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Expression and Purification of Dengue Virus Type 2 Envelope Protein as a Fusion with Hepatitis B Surface Antigen in *Pichia pastoris*

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The methylotrophic yeast, *Pichia pastoris*, has been used as a host to express the envelope protein (Den2E) of dengue type 2 virus (NGC strain) as a chimera with hepatitis B surface antigen (HBsAg): a protein known to self assemble into virus-like particles (VLPs) and to be efficiently expressed in *P. pastoris*. The *Den2E* gene used in this study is a truncated version encoding the first 395 amino acid (aa) residues of the mature Den2E protein; the *HBsAg* gene encodes the full length 226 aa HBsAg protein. Two in-frame gene fusions were constructed for intracellular expression in *P. pastoris*. The first one contains the *HBsAg* gene as the 5′ partner and the *Den2E* gene as the 3′partner (*HBsAg–Den2E*). In the second one, the relative positions of the two partners of the gene fusion were reversed to create the hybrid *Den2E–HBsAg* gene. These fusion genes were integrated into the genome of *P. pastoris* under the control of the methanol-inducible alcohol oxidase (*AOX1*) promoter. Of the two fusions, the *Den2E–HBsAg* gene was expressed at higher levels in *P. pastoris* based on Northern analysis. The hybrid protein (~68 kDa) expressed by this clone was purified to near homogeneity using a combination of acid precipitation, hydrophobic interaction, and immunoaffinity chromatographic steps. Final purification achieved was ~1400-fold with a yield of ~26%. The chimeric protein was found to possess the ability to assemble into high molecular weight aggregates (akin to HBsAg particles). The recombinant fusion protein eluted close to the void volume of a Sepharose CL-4B column indicating its macromolecular nature. On a CsCl density gradient the recombinant fusion protein sedimented to a position very similar to that of HBsAg VLPs. The hybrid protein is recognized by the two neutralizing monoclonals against the two components of the chimeric protein.

Key Words: dengue envelope protein; hepatitis B surface antigen; *Pichia pastoris*.

Dengue is an acute flaviviral disease prevalent in over a hundred countries, mostly in tropical and subtropical areas of the world. Infection with dengue viruses, of which there are four closely related serotypes, is responsible for 50–100 million cases a year (1). Infection with dengue virus of one serotype does not offer cross-protection against other serotypes. A first infection sensitizes a person so that approximately 2% of second infections (with a different serotype) can develop into potentially fatal dengue hemorrhagic fever. The relative risk of experiencing the most severe form of the disease is 100-fold higher after a secondary than after a primary infection (2). A useful dengue vaccine, therefore, should ideally protect against all four serotypes of dengue viruses. The availability of the complete nucleotide sequences of all four dengue virus serotypes in recent years (3) has greatly stimulated efforts to develop candidate subunit vaccines by recombinant DNA technology as an alternate to live attenuated dengue virus vaccine (4).

Available data in the literature show that both structural and nonstructural proteins of dengue virus can serve as potential vaccine candidates (4, 5). Among the

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several dengue viral proteins, the envelope (E)[2] protein appears to be very promising vaccine candidate for several reasons. It is the most important antigen from the viewpoint of virus biology and humoral immunity. It is a multifunctional protein involved in virion assembly, host cell surface receptor binding, and membrane fusion (6, 7). It is the major component of the virion envelope and is responsible for eliciting the first and longest lasting, protective antibody response to dengue infection (8). Anti-E-monoclonal antibodies (9) and purified E protein (10, 11) are capable of conferring protection against lethal dengue virus challenge, by passive immunization and induction of neutralizing antibodies, respectively.

The E protein has been expressed in several heterologous systems including *Escherichia coli* (12, 13), yeast (12, 14), insect (11, 15), and mammalian cells (16, 17). Potent flaviviral E protein antigens must be properly folded in a conformation that maintains the integrity of its neutralizing epitopes (18–20). In view of this constraint, the bacterial host system with a reducing intracellular environment is not very conducive to protein folding. The other expression systems have the potential to generate properly folded E protein. However, the production of recombinant protein, using the insect and mammalian cell based tissue culture systems, can be very expensive compared to the yeast based expression systems.

Yeast based expression system is unique in that it combines the advantages of both prokaryotic (high expression levels, easy scale-up, inexpensive growth media) and eukaryotic (capacity to carry out most of the posttranslational modifications characteristic of higher eukaryotes) expression systems. In recent years, the methylotrophic yeast, *Pichia pastoris*, has emerged as a powerful and inexpensive heterologous expression system for the production of high levels of functionally active recombinant proteins (21). From an expression perspective, the existence of well-established fermentation methods, that can generate very high cell densities using purely defined media (22) and the strong, tightly regulated methanol-inducible alcohol oxidase (*AOX1*) promoter (23), make *P. pastoris* a very valuable host for heterologous protein expression.

In this study, we have sought to evaluate *P. pastoris* as a host to express the E proteins of dengue viruses. Our goal is to develop a *P. pastoris* vector capable of simultaneously expressing the E proteins of all four dengue virus serotypes. Our experience shows that it should be possible to express all the four E proteins in a single recombinant *P. pastoris* expression vector. Large inserts of up to 16 kb (containing up to 8 copies of the *HBsAg* gene) have been successfully cloned and expressed in this system (24). As a first step toward realizing this objective, we have focused on the E protein of dengue virus type 2. Since work reported in the literature suggests that dengue E protein may be susceptible to proteolysis in *P. pastoris* (12), we chose to express it as a hybrid protein with a suitable fusion partner in an attempt to minimize this potential problem. We chose the surface antigen of Hepatitis B virus (HBsAg) as the fusion partner for the following reasons. First, it can be expressed efficiently in *P. pastoris* using the *AOX1* promoter (24, 25). Second, HBsAg expressed in *P. pastoris* is very stable as it spontaneously assembles into ~20 nm particles (24, 25). Such virus-like particles (VLPs) are highly immunogenic. Third, this inherent capacity of HBsAg to generate particulate structures is likely be retained in its fusion derivatives. Last, HBsAg has been used successfully as a carrier in the development of a circumsporozoite protein based malarial vaccine candidate (26). The specific objectives of this study were to (i) express a hybrid protein containing the E protein of dengue virus type 2 and HBsAg in *P. pastoris*, (ii) purify the hybrid protein, (iii) determine if the hybrid molecule assembles into particles, and (iv) determine if the hybrid protein is recognized by antibodies specific to the Den2E and HBsAg precursors.

MATERIALS AND METHODS

Host Strain and Plasmid Vectors

The *P. pastoris* host strain used in this study was the histidine requiring auxotroph, GS115 (*his4*). *In vitro* protein expression studies were performed using constructs created by inserting the recombinant gene downstream of the T7 promoter of the pVAX1 plasmid vector. For intracellular expression in *P. pastoris*, the plasmid pAO815 was employed. This plasmid contains the *P. pastoris AOX1* promoter and transcription termination sequences separated by a unique *Eco*RI restriction site for insertion of the foreign gene of interest. It also contains a wild-type copy of the histidinol dehydrogenase (*HIS4*) gene necessary for selection of *P. pastoris* transformants. Both GS115(*his4*) host strain as well as the plasmids (pVAX1 and pAO815) were purchased from Invitrogen.

PCR Primers and Templates

The plasmid pAO815 + HBsAg described previously (24) was used as the template for the amplification of the *HBsAg* gene (0.7 kb). This plasmid contains one copy of the *HBsAg* gene inserted into the unique *Eco*RI site of pAO815 described above. Dengue type 2 envelope (*Den2E*) gene (~1.3 kb) was amplified from a plasmid

[2] Abbreviations used: *AOX1, Alcohol Oxidase 1*; BMGY, buffered minimal glycerol medium with yeast nitrogen base; BMMY, buffered minimal methanol medium with yeast nitrogen base; Den2E, Dengue type 2 envelope protein; E, envelope protein; HBsAg, hepatitis B surface antigen; VLP, virus-like particle.

containing a DNA copy of the first ~2.2 kb of the RNA genome of dengue type 2 virus (NGC strain) (27), a kind gift from Professor. R. Padmanabhan (University of Kansas Medical Center, KS). Nucleotide sequences of the primers for PCR amplification of these two genes are shown below.

1. GCGGAATTCTACGTACCATGGAGAACATCAC ATCAGGA
   *Eco*RI
2. AACGCATTGCGGCCGCAATGTATACCCAGA GACAAAAGAAAATTGG
   *Not*I
3. GGTATACATTGCGGCCGCAATGCGTTGCATA GGAATATC
   *Not*I
4. CCGGAATTCCTCGAGTCACTATCCTTTCTTA AACCAGTTGAG
   *Eco*RI, *Xho*I
5. GCGGAATTCTACGTACCATGCGTTGCATAGG AATATCA
   *Eco*RI
6. GTTCTCCATTGCGGCCGCTCCTTTCTTAAAC CAGTT
   *Not*I
7. TAAGAAAGGAGCGGCCGCAATGGAGAACAT CACATC
   *Not*I
8. CCGGAATTCCTCGAGTCACTAAATGTATACC CAGAGACAAAAG
   *Eco*RI, *Xho*I

