitis B core antigen in a hepatitis B e antigen polypeptide (P19). J Gen Virol 57:325.

Takahashi, K., A. Machida, G. Funatsu, M. Nomura, S. Usuda, S. Aoyagi, K. Tachibana, H. Miyamoto, M. Imai, T. Nakamura, Y. Miyakawa, and M. Mayumi. 1983. Immunochemical structure of hepatitis B e antigen in the serum. J Immunol 130:2903.

Tedder, R., and F. Bull. 1979. Characterization of e antigen associated with hepatitis B. Clin Exp Immunol 35:380.

Tiollais, P., C. Pourcel, and A. Dejaean. 1985. The hepatitis B virus. Nature 317:489.

Tiollais, P., P. Charnay, and G. Vyas. 1981. Biology of hepatitis B virus. Science 213:406.

Tjia, S., E. Carstens, and W. Doerfler. 1979. Infection of Spodoptera frugiperda cells with Autographa californica nuclear polyhedrosis virus: II. The viral DNA and the kinetics of its replication. Virology 99:399.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. PNAS 76:4350.

Valenzuela, P., M. Quiroga, J. Zaldivar, P. Gray, and W. Rutter. 1980. The nucleotide sequence of the hepatitis B viral genome and the identification of major viral genes. Page 57, in B. Fields et al (ed.), Animal virus genetics. Academic Press, New York.

Valenzuela, P., A. Medina, and W. Rutter. 1982. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature 298:347.

Valenzuela, P., D. Coit, and C. Kuo. 1985. Synthesis and assembly in yeast of hepatitis B surface antigen particles containing the polyalbumin receptor. BioTechnology 3:317.

Van der Beek, C., J. Saaijer Riep, and J. Vlak. 1980. On the origin of the polyhedral protien of Autographa californica nuclear polyhedrosis virus. Virology 100:326.

Zelent, A., M. Sells, P. Price, A. Mohamad, G. Acs, and J. Christman. 1987. Murine cells carrying integrated tandem genomes of hepatitis B virus DNA transcribe RNAs from endogenous promoters on both viral strands and express middle and major viral envelope proteins. J Virol 61:1108.