Co-expression of the Hepatitis B Surface and Core Antigens Using Baculovirus Multiple Expression Vectors

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(Accepted 28 July 1988)

SUMMARY

The hepatitis B (HB) virus DNA sequences coding for the pre-core (preC) or C antigens (HBpcAg, HBcAg) have been inserted into the baculovirus plasmid transfer vector, pAcYM1, such that the HB viral sequences are under the control of the polyhedrin promoter of Autographa californica nuclear polyhedrosis virus (AcNPV). Spodoptera frugiperda cells infected with either of the derived recombinant plasmids in the presence of infectious AcNPV DNA yielded recombinant, polyhedrin-negative viruses that expressed high levels of the respective HBpcAg or HBcAg (representing approx. 5 to 10% and approx. 40% of the stained cellular proteins, respectively). The particulate 27 nm HBcAgs have been purified to homogeneity from infected cell extracts by density gradient centrifugation. Dual expression transfer vectors containing the HBcAg gene sequences and the coding sequences of the HB viral S antigen (HBsAg), each gene under the control of its own copy of the polyhedrin promoter, have also been constructed and used to derive recombinant viruses. The recombinant with the HB C and S genes expressed high levels of the HBcAg (approx. 40% of the cellular proteins) and low levels of the HBsAg (approx. 2% of the stained cellular proteins). Dual expression, occluded, recombinant baculoviruses that make HBsAg, as well as the AcNPV polyhedrin protein, have been prepared that are highly infectious for Trichoplusia ni caterpillars, allowing reproducible preparation of the antigen in larvae. Using radioimmunoassays (RIAs) and ELISAs, the recombinant HBcAg (RIA) and HBsAg (ELISA) have been used to identify human antibodies to HB virus with results that compare favourably with the data obtained with non-recombinant antigens.

INTRODUCTION

Hepatitis B (HB) is a disease of major significance world-wide (Hollinger & Melnick, 1985). Infected individuals may experience only an acute infection, or may develop a long term infection that is associated with liver cirrhosis and hepatocellular carcinoma, chronic active HB, chronic persistent HB and chronic lobular HB (Hollinger, 1985). In certain regions of the world, particularly but not exclusively in south-east Asia and Africa, persistent infections may occur in up to 15% of the population with as many as 40% of those individuals liable to die from one or another form of the disease. Estimates have been made that world-wide up to 300 million people are carriers of the disease (i.e. are persistently infected with the virus). Plasma- and recombinant DNA-derived preparations that contain the HBsAg have been used successfully to vaccinate people against the disease.

Baculoviruses have been shown to be efficient eukaryotic vectors for foreign genes. A variety of genes have been expressed in baculoviruses using vectors based on the Autographa californica nuclear polyhedrosis virus (AcNPV) polyhedrin gene promoter system that was originally
described by Smith et al. (1983). One of the most efficient vectors (pAcYM1; Matsuura et al., 1987) has the complete upstream promoter sequence of the AcNPV polyhedrin gene. It has been used to make both processed and unprocessed foreign gene products (e.g. glycoproteins and unmodified proteins) with yields that, depending on the protein, can represent up to 40% of the stained cellular proteins (i.e. levels comparable to that of the AcNPV polyhedrin protein). Lower levels of expression have been reported for vectors that do not have all of the upstream sequences of the polyhedrin promoter (Matsuura et al., 1986, 1987). Low level expression has been observed for certain gene products irrespective of the vector used (including the HBsAg, Kang et al., 1987; see below); the reason for the lower expression is not known.

Multiple expression vectors that make a foreign gene product as well as polyhedrin protein have recently been described (Emery & Bishop, 1987). Since such recombinant viruses are occluded they are highly infectious in caterpillar hosts that are permissive for the parent virus, allowing the production of the foreign gene product in a cost-effective manner. In this paper multiple expression vectors are described in which two foreign gene products are made by the same recombinant virus [i.e. HB core antigen (HBcAg) and HB surface antigen (HBsAg)], as well as occluded recombinant viruses that make HBsAg, and single expression vectors that make high levels of HBcAg, or HB pre-core antigen (HBpcAg).

METHODS

Viruses and cells. AcNPV and recombinant virus stocks were grown and assayed in confluent monolayers of Spodoptera frugiperda cells in medium containing 10% foetal calf serum (FCS) according to the procedures described by Brown & Faulkner (1977). On occasion, virus stocks were made using spinner cultures of S. frugiperda cells in medium containing 5% FCS.

DNA manipulations. Plasmid DNA manipulations were carried out essentially as summarized by Maniatis et al. (1982). Restriction enzymes, (except AccI), T4 DNA ligase and the Klenow large fragment of DNA polymerase were purchased from Biolab (Beverley, Mass., U.S.A.). The enzyme AccI was purchased from Amersham. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim.