Generation of Fusion Constructs

Two gene fusions, shown in Fig. 1, were constructed for this study. The *Den2E* gene used in our experiments was a truncated version encoding the first 395 amino acid (aa) residues of the Den2E protein; the *HBsAg* gene encoded the full-length 226 aa HBsAg protein. The first one contained the *HBsAg* gene as the 5′ partner and *Den2E* gene as the 3′ partner (referred to as *HBsAg–Den2E* in this paper). In the second one, the relative positions of the two partners of the gene fusion were reversed to create the hybrid *Den2E–HBsAg* gene. In both cases, the two partners were fused in-frame *via* a *Not*I site. The components of each gene fusion were amplified separately by PCR, restricted with *Not*I and then ligated together to generate the full-length hybrid genes. To construct *HBsAg–Den2E*, the *HBsAg* (amplified with primers 1 and 2) and *Den2E* (primers 3 and 4) PCR products were digested with *Not*I and ligated together to create the full-length chimeric gene. The ligated product was then digested with *Eco*RI and *Xho*I for insertion into the *Eco*RI and *Xho*I sites of pVAX1 (Fig. 2A) or with *Eco*RI alone for insertion into the unique *Eco*RI site of pAO815 (Fig. 3A). The reverse fusion construct, *Den2E–HBsAg*, was created using a similar strategy. The *Den2E* and *HBsAg* PCR products, amplified with primers 5 + 6 and 7 + 8, respectively, were cut with *Not*I and then ligated together. The full-length hybrid gene was then cut with *Eco*RI and *Xho*I as before prior to cloning into pVAX1 (Fig. 2A) or with *Eco*RI alone for cloning into pAO815 (Fig. 3A). Each of the two hybrid proteins encoded by the fusion genes described above is designed to carry the first 395 aa residues of Den2E and all 226 aa residues of HBsAg, with three in-frame alanyl residues linking the two fusion partners. Thus, both fusion genes are expected to encode hybrid proteins of 624 aa each, corresponding to a molecular weight of ~68 kDa.

In Vitro Transcription and Translation

The TNT quick coupled transcription/translation system from Promega was used for the *in vitro* expression of the recombinant constructs as per the manufacturer's instructions. In brief, 1 μl (1.0 μg) of supercoiled plasmid carrying the recombinant gene of interest under the T7 promoter was used in a 25 μl TNT reaction containing 20 μl TNT Quick Master Mix, 2 μl Redivue L-[^{35}S]methionine (1000 Ci/mmol at 10 mCi/ml from Amersham), 0.5 μl cold, 1 mM methionine, and 1.5 μl RNase-free water. The reaction was incubated at 30°C for 90 min and the products analyzed on a 12.5% denaturing polyacrylamide gel, followed by fluorography.

Immunoprecipitation

The *in vitro*-synthesized proteins were analyzed by immunoprecipitation as follows. About 20 μl of protein G–Sepharose was incubated with 20 μg anti-HBsAg mAb in 100 μl phosphate-buffered saline (PBS) for 15 min at room temperature (RT) and washed three times with 1 ml of PBS. Next, 5 μl TNT reaction was added to the anti-HBsAg mAb/Protein G complex in 100 μl and allowed to incubate 30 min at RT. The resultant immunecomplexes were washed with 1X radioimmunoprecipitation assay buffer, boiled in 25 μl Laemmli sample buffer and analyzed on SDS–12.5% PAGE followed by fluorography.

P. pastoris Transformation

Electrocompetent *P. pastoris* GS115 cells (80 μl) prepared from a culture growing in log phase were mixed with 5–20 μg of *Bgl*II linearized expression vector in a 0.2-cm electroporation cuvette. The cells were then pulsed for ~10 ms with a field strength of ~7500 V/cm using a Bio-Rad Gene Pulser. Transformants harboring the plasmid-borne *HIS4* marker were selected on minimal plates lacking histidine and screened by direct PCR analysis as described previously (28). Briefly, single colonies were digested with zymolase (7.5 ng/μl) for 1 h at 30°C [in 35 μl of 10 mM Tris–HCl (pH 8) 10 mM

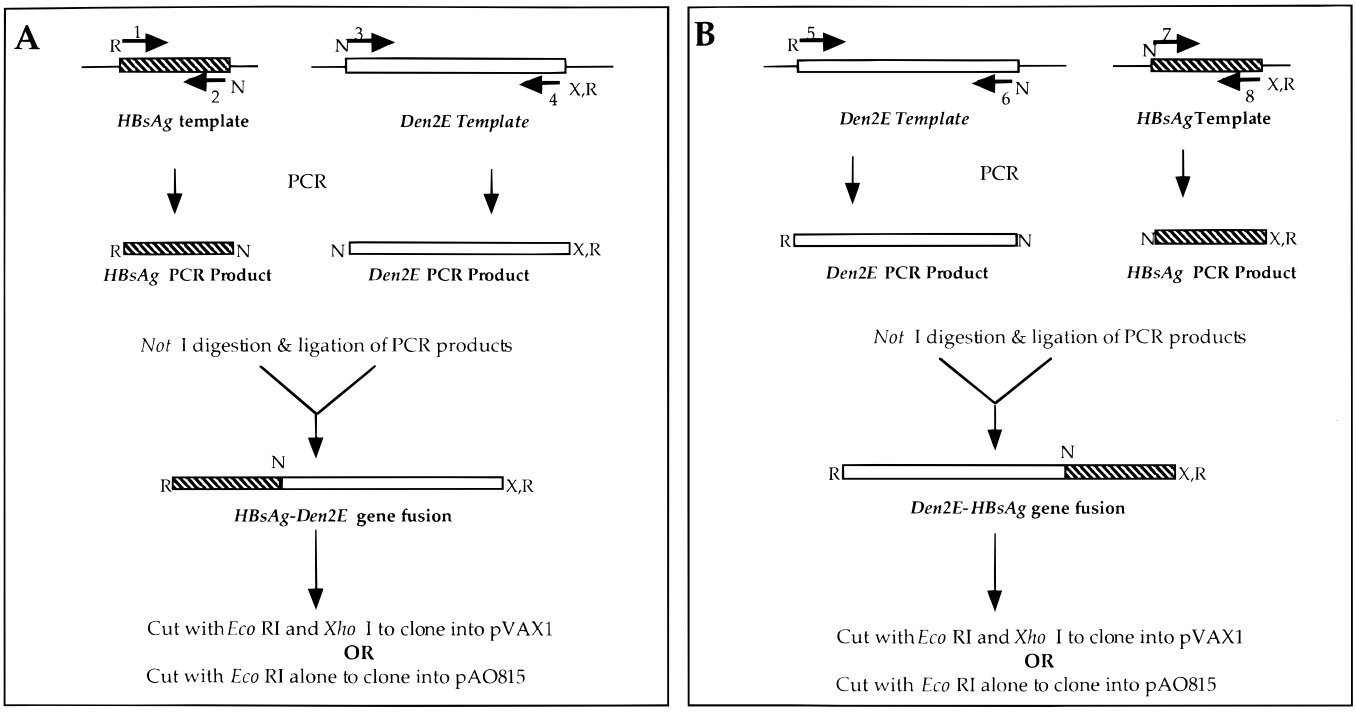

FIG. 1. Strategy for the creation of *HBsAg-Den2E* (A) and *Den2E-HBsAg* (B) fusion constructs. The *HBsAg* (hatched box) and *Den2E* (empty box) genes were PCR amplified using appropriate primers (indicated by the numbered arrows) from plasmids (templates) carrying these genes. The resultant PCR products were fused through a *Not*I site that was engineered into the relevant primers. The resultant fusion products were then restricted with appropriate enzymes to facilitate cloning into either pVAX1 or pAO815 vectors. Restriction enzyme recognition sites are indicated by capital alphabets (N, *Not*I; R, *Eco*RI; X, *Xho*I).

DTT], boiled for 10 min and used in 50 μl PCR reactions containing specific primers. Primer pairs used were 1 + 4 and 5 + 8, respectively, for identifying the *HBsAg–Den2E* and *Den2E–HBsAg* fusion genes. Selected clones were analyzed for expression of the recombinant fusion proteins by ELISA, as described below.

Shake-Flask Cultures

For most routine experiments, small-scale cultures of 100 ml were grown in 500 ml baffled flasks in BMGY medium [100mM potassium phosphate buffer (pH 6)/ 1.34% (w/v) yeast nitrogen base/1% yeast extract/ 2%peptone/4 $\times$ 10^{-5}% biotin/1%(v/v) glycerol] at 30°C with shaking (250 rpm) for 16–18 h to logarithmic phase. For routine protein expression experiments, cultures were induced after 16–18 h with 1% methanol. At the time of induction, the optical density of the cells at 600 nm (OD_{600}) was in the range of 2–6. To initiate induction, cells growing in logarithmic phase, in BMGY, were pelleted down (5000 rpm/5 min/4°C/Sorvall GSA rotor) in sterile centrifuge bottles and resuspended in 10 ml BMMY, transferred to the culture flask, and returned to the shaking incubator. The composition of BMMY is similar to BMGY except that 1% methanol replaces 1% glycerol. Induction was typically carried out for 72 h. During the course of the induction phase, methanol was added every 24 h to a final concentration of 1% (v/v).

Preparation of Soluble Extracts for ELISA

Aliquots of cells corresponding to 100 OD_{600} units were transferred into borosilicate tubes, pelleted by low-speed centrifugation (6000 rpm, 10 min) and washed twice with 10 ml lysis buffer [10 mM phosphate buffer (pH 7.2)/5 mM EDTA/0.1% (v/v) TritonX-100/1 mM PMSF]. Washed cells were resuspended in 0.35 ml lysis buffer containing 0.6-g zirconia glass beads (0.45 μm) and lysed by 10 1-min bursts of vortexing at maximum speed, with chilling on ice for 1-min between bursts. The lysate was spun down and the supernatant collected. The levels of recombinant Den2E–HBsAg hybrid protein in this clarified lysate (soluble extract) were determined by using a commercially available ELISA kit (see below).

ELISA for Soluble Recombinant Den2E-HBsAg Hybrid Protein

Recombinant Den2E–HBsAg hybrid protein in soluble cell extracts, column fractions and CsCl-gradient

fractions were analyzed using the Hepanostika micro-ELISA system (specific for HBsAg 22-nm particles) purchased from Organon Teknika (The Netherlands). Sample aliquots ranging from 10–100 μl (after appropriate dilution when necessary) were assayed as per the manufacturer's instructions. Den2E–HBsAg hybrid protein in fractions eluted from immunoaffinity column was detected using a sandwich ELISA. In this sandwich assay, the hybrid protein was captured on the ELISA plate using anti-Den2E-monoclonal antibody, 3H5 mAb (neutralizing mAb, purchased from ATCC, Catalog No. HB46), and revealed by a particle-specific horse-radish peroxidase (HRPO) conjugated anti-HBsAg polyclonal antibody.

Northern Analysis

Total RNA was prepared from *P. pastoris* transformants harboring the two gene fusions using the RNeasy midi kit purchased from Qiagen. Twenty micrograms of each RNA sample was loaded on a 1.2% agarose gel containing 6% formaldehyde, transferred on to a nylon membrane, and probed separately with $\alpha[^{32}P]$-labeled *HIS4* or *Den2E* DNA. The blots were visualized by autoradiography and scanned using Kodak Digital Science software.

Purification of the Den2E-HBsAg Hybrid Protein

A 2-liter culture of logarithmically growing *P. pastoris* transformant harboring the *Den2E–HBsAg* fusion gene was spun down and induction initiated by resuspending the cell pellet in 200 ml BMMY. Cells were harvested 72 h postinduction. The pellet (~50 g wet weight) was washed twice with 200 ml cold distilled water, once with 100 ml incomplete lysis buffer [10 mM phosphate buffer (pH 7.2)/5 mM EDTA/0.1% Triton 100], and finally resuspended in 100 ml complete lysis buffer (in addition to the ingredients listed for the incomplete buffer, this also contains 0.1% Chaps and 1 mM PMSF). This suspension was mixed with ~300 g of glass beads (0.45 μm) and the cells disrupted using a bead beater (Biospec) in 15 1-min pulses with 1 min off-time between pulses. The lysate was separated from the beads using a sintered glass funnel. The beads were washed twice with ~50 ml cold complete lysis buffer and pooled with the initial filtrate. The volume of the lysate was made up to 250 ml with cold complete lysis buffer. The lysate was then clarified by centrifugation in a Sorvall GSA rotor at 10,000 rpm for 30 min at 4°C. The supernatant was further clarified by acid precipitation as described. The pH of the lysate was adjusted to 5 using ~2–3 ml 1 N phosphoric acid and stirred for 30 min at room temperature. This was centrifuged at 10,000 rpm for 50 min at 4°C. The resultant supernatant was adjusted to pH 6.5 with 1 N NaOH and allowed to bind to 10 grams of aerosil [50% (v/v) suspension in water] at room temperature for 10–30 min with stirring. The unbound proteins were removed by centrifugation at 10,000 rpm for 15 min at 4°C. The bound proteins were eluted from aerosil by gently shaking with 75 ml 0.5 M ammonia for 45 min at room temperature followed by centrifugation at 10,000 rpm for 30 min at 4°C in a GSA rotor. The ammonia elution was repeated a second time and the pooled aerosil eluate (150 ml) was dialