Secretion of Particles of Hepatitis B Surface Antigen from Insect Cells Using a Baculovirus Vector

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SUMMARY

The coding sequences of the hepatitis B virus surface antigen were inserted into a baculovirus transfer vector produced from Autographa californica nuclear polyhedrosis virus (AcNPV) so that the foreign gene was under the control of the AcNPV polyhedrin promoter. Spodoptera frugiperda cells infected with the derived recombinant baculovirus produced and secreted 22 nm particles containing the hepatitis B surface antigen. The particles had morphological and antigenic properties identical to those of 22 nm particles isolated from the plasma of chronic active hepatitis patients.

INTRODUCTION

An important area in recombinant DNA technology is the development of expression systems which produce and secrete large quantities of the desired gene products with the appropriate post-translational modifications. Both prokaryotic and eukaryotic host/vector systems have been utilized. Recently, Autographa californica nuclear polyhedrosis virus (AcNPV) and other baculoviruses have been shown to be suitable helper-independent viral expression vectors for the efficient production of foreign proteins in cultured insect cells. This system has been used for the production of human $\beta$ interferon (Smith et al., 1983), $\beta$-galactosidase (Pennock et al., 1984), human interleukin-2 (Smith et al., 1985), human c-myc protein (Miyamoto et al., 1985), human $\alpha$ interferon (Maeda et al., 1985), influenza virus haemagglutinin (Possee, 1986; Kuroda et al., 1986), the S RNA-coded genes of lymphocytic choriomeningitis arenavirus (Matsuura et al., 1986) and Punta Toro phlebovirus (Overton et al., 1987) as well as cDNA clones of bluetongue virus (Inumaru & Roy, 1987). For the interferon gene expression, it was shown that the products made by the recombinant baculoviruses were glycosylated by an N-linked glycan, the pre-interferon signal peptide was removed and the protein was efficiently secreted (Smith et al., 1983). It has also been shown that human interleukin-2 was secreted and that the extracellular product stimulated the growth of interleukin-2-dependent cell lines (Smith et al., 1985). In this communication, we report the production of human hepatitis B virus (HBV) surface antigen (HBsAg) in insect cells using AcNPV as an expression vector. Spodoptera frugiperda cells infected with recombinant AcNPV containing the HBV gene expressed and secreted large quantities of HBsAg.

METHODS

Viruses and cells. AcNPV and recombinant virus stocks were grown in suspension cultures of S. frugiperda cells in TC 100 medium containing 10% foetal bovine serum (Possee, 1986; Matsuura et al., 1986).

Construction of pAcRP6-HBsYK14. A pUC8-based plasmid (pUC8-HBs) containing the entire HBsAg coding region of the adw strain of HBV was constructed from the 1400 bp BamHI fragment of HBV DNA (Fig. 1; Ono et al., 1983). The fragment was isolated from the recombinant plasmid and digested with AcI. The HBsAg gene,
having BamHI and AccI sticky ends, was then ligated to a synthetic oligonucleotide containing an AccI-compatible end sequence, the translation termination signal of the HBsAg gene, a PstI site, a BamHI site and a HindIII-compatible end sequence (see Fig. 1). The derived DNA was inserted between the BamHI and HindIII sites of pUC8 to give the plasmid pHBsYK2 (Fig. 1). pHBsYK2 plasmid DNA was then digested with BamHI and the 828 bp BamHI fragment containing the entire coding region of HBsAg was isolated from an agarose gel. This BamHI fragment was inserted into the BamHI site of the AcNPV transfer vector, pAcRP6 (Possee, 1986; Matsuura et al., 1986). All the junction DNA sites were sequenced using the method of Maxam & Gilbert (1980).

Isolation of recombinant viruses. S. frugiperda cells were transfected with a mixture of wild-type AcNPV DNA and pAcRP6-HBsYK14 recombinant plasmid DNA by procedures described previously (Possee, 1986; Matsuura et al., 1986). After 4 days of incubation at 28 °C, the supernatant fluids were harvested and virus was titrated in confluent monolayers of S. frugiperda cells. Plaques showing no occlusion bodies were picked and titrated to obtain recombinant, polyhedrin-negative viruses. Following a third plaque picking, stocks of the recombinant viruses were obtained (10^7 to 10^8 p.f.u./ml).

Southern blot analyses. A large quantity of the AcNPV-HBsYK14 recombinant virus was prepared from spinner cultures of S. frugiperda cells and the viral DNA extracted. DNAs from AcNPV-HBsYK14 and wild-type AcNPV were resolved by electrophoresis on a 1 % agarose gel and probed with nick-translated HBsAg-specific DNA.

Northern blot analyses. Total cellular RNA was extracted from S. frugiperda cells infected with either the recombinant AcNPV-HBsYK14 or wild-type AcNPV. The total cellular RNA was denatured with glyoxal and
resolved by electrophoresis on a 1.5% agarose gel. The RNA was electroblotted onto Genescreen (New England Nuclear), the filter was baked and the blots were cut into 5 mm wide longitudinal strips and hybridized with the nick-translated BamHI fragment of pHBsYK2 DNA (see Fig. 1), or with nick-translated pAcRP6 DNA.

Radioimmunoassay of HBsAg secreted from *S. frugiperda* cells. *S. frugiperda* cells were infected with either the recombinant AcNPV-HBsYK14 or wild-type AcNPV, at a m.o.i. of 3 p.f.u./cell. After 1 h of adsorption, cells were resuspended in TC 100 culture media to give a cell density of 2 × 10^6 cells/ml and incubated at 28 °C on a reciprocal shaker. Samples (0.2 ml) of culture fluid were harvested every 12 h post-infection and cells removed by centrifugation at 3000 r.p.m. for 10 min. The cell-free culture fluid was assayed for HBsAg using a radioimmunoassay kit (Austria II-125; Abbott Laboratories, North Chicago, Ill., U.S.A.).

Protein analyses. *S. frugiperda* cells were infected at a m.o.i. of 10 p.f.u./cell in 35 mm tissue culture dishes and labelled with 10 μCi [35S]methionine (Amersham, 1131 Ci/mmol) for 5 h using methionine-free medium. Prior to labelling, the cells were incubated for 1 h in methionine-free medium to reduce the intracellular pools of the precursor. After the labelling periods, the media were removed, the cells rinsed with cold phosphate-buffered saline and the cells lysed in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.05 M Tris-HCl, 0.1% SDS pH 7.4). Aliquots of 100 μl of the extracts were incubated with 10 μl of anti-HBsAg antiserum for 1 h at 37 °C before addition of 25 μl of a suspension of 100 mg Protein A-Sepharose CL-4B beads (Sigma) in RIPA buffer. Following a further 1 h incubation at 4 °C, the immune complexes that were bound to the beads were removed by boiling for 5 min in dissociation buffer (2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62.5 mM Tris–HCl, 0.01% bromophenol blue pH 6-8) and the eluants subjected to electrophoresis in a 10% polyacrylamide gel (Matsuura et al., 1986). After electrophoresis, the gels were impregnated with 2,5-diphenyloxazole and exposed at -70 °C to X-ray film.

RESULTS

We have cloned the BamHI fragments of the adw subtype of the HBV genome using standard cloning techniques (Maniatis et al., 1982). A 1400 bp BamHI fragment containing coding sequences of part of the pre-S2 region of HBV and the entire HBsAg was cloned into the BamHI site of plasmid pUC8. This 1400 bp fragment was then modified by cutting the fragment with AccI to eliminate downstream HBV sequences and by adding a synthetic oligonucleotide which contained the original HBsAg translation termination signal plus PstI. BamHI and HindIII restriction enzyme sites (Fig. 1). Sequence analysis showed that the resulting BamHI fragment contained the entire coding region of the HBsAg gene including the complete S antigen-coding region, as well as translation initiation and translation termination sites (Fig. 1). Some 150 pre-S nucleotides precede the HBsAg ATG codon; however, these pre-S sequences do not include an ATG codon and consequently cannot be translated. The modified 828 bp BamHI fragment was inserted into a baculovirus transfer vector, pAcRP6 (Possee, 1986; Matsuura et al., 1986) as shown in Fig. 1. The recombinant plasmid, pAcRP6-HBsYK14, therefore contains coding sequences for the entire surface antigen. The recombinant plasmid DNA was purified and used with wild-type AcNPV DNA to co-transfect *S. frugiperda* cells. Polyhedrin-negative plaques were isolated and subsequently plaque-purified three times to obtain a recombinant virus stock that was free of wild-type virus.

Recombinant virus DNA was extracted from gradient-purified virus and analysed by agarose gel electrophoresis. The DNA was electroblotted onto Genescreen (New England Nuclear) and the filter was hybridized with nick-translated DNA specific for either the HBsAg gene or the AcNPV polyhedrin gene. As shown in Fig. 2, the recombinant AcNPV-HBsYK14 contained HBsAg gene-specific sequences. By contrast, wild-type AcNPV showed no sequence homology with the HBsAg gene.

In order to determine the size of HBsAg-specific mRNA species present in AcNPV-HBsYK14 recombinant virus-infected cells, mRNA was isolated from infected *S. frugiperda* cells. The virus-infected cellular RNAs were denatured with glyoxal, electrophoresed in an agarose gel, blotted onto Genescreen and hybridized with either a 32P-labelled HBsAg gene-specific probe (the BamHI fragment from pUC8-HBs), or with pAcRP6 to detect residual polyhedrin and other AcNPV gene sequences. As shown in Fig. 3, RNA recovered from the recombinant virus-infected cells hybridized strongly with the HBsAg-specific probe and only weakly with the pAcRP6 DNA. By contrast, RNA from wild-type virus-infected cells hybridized only with pAcRP6 DNA. These results indicated that HBsAg-specific mRNA...
Fig. 2. Southern blot analyses of recombinant AcNPV DNA. DNA samples derived from recombinant AcNPV-HBsYK14 and wild-type AcNPV were electrophoresed, transferred onto the membrane and hybridized with an HBsAg gene-specific probe. (a) Ethidium bromide staining of recombinant (lane 2) and wild-type (lane 3) viral DNAs. Lane 1, λDNA/HindIII markers. (b) Hybridization of recombinant (lane 1) and wild-type (lane 2) viral DNAs with the BamHI fragment of pHBsYK2 (Fig. 1).

Fig. 3. Northern blot analyses of RNAs obtained from recombinant virus- and wild-type AcNPV-infected S. frugiperda cells. Total RNA preparations obtained from virus-infected cells were electrophoresed, electroblotted onto the membrane and hybridized with either an HBsAg gene-specific probe (HBsAg BamHI fragment) (lanes 1 and 2) or an AcNPV-specific probe (pAcRP6) (lanes 3 and 4). 14C-labelled 28S and 18S ribosomal RNAs were used as size markers (lane 5). Lanes 1 and 3, strips containing RNA from wild-type AcNPV-infected cells. Lanes 2 and 4, strips containing RNA from recombinant AcNPV-HBsYK14-infected cells.

species were present in the recombinant virus-infected cells. The size heterogeneity of mRNA species representing foreign genes expressed by recombinant baculoviruses has been reported previously and is presumably due to indefinite termination of RNA transcripts and heterogeneous 3' ends (Matsuura et al., 1986). Size heterogeneity is also apparent with the HBsAg-specific mRNAs (Fig. 3).

S. frugiperda cells were infected with the polyhedrin-negative AcNPV-HBsYK14 recombinant virus and the expression of HBsAg was investigated. The extracellular fluid was monitored for the presence of HBsAg using a commercially available radioimmunoassay kit (Abbott Laboratories). The kinetics of HBsAg production secreted from the infected cells is shown in Fig. 4. Antigen was detected in the extracellular fluid as early as 12 h post-infection. The amount of total HBsAg produced in a 1 ml culture containing 6 × 10^6 S. frugiperda cells 4 days after infection was estimated using the standards provided with the commercial kit to be of the order of 8 to 10 μg. Confirmation of these yields by purification of the antigen and direct protein assays was not undertaken. As shown in Fig. 5, electron microscopic examination revealed the presence of 22 nm particles in the extracellular fluid of the recombinant virus-infected S.
HBsAg synthesis by recombinant baculoviruses

Fig. 4. Kinetics of HBsAg synthesis and secretion from *S. frugiperda* cells infected with the recombinant AcNPV-HBsYK14. Aliquots of 0.2 ml of infected cell culture fluids were assayed for HBsAg using a commercial radioimmunoassay kit (Abbott Laboratories). ●, AcNPV-HBsYK14 and ○, AcNPV wild-type.

Fig. 5. Electron micrographs of HBsAg particles produced by *S. frugiperda* cells infected with the recombinant AcNPV-HBsYK14. The recombinant AcNPV-HBsYK14-infected cell culture medium was harvested at 4 days after infection. Cells and cell debris were removed by centrifugation at 3000 r.p.m. for 10 min. Cell-free supernatant fluids were examined by negative staining using 1% phosphotungstic acid. Bar markers represent 100 nm. (a), Cell-free supernatant containing both AcNPV and 22 nm particles. (b) Supernatant material after centrifugation at 25000 r.p.m. for 1 h, to remove AcNPV particles. (c) Twentyfold concentration of the particles shown in (b). (d) Twenty-two nm particles purified from blood donated by a chronic active hepatitis patient.

*frugiperda* cells that were indistinguishable from the 22 nm particles isolated from the plasma of chronic active hepatitis patients. Purification of the extracellular particles by equilibrium gradient centrifugation, demonstrated that they reacted with reference hepatitis B antisera and yielded immune complexes (see Fig. 6). When the relative amounts of HBsAg associated with frozen and thawed extracts of infected cells were compared to that of the secreted form of the antigen at different times post-infection, it was found that approximately 90% of the antigen was extracellular. However this was only true for cells that were stirred or otherwise agitated. For resting cells, although the overall amounts of antigen (internal + external) were the same, the proportions were reversed. The reason for this is not known.

Proteins in the recombinant AcNPV-HBsYK14-infected *S. frugiperda* cells and in extracellular 22 nm particles, were analysed by PAGE. *S. frugiperda* cells were labelled with $^{35}$S]methionine and the cells were lysed in RIPA buffer after various labelling periods.
Fig. 6. SDS-PAGE of HBsAg produced from recombinant AcNPV-HBsYK14-infected *S. frugiperda* cells. (a) Lanes 1 (24 h post-infection) and 2 (48 h post-infection) show the [35S]methionine-labelled proteins derived from the recombinant AcNPV-HBsYK14-infected cells; lane 3 (24 h post-infection) and lane 4 (48 h post-infection) show [35S]methionine-labelled proteins derived from wild-type AcNPV-infected cells; lane 5 shows proteins from uninfected *S. frugiperda* cells. (b) Lane 2 shows immunoprecipitated proteins from a preparation of 22 nm particles from the infected cell culture fluid using anti-HBsAg antibody; (a) lane 6 and (b) lane 1 show molecular weight markers. P represents the position of AcNPV polyhedrin protein. S represents HBsAg and S' may represent a more highly glycosylated form of HBsAg.

(Matsuura *et al.*, 1986). Samples of the extracts were analysed by SDS-PAGE (Fig. 6a). In addition, proteins in the 22 nm particles recovered from the infected cell culture media were immunoprecipitated with anti-HBsAg antibody and analysed by SDS-PAGE (Fig. 6b). Recombinant virus-infected cells produced a major 22000 *M*$_r$ protein (Fig. 6a, lanes 1 and 2) while wild-type AcNPV-infected cells synthesized the expected 33000 *M*$_r$, polyhedrin protein (Fig. 6a, lanes 3 and 4). Neither protein was present in uninfected *S. frugiperda* cells (Fig. 6a, lane 5). Anti-HBsAg antibody precipitated the 22000 *M*$_r$ protein and minor amounts of a 24000 *M*$_r$ protein from the material recovered from the extracellular fluids as 22 nm particles (Fig. 6b, lane 2). Similar results were obtained for 22 nm particles centrifuged to equilibrium in gradients of sucrose. The minor protein may well represent an alternative glycosylated form of the major protein, but this was not investigated further. None of the proteins produced in the wild-type AcNPV-infected cells was precipitated with the anti-HBsAg antibody (data not shown). In summary, the data indicated that HBsAg sequences were expressed by the recombinant baculovirus and encoded protein that was reactive with hepatitis B antisera.

**DISCUSSION**

It has been demonstrated that recombinant baculoviruses into which the HBsAg gene has been inserted under the control of the polyhedrin gene promoter express and secrete into the extracellular fluids 22 nm particles that are antigenically and physically like those made in natural virus infections in man. The level of expression may not be optimal in view of the recent
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report of the importance of the upstream sequences on the expression of the S-coded genes of lymphocytic choriomeningitis virus (Matsuura et al., 1987). This is currently under investigation.

It has been claimed that the HBV pre-S2 region (Milich et al., 1985; Valenzuela et al., 1985) enhances the immunogenicity of the HBsAg. A recombinant baculovirus (AcNPV-PsSYK27) has therefore been constructed containing the coding region of the pre-S2 region (55 amino acids) as well as the HBsAg gene. By comparison with the AcNPV-HBsYK14 virus, the AcNPV-PsSYK27 recombinant virus-infected S. frugiperda cells synthesized a similar amount of 22 nm particles. These particles were antigenically and morphologically indistinguishable from the 22 nm particles shown in Fig. 5. Whether they will elicit an enhanced immunological response when made or used in conjunction with the baculovirus-expressed HBsAg is under investigation.

In summary, human HBV surface antigen has been synthesized in insect cells using AcNPV as an expression vector. The antigen is expressed in large quantities and secreted in the form of 22 nm particles. The 22 nm particles contain predominantly a 22000 Mr protein exhibiting a morphology and antigenicity closely resembling the 22 nm particles isolated from the plasma of chronic active hepatitis patients.

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REFERENCES


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