Construction of recombinant transfer vectors for single gene expression. A 581 bp DNA fragment containing the coding region of the HB virus C gene and a 1005 bp fragment containing the coding region of the preC plus C gene were excised with StyI and HinPI, respectively, from plasmid pSCK102 (representing an adw serotype of HB virus) kindly supplied by C.-Y. Kang (University of Ottawa, Canada). Each fragment was repaired with the Klenow fragment of DNA polymerase, then cloned into the BamHI site of the baculovirus vector pAcYM1 (Matsuura et al., 1987). The recombinant transfer vectors were designated pAcYM1KTc and pAcYM1KTpc respectively (Fig. 1). The entire S gene of HB virus was prepared by AccII digestion of plasmid pAcRP6-HBsYK14 (Kang et al., 1987), ligated into the BamHI site of pAcYM1, then digested with BamHI. The resulting 725 bp DNA fragment containing the S gene was inserted into the BamHI site of the vector pAcYM1 to construct the recombinant transfer vector pAcYM1KTs (Fig. 1).

Construction of recombinant transfer vectors for dual gene expression. Plasmid pAcYM1KTs was digested with AccII and the fragment containing the S gene, its associated polyhedrin promoter and transcription termination sequences was ligated into the dephosphorylated EcoRV digestion product of plasmid pAcYM1KTc to give the dual expression, recombinant transfer vector pAcVCKTsc (Fig. 1), or into the EcoRV digestion product of pAcYM1KTpc to give the dual expression vector pAcVCKTspc (Fig. 1). Insertion of the same HB S gene fragment into the 7.3 kb EcoRI I fragment of AcNPV (in a modified pUC8 plasmid; Possee, 1986) yielded the recombinant transfer vector pAcVCKTs (Fig. 1).

Transfections and selection of recombinant viruses. To obtain recombinant viruses that would express foreign gene(s) S. frugiperda cells were transfected essentially as described by Overton et al. (1987), with mixtures of infectious AcNPV DNA and plasmid DNA representing the individual recombinant transfer vectors. Recombinant virus YM1KTc was obtained from cotransfection with the pAcYM1KTc plasmid DNA, likewise recombinant YM1KTpc from pAcYM1KTpc, recombinant YM1KTs from pAcYM1KTs and recombinant VCKTsc from pAcVCKTsc. To derive an occluded recombinant virus that expressed both the HB S antigen and AcNPV polyhedrin protein, S. frugiperda cells were cotransfected with a mixture of plasmid pAcVCKTs DNA and DNA representing a polyhedrin-negative virus derived from pAcSI. 10.2 that contained the bluetongue virus serotype 10 segment DNA 2 (Inumaru & Roy, 1987). Plaques of recombinant viruses (VCKTs) that contained visible occlusion bodies were recovered as described by Emery & Bishop (1987).

Extraction and characterization of viral DNA. Viral DNA was prepared as described previously (Overton et al., 1987). DNA samples were digested to completion with EcoRI and the products were subjected to Southern analyses as described by Matsuura et al. (1986).
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Fig. 1. Schematic diagram of the transfer vectors pAcYM1KTPc, pAcYM1KTC, pAcYM1KTS, pAcVCKTspc, pAcVCKTsc and pAcVCKTs as described in Methods. In the sequences shown the BamHI insertion sites and the initiation codons of the HBpcAg, HBcAg and HBsAg are underlined.

Labelling and analyses of infected cell polypeptides. S. frugiperda cells were infected with virus, usually at a multiplicity of 10 p.f.u./cell, in 35 mm tissue culture dishes and incubated at 28 °C for 48 h. At the required times the cells were treated for 1 h with methionine-free medium, then labelled for 3 h with 15 μCi of [35S]methionine (Amersham; 1131 Ci/mmol) in the same medium. On occasion, the cells were not labelled (see text). The cells were rinsed three times with phosphate-buffered saline (PBS) and lysed in 150 μl of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.5 M-NaCl, 0.5 M-Tris-HCl, 0.01 M-EDTA, 0.1% SDS, pH 7-4). Portions of the protein samples were boiled 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 subjected to SDS-PAGE in a discontinuous gel of 10 to 20% polyacrylamide as described by Laemmli (1970). After electrophoresis the gel was fixed in 10% (v/v) acetic acid and stained with Kenacid Blue, dried, then exposed at -70 °C to X-ray film.

Immunoblotting analysis. After SDS-PAGE, proteins were transferred electrophoretically to nitrocellulose membranes for 4 h at 150 mA. The membranes were soaked at 4 °C overnight in TBS (20 mM-Tris-HCl pH 7.4, 0.15 M-NaCl containing 10% FCS). After another wash with TBS, the membranes were treated for 1 h at 20 °C with rabbit anti-HBcAg serum in TBS containing 5% FCS and 0.05% Tween 20 (TTBS). Following further washes with TTBS, bound antibodies were detected with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma), Fast Blue BB salt and fl-naphthyl phosphate (Sigma) as substrate.

Purification of HBcAg and HBsAg by gradient centrifugation. Cells were infected with recombinant baculoviruses containing the HBsAg gene (YM1KTs) and the supernatant fluids recovered 4 or 5 days post-infection (p.i.). The cell culture supernatant fluids containing HBsAg were clarified following treatment with 7% polyethylene glycol 6000 (PEG) and the HBsAg was recovered by precipitation with 9% PEG. The pelleted material was resuspended in TNE buffer and centrifuged at 150000 g for 15 h at 4 °C using an SW41 Ti rotor (Beckman). After centrifugation, the gradients were fractionated and peak fractions containing HBsAg identified by radioimmunoassay (RIA) (see later), pooled and pelleted by centrifugation (Ti 50 rotor, 100000 g for 15 h at 4 °C). The products were resuspended in H2O, and samples were analysed by electron microscopy. Cells infected with recombinant viruses containing HBcAg were extracted 4 days p.i. by sonication, or by freezing and thawing three times, the products were subjected to centrifugation to remove cell debris and the derived supernatant fluids were centrifuged through a 30% sucrose cushion using a 42.1 rotor (Beckman) at 106000 g for 16 h, then resuspended in TNE and purified by CsCl isopycnic centrifugation using an SW41 rotor at 160000 g for 36 h. The gradient was fractionated and the peak fractions containing HBcAg were identified by SDS-PAGE, pooled, pelleted by centrifugation and resuspended in H2O.

Infection of Trichoplusia ni with recombinant viruses that express HBsAg. Groups of 10 to 20 fourth instar T. ni caterpillars, grown on semi-synthetic media (Hoffman et al., 1966), were individually infected orally with the non-occluded recombinant virus YM1KTs (5 x 10⁵ p.f.u./larva), or by polyhedral inclusion bodies (PIBs) representing...
the recombinant VCKTs virus (4 × 10^4 PIBs/caterpillar). After 4 to 5 days of extrinsic incubation the visibly infected and moribund caterpillars were harvested, homogenized in 200 μl of 10 mM-Tris-HCl pH 7.4 containing 0.02% sodium diethyldithiocarbamate. Portions (5 μl) of the homogenates were assayed for HBsAg by RIA.

**RIA for detection and quantification of HBsAg.** Recombinant virus-derived HBsAg was measured by solid-phase RIA (AUSRIA II; Abbott Laboratories, North Chicago, Ill., U.S.A.) using the HBsAg supplied by the manufacturer as the standard (20 ng/ml).

**SeroLOGY.** Immunofluorescence was used to detect recombinant virus-derived HBsAg and HBeAg in infected cells.

Antibodies to HBsAg (anti-HBs) in human sera were measured using a commercially available RIA (AUSAB; Abbott Laboratories) and by two solid-phase sandwich ELISA procedures. ELISA 1 used human plasma-derived HBsAg for antibody capture and detection; ELISA 2 used recombinant virus-derived HBsAg for antibody capture and human plasma-derived HBsAg for antibody detection. Antibodies to HBeAg (anti-HBe) were measured using two solid-phase competitive RIAs. RIA 1 used human liver-derived HBsAg; RIA 2 used recombinant virus-derived HBeAg.

**Immunofluorescence analyses.** *S. frugiperda* cells were infected with virus at a multiplicity of 1 p.f.u./cell in 35 mm tissue culture dishes. At 72 h p.i. portions of 5 μl were spotted onto polytetrafluoroethylene-coated multipot microscope slides and fixed with cold acetone for 10 min. Uninfected (control) cells were treated similarly. Sera and ascitic fluids were diluted 10-fold with PBS. Samples (10 μl) of each preparation were pipetted on to one area of infected cells and one area of control cells. Slides were incubated for 30 min at 37 °C in a sealed box at high humidity. Excess antibody was rinsed from the slides with PBS, the slides were drained and any remaining liquid was removed. Appropriate conjugates were diluted in PBS containing 0.005% Evans Blue to mask non-specific (background) fluorescence. Sheep anti-human Ig-fluorescein isothiocyanate (FITC) (Wellcome) and goat anti-mouse IgG and IgM-FITC (Tago, Burlingame, Ca., U.S.A.) were diluted to provide bright fluorescence with positive sera and low background staining with negative sera and on control cells. Portions (10 μl) of diluted conjugate were pipetted on to each area of cells and the slides were incubated at 37 °C for 30 min in a sealed box that provided a humid environment. The stained slides were rinsed and washed as before and finally rinsed for 1 min in distilled water. After drying in air the slides were examined using a fluorescence microscope.

**RIA to measure anti-HBs.** Antibodies to HBsAg were measured in a commercially available RIA (AUSAB) with comparison to the WHO reference anti-HBs preparation, which was diluted in negative human serum to contain 100, 50 and 10 international units (IU)/l.

**ELISA to measure anti-HBs.** The solid-phase sandwich ELISA used to measure anti-HBs included a novel enhancement system developed in the Division of Microbiological Reagents and Quality Control [Central Public Health Laboratory (CPHL), Colindale]. FITC-conjugated antigens or antibodies are detected by a peroxidase conjugate of the monoclonal anti-FITC antibody described by Samuel et al. (1988). In this anti-HBs assay, test antibody was sandwiched in microwells between coating HBsAg and an HBsAg–FITC conjugate. Human plasma-derived HBsAg was conjugated to FITC as described by Samuel et al. (1988) using 1 mg HBsAg and 37.5 μg FITC in 0.5 ml of 0.1 M-sodium carbonate buffer pH 9.3, containing 0.1 M-NaCl. An anti-FITC–peroxidase conjugate prepared by the peridate method of Wilson & Nakane (1978) was used to detect bound antigen–FITC.

Human plasma-derived HBsAg was purified from human serum and recombinant virus-derived HBsAg was purified from cell culture supernatant fluids by affinity chromatography using a column of Protein A–Sepharose CL-4B (Pharmacia) to which monoclonal anti-HBs had been cross-linked using dimethylsuberimidate (Davies & Stark, 1970; Parkhouse, 1984). The antigen was recovered and diluted in 0.1 M-glycine–HCl buffer pH 7.5 containing 0.1% sodium azide, to give a concentration of 1 μg/ml and used to coat polystyrene microtitre plates (Nunc-Immuno plate Maxisorp F96; Nunc). Antigen suspension (100 μl) was pipetted into each well and the plates were incubated at room temperature for 3 days. They were then washed, and unused binding sites were blocked with 1% bovine serum albumin (BSA) in 0.02 M-Tris–HCl buffer pH 7.6, containing 0.1% sodium azide. Plates were stored at 4 °C until required. Patient sera (100 μl), either from HBsAg vaccinees, or from patients with a history of HB infection, as well as appropriate positive and negative controls, were added to the HBsAg-coated wells and incubated at 37 °C for 1 h. The wells were washed to remove unbound material and 100 μl of human plasma-derived HBsAg–FITC conjugate buffer (PBS containing 0.05% Tween 20, 5% negative human serum and 1% BSA) was added. Following incubation for 1 h at 37 °C and a final washing step, a substrate solution containing hydrogen peroxide and the chromogen tetramethylbenzidine (TMB) was added (0·001 g TMB dissolved in 100 μl DMSO, and 7.5 μl of 6% w/v hydrogen peroxide in 10 ml 0·1 M-citrate–acetate buffer pH 6·0). The enzyme reaction was stopped after 30 min by the addition of 100 μl of 2 M-H₂SO₄. The absorbances of the controls and the test samples were measured at 450 nm.

**RIA to measure anti-HBe.** The solid-phase competitive RIAs used were modifications of the method described by Cohen et al. (1981). Human liver-derived HBeAg was diluted 1·4 × 10³-fold and recombinant virus-derived
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HBCAg was diluted 2 × 10⁵-fold in 0.02 M-Tris-HCl buffer pH 7.6, containing 0.1% sodium azide. Two-hundred-and-ten etched polystyrene beads (Northumbria Biologicals, Cramlington, U.K.) were added to 35 ml of each diluted antigen in small flasks which were first shaken at room temperature for 2 h, then incubated at room temperature in the dark for a further 2 to 3 days. The HBCAg–beads were washed three times in PBS and stored until required at 4 °C in PBS containing 0.5% BSA and 0.1% sodium azide. Test serum (20 µl) was added to each well of an assay plate followed by 180 µl of ¹²⁵I-labelled anti-HBc diluted in PBS containing 10% FCS. A negative serum, a strong positive and a weak positive serum served as controls and were similarly processed. Excess buffer was removed from the HBCAg–beads by blotting, and one bead was added to each well. The wells were sealed and the plates incubated overnight in a humid atmosphere at room temperature. The wells were washed with distilled water using a Qwikwash (Abbott Laboratories) and then the beads were transferred to counting tubes and counted for radioactivity in a 16-well gamma-counter (NE 1600, Nuclear Enterprises, Edinburgh, U.K.). Background radiation was also measured. The percentage inhibition of the ¹²⁵I-anti-HBc binding was expressed as:

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100 - \left\{ \frac{\text{(count - background) × 100}}{\text{mean negative count - background}} \right\}.
\]

In these assays, strong positive control sera gave 94% inhibition and weak positive control sera gave 76% inhibition. Similar values were obtained for both the recombinant virus-derived and the liver-derived antigen. The positive cut-off value was 67% inhibition and the negative cut-off value was 50% inhibition.

RESULTS

Sequence analyses of HB virus C and preC genes

DNA subclones encompassing the C and preC antigens of an adw serotype of HB virus were obtained as described in Methods. The sequences of the HBCAg and HBpcAg gene products were deduced from DNA analyses of these clones (Fig. 2). Indicated in the figure are the methionine residues (boxed) that initiate the preC and C gene products. Also indicated are the positions of nucleotide changes (open triangles) and amino acid changes (filled triangles) by comparison with the HB virus adw sequence published by Ono et al. (1983). The sequences of the indicated gene products were comparable to those published for other isolates of HB virus (see Galibert et al., 1979; Pasek et al., 1979; Valenzuela et al., 1980; Fujiyama et al., 1983; Ono et al., 1983; Bichko et al., 1985).

Recombinant baculoviruses that express HBCAg or HBpcAg or HBsAg

The coding sequences of HBpcAg and HBCAg were inserted into the AcNPV transfer vector pAcYM1 (Matsuura et al., 1987) as described in Methods (Fig. 1). The derived recombinant transfer vectors (pAcYM1KTc, pAcYM1KTc) were analysed by restriction endonuclease digestion and the junction sequences determined to be those shown in Fig. 1. Following cotransfection with infectious AcNPV DNA, recombinant baculoviruses were obtained (YM1KTpc, YM1KTc), plaque-purified and high titre virus stocks were obtained. The levels and identities of the Mr 24.6K HBpcAg and 21.4K HBcAg were determined by the incorporation of [³⁵S]methionine (Fig. 3 a) and by Western analyses (Fig. 3 b). Essentially all the HBCAg and HBpcAg was cell-associated. Electron micrographs of HBCAg extracted from infected cells and purified by CsCl gradient centrifugation are shown in Fig. 4 (a). From analyses of stained protein gels of the gradient-purified HBCAg, by comparison with known amounts of BSA, the yield of purified HBCAg was determined to be of the order of 5 mg/l of 1 × 10⁹ infected cells.

Using small volume cell cultures (e.g. 35 mm dishes), the level of HBsAg synthesis by the recombinant baculovirus HBsYK14 (Kang et al., 1987) has been reported to be equivalent to 1-5 mg per 10⁹ infected cells. In large culture volumes (100 to 1000 ml), expression levels of the order of 0.3 to 0.5 mg per 10⁹ infected cells are obtained with the same virus. The reason for this difference in expression level is not known. In order to improve this, the HB S gene was transferred from the pAcRP6 vector to the pAcYM1 vector since higher expression has been observed with the latter for a variety of gene products (see Matsuura et al., 1987). In an initial study, the HBsAg gene was recovered from the transfer vector pAcRP6-HB sYK14 (Kang et al., 1987) by BamHI digestion and inserted directly into the BamHI site of transfer vector pAcYM1. The derived transfer vector was used to prepare a recombinant virus and the level of HBsAg synthesis was measured. The data obtained (not shown) indicated that the level of expression
Fig. 2. The sequences of the HBcAg and HBpcAg genes and amino acids. The methionine codons that initiate the preC and C gene products are boxed. Open and filled triangles show nucleotide and amino acid differences, respectively, by comparison with sequences published by Ono et al. (1983).

Fig. 3. Metabolic labelling (a) and Western blot analysis (b) of proteins expressed by recombinant baculoviruses. S. frugiperda cells were infected with recombinant viruses or wild-type AcNPV: lanes 1, YM1KTc; lanes 2, YM1KTpc; lanes 3, YM1KTs; lanes 4, VCKTsc; lanes 5, VCKTs; lanes 6, AcNPV. Proteins were labelled 48 h p.i. for 3 h with [35S]methionine and the 35S-labelled proteins were recovered, resolved by gel electrophoresis, blotted to nitrocellulose membranes and fluorographed (a) or detected immunologically with anti-HBcAg serum (b). Uninfected cells were treated similarly (lanes 7). The positions of HBcAg (C), HBpcAg (pC), HBsAg (S) and AcNPV polyhedrin protein (P) are indicated. The positions of M, marker proteins (M, x 10^-3) are indicated on the left.

was equivalent to 0.3 mg per 10^9 infected cells and similar to that of the recombinant HBsYK14 run in parallel.

One reason for the low level of expression could have been the presence of some 100 nucleotides of the HB virus genome upstream of the HBsAg coding region (i.e. between the HB
Fig. 4. Electron micrographs of HBcAg and HBsAg particles (approx. 30 nm) produced by *S. frugiperda* cells infected with recombinant viruses. (a) HBcAg was extracted by sonication from YM1KTc virus-infected cells (alternatively, on occasion, by freeze-thawing the cells) and purified by CsCl isopycnic centrifugation as described in Methods, then stained with 1% uranyl acetate. (b) HBsAg was collected from the supernatant fluids of YM1KTs virus-infected cells and clarified by centrifugation (2000 g) after treatment with 7% PEG; the antigen was pelleted after adjusting the supernatant fluids with 9% PEG, purified by sucrose gradient centrifugation and stained with 1% uranyl acetate.

**Recombinant baculoviruses that co-express HBcAg and HBsAg**

A sequence containing the polyhedrin promoter, the HBsAg gene and the polyhedrin transcription termination signal was recovered from the HBsAg transfer vector pAcYM1KTs by *AccII* digestion (Fig. 1). It was inserted into the *EcoRV* sites of the transfer vectors pAcYM1KTpc and pAcYM1KTc in order to make dual recombinant vectors (Fig. 1; Emery & Bishop, 1987). The recombination vectors were characterized and the vector with the HBsAg gene in the opposite orientation to the C gene (pAcVCKTsc, Fig. 1) was used to derive recombinant viruses by cotransfection of *S. frugiperda* cells in the presence of infectious AcNPV DNA. The recombinant virus VCKTsc was recovered, plaque-purified and grown to a high titre. The antigens made by the VCKTsc recombinant were analysed. The synthesis of HBcAg by VCKTsc was demonstrated by the incorporation of [35S]methionine and by Western analyses (Fig. 3). The level of synthesis of HBcAg was essentially similar to that obtained with the recombinant YM1KTc.

The synthesis and secretion of HBsAg by cells infected with the dual recombinant VCKTsc and the residual cell-associated HBsAg were determined by RIA (Fig. 5). The HBsAg protein was also identified in infected cell extracts by the incorporation of [35S]methionine (Fig. 3). Based on the RIAs, the total yield of HBsAg was estimated to be of the order of 0.4 mg/l of
Fig. 5. Kinetics of HBsAg synthesis from *S. frugiperda* cells infected with the recombinant YM1KTs (a) or VCKTsc (b). Infected cells (5 x 10⁹) were cultured in 500 ml medium with stirring. Samples (5 ml) were collected every day and the medium and cells separated by low speed centrifugation. Cells were lysed by sonication after rinsing gently with PBS. Viral HBsAg in the cell lysates (A) or in the clarified culture medium (△) were measured by solid-phase RIA (AUSRIA II, Abbott Laboratories) with the human-derived HBsAg as a positive control and to quantify the yields (see Methods); total yield (●) is also shown. The data are expressed as the amount of antigen synthesized per ml of original medium.

1 x 10⁹ infected cells by 5 days p.i. (Fig 5), slightly higher than that obtained with the single expression recombinant virus, YM1KTs, analysed in parallel. The level of secreted HBsAg (Fig. 5) was almost double that obtained with the recombinant YM1KTs, although the cell-associated HBsAg was essentially the same. The significance, if any, of these differences is not known. Overall, though, the level of HBsAg synthesis represented only a small fraction of the amount of HBCAg synthesis.

Recombinant baculoviruses that co-express HBsAg and AcNPV polyhedrin protein

The sequence containing the polyhedrin promoter, the HBsAg gene and the polyhedrin transcription termination signal was recovered from the HBsAg transfer vector pAcYM1KTs by AccI digestion (Fig. 1). It was inserted into the *EcoRV* site of the *EcoRI* I fragment in order to make a dual recombinant vector that contained the HBsAg gene in addition to the AcNPV polyhedrin gene (Emery & Bishop, 1987). The recombinant vectors were characterized, and those with the HBsAg gene in the opposite orientation to the polyhedrin gene (pAcVCKTs, Fig. 1) were used to derive recombinant viruses by cotransfection of *S. frugiperda* cells in the presence of infectious AcNPV DNA. The proteins made in infected *S. frugiperda* cells by recombinant VCKTs are shown in Fig. 3 in comparison with an AcNPV infection (so that the polyhedrin protein could be identified). Although for the latter only small amounts of polyhedrin were made (the m.o.i. was < 1 p.f.u./cell), the data served to identify the polyhedrin protein in the VCKTs-infected cells. As in the other recombinants that contained the HB S gene, the HBsAg was a minor component of the total labelled proteins. From RIA it was estimated to represent some 0-4 mg/l of 1 x 10⁹ infected cells by 5 days p.i.

Levels of synthesis of HB proteins in recombinant virus-infected *S. frugiperda* cells

In Fig. 6 are shown the profiles of stained proteins recovered from cells infected with the single expression recombinant viruses YM1KTc, YM1KTpc and YM1KTs, or the dual expression recombinants, VCKTsc and VCKTs, in comparison with an AcNPV infection and mock virus-infected cells. The AcNPV and VCKTs infections were initiated at multiplicities of approx. 1 p.f.u./cell (accounting for the low levels of polyhedrin protein), the other viruses at 10 p.f.u./cell, and the proteins were recovered 2 days p.i. It was evident that for viruses containing the HB C gene, the HBCAg was present in the cell extracts in substantial quantities (estimated by scanning to be approx. 40% of the total protein in the cell extracts; see Matsuura *et al.*, 1987).
Co-expression of HBsAg and HBcAg

Fig. 6. Expression of HBcAg, HBpcAg, HBsAg and polyhedrin protein by recombinant baculoviruses: lane 1, YM1KTc; lane 2, YM1KTpc; lane 3, YM1KTs; lane 4, VCKTsc; lane 5, VCKTs; lane 6, AcNPV; lane 7, mock-infected. Cell extracts from recombinant virus- or AcNPV-infected cells were obtained at 2 days p.i. After resolution by SDS–PAGE, the gel was stained with Kenacid Blue. The positions of the HBpcAg, HBcAg, HBsAg and polyhedrin protein are indicated.

The preC antigen was observed in lower amount (estimated to be approx. 5 to 10% of the stained proteins). Between multiplicities of 1 and 10 p.f.u./cell results essentially similar to those shown in Fig. 6 were obtained for recombinants containing either the HB C or the HB pC gene.

Despite the fact that the HBsAg was located in a position in the gel where a cellular protein migrated (see Fig. 6, lane 7), it could be visualized as an enhanced band in the stained gels, although in considerably lower amounts than HBcAg. Between multiplicities of 1 and 10 p.f.u./cell essentially similar levels of cell-associated HBsAg were observed by 2 days p.i., although for the VCKTs virus there were enhanced levels of polyhedrin protein at the higher multiplicities (estimated to be approx. 40% of the cellular protein, see Fig. 3). Even at the higher multiplicities, it was estimated that the cell-associated HBsAg represented no more than around 2% of the stained proteins.

Immunofluorescence using recombinant virus-infected cells expressing HBcAg and HBsAg to identify human antibodies to those antigens

Human sera were provided by the virus Reference Laboratory of the CPHL, Colindale. Eight human sera that were strongly positive for antibody to HBcAg and negative for antibody to HBsAg in RIAs were used to stain the slides. Each serum produced strong fluorescence on the recombinant VCKTsc virus-infected insect cells (Fig. 7a), but there was no reaction to control, uninfected cells (Fig. 7c), indicating that by this procedure the antigens in the infected cells were suitable substrates for the detection of human antibodies to HBcAg.

Human vaccinee sera with high levels of antibody to HBsAg (up to 500 IU/l), but which contained no anti-HBcAg as judged by the appropriate RIA, gave no fluorescence on the same recombinant virus-infected cells. The presence of HBsAg in these virus-infected cells was confirmed, however, using a mouse monoclonal anti-HBsAg, producing strong fluorescence.
Fig. 7. Immunofluorescence of recombinant baculovirus-infected *S. frugiperda* cells. *S. frugiperda* cells infected 72 h previously with a recombinant baculovirus, VCKTsc, were fixed with acetone and examined by indirect immunofluorescence assay as described in Methods, using (a) human anti-HBc serum or (b) mouse monoclonal anti-HBs ascitic fluids. Uninfected *S. frugiperda* cells were treated similarly and examined using mouse monoclonal anti-HBs (c). Bar marker represents 50 μm.
Co-expression of HBsAg and HBcAg

Table 1. Anti-HBs levels in human sera tested by RIA and ELISA

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<tr>
<th>Antibody levels</th>
<th>RIA (ELISA 1)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA 2†</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>&lt;10 IU/l</td>
</tr>
<tr>
<td>10 to 50 IU/l</td>
<td>5</td>
</tr>
<tr>
<td>&gt;50 IU/l</td>
<td>&gt;100 IU/l</td>
</tr>
<tr>
<td>5 (90)</td>
<td>1</td>
</tr>
<tr>
<td>(1)</td>
<td>3</td>
</tr>
</tbody>
</table>

*Human antibody titres to HBsAg were determined using human plasma-derived HBsAg for antibody capture (and detection) and reference anti-HBs from WHO in RIAs, or from BPL (Elstree, U.K) in ELISA 1.
†The data were compared to the human antibody titres determined using the recombinant HBsAg for antibody capture (and human plasma-derived HBsAg for detection) and reference antibody from BPL in ELISA 2.

Table 2. Anti-HBc analyses* of human sera by RIA using liver- or recombinant virus-derived antigen

<table>
<thead>
<tr>
<th>Antibody levels</th>
<th>RIA 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA 2</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>76</td>
</tr>
<tr>
<td>Weak positive</td>
<td>4</td>
</tr>
<tr>
<td>Strong positive</td>
<td>2</td>
</tr>
</tbody>
</table>

*Human antibody titres to HBcAg were determined using liver- (RIA 1) or recombinant virus-derived HBcAg (RIA 2).

(Fig. 7b). It was concluded that the cell-associated HBsAg in the virus-infected cells was not adequate for identifying such levels of human antibodies to HBsAg.

ELISAs using purified recombinant virus-derived HBsAg to identify human antibodies to HBsAg

Eighty-eight sera sent to the Virus Reference Laboratory (CPHL) for anti-HBsAg determination and 91 sera from HBsAg-negative blood donations (North London Blood Transfusion Centre, Edgware, U.K.) were tested by RIA and ELISA. The RIA used a non-recombinant HBsAg from Abbott Laboratories. ELISA 1 used the human plasma-derived HBsAg for both antibody capture and detection. ELISA 2 used the recombinant HBsAg purified as described in Methods for antibody capture and a human plasma-derived HBsAg for detection. The results were compared to positive control sera prepared by dilution in normal human serum of WHO anti-HBsAg reference serum (RIA), or Blood Products Laboratory (BPL, Elstree, U.K.) anti-HBsAg reference serum (ELISA). The control sera contained 10, 50 and 100 IU/l of anti-HBsAg antibody. There was good agreement between the tests using the two different antigen sources for antibody capture as shown in Table 1.

Competitive RIA using purified recombinant virus-derived HBcAg to identify human antibodies to HBcAg

A total of 150 samples of serum sent to the Virus Reference Laboratory (Colindale) for anti-HBcAg determination were tested by competitive RIA using either the recombinant virus-derived HBcAg, or human liver-derived HBcAg. Six of the sera tested gave weak positive reactions with one test but negative reactions with the other (see Methods and Table 2). Two sera gave a positive reaction in the RIA using liver-derived material and a negative reaction using the recombinant antigen. Competitive RIA with human liver-derived HBcAg is known to give some false positive results, possibly due to the serum reacting against liver
proteins. In view of this, the agreement between the two tests was considered to be excellent; however, many more sera will have to be tested in the RIA (or by ELISA) with the recombinant virus-derived HBcAg to determine the suitability and specificity of such tests.

Expression of HBsAg in T. ni larvae

When the single expression vector for HBsAg (recombinant YM1KTs) was used to infect fourth instar larvae of T. ni (1 × 10^5 to 2 × 10^5 p.f.u./larva) it was found that the quantity of HBsAg in moribund larvae was of the order of 1 to 2 μg per larva. However, at this late larval development stage only 30% of the larvae became infected. Reproducible infection was obtained using earlier instar larvae, but the yields of antigen were correspondingly lower (<0.1 μg per larva). The use of higher titres of virus to circumvent the problem with fourth instar larvae was not considered practicable without concentrating the virus. Stock virus titres were usually of the order of 10^7 to 10^7.7 p.f.u./ml tissue culture fluids.

It has been demonstrated that occluded AcNPV are more effective and reproducible in establishing infections in T. ni larvae than non-occluded AcNPV (Emery & Bishop, 1987). Individual fourth instar T. ni larvae were therefore infected with 4 × 10^4 PIBs representing the occluded recombinant VCKTs (see Methods). All the larvae became infected. The levels of HBsAg obtained in these larvae were of the order of 2 to 4 μg per larva.

Electron microscopic analyses of cells infected with recombinants that express the HBcAg

In view of the amounts of HBcAg synthesized in S. frugiperda cells infected with the recombinant virus YM1KTc, an examination of electron micrographs of cells infected with the virus was undertaken (Fig. 8). Numerous spherical particles were evident in the cells, primarily in the vicinity of the cell nucleus. The sizes of the particles were comparable to those of gradient-purified HBcAg (see Fig. 4).

DISCUSSION

A clone of HB virus (adw serotype) has been used to provide HBc and HBpc gene sequences. The sequences of these genes have been determined and found to be similar to those reported by others (Galibert et al., 1979; Pasek et al., 1979; Valenzuela et al., 1980; Fujiyama et al., 1983; Ono et al., 1983; Bichko et al., 1985). Together with an HBs clone, the clones were used to derive both single and multiple expression vectors employing the baculovirus AcNPV.

Data have been presented which show that the HBcAg is expressed to high levels in insect cells infected with the appropriate recombinant baculovirus. The HBcAg was identified in infected cells in the form of particles, which could be purified to homogeneity by CsCl gradient centrifugation. Data have been presented showing that either recombinant virus-infected cells, or purified HBcAg, can serve as antigen source for the identification of human antibodies, giving results comparable to those obtained using human liver-derived antigens.

Expression of the HBpcAg has also been reported. Although present at a lower level than the HBcAg, the amount was estimated to represent some 5 to 10% of the total cellular protein by the end of an infection course. The reason for the lower expression was not investigated, nor were any analyses undertaken of the antigenicity or other characteristics of the HBpcAg, or whether HBeAg was secreted from the virus-infected cells.

Analyses of the quantity of HBsAg expressed from recombinants derived from the pAcYM1 vector were not significantly enhanced in comparison with that reported previously using recombinants derived from the pAcRP6 vector (see Kang et al., 1987). The levels of cell-associated HBsAg were no more than an estimated 2% of the total cellular protein, significantly lower than the level of HBcAg, or HBpcAg, expression from their respective recombinant viruses. The pAcYM1 transfer vector has the complete upstream sequence of the AcNPV polyhedrin gene (including the putative promoter region), whereas the pAcRP6 transfer vector lacks some eight nucleotides. Previous studies have indicated that recombinants derived from the pAcYM1 vector give higher levels of expression of most foreign genes (see Matsuura et al., 1987). This does not appear to be the case for the HB S gene. The reason why the pAcYM1-derived virus was not significantly more effective in the expression of the HBsAg is not known,
Fig. 8. Electron micrographs of virus-infected *S. frugiperda* cells. In (a) a thin section of a cell infected with recombinant YM1KTc is shown, in (b) the same cell at a fourfold higher magnification. The arrowhead points to the massed HBcAg surrounding the infected cell nucleus. Bar marker represents 2 μm.
but could relate to either restricted processing events of the antigen in insect cells or to other factors such as mRNA transcription levels. Further investigation is required, perhaps using other baculovirus promoters and other cell systems.

When the YM1KTs recombinant was used to infect fourth instar *T. ni* larvae, yields of some 1 to 2 μg of the antigen were obtained by the time the larvae were moribund. However the infection rate with this non-occluded virus was low. With the occluded recombinant, VCKTs, the infection rate was reproducibly 100%. The level of HBsAg expression was of the order of 2 to 4 μg per larva by the time of death. Although low by comparison with other antigens, the fact that infection was reproducible allows the preparation of the antigen from this cost-effective source.

We are grateful for the expert technical assistance of B. Megson who did the anti-HBs RIA (AUSAB) and the anti-HBc RIA using liver-derived HBcAg. Human sera were provided by the Virus Reference Laboratory, Colindale.

REFERENCES


(Received 13 May 1988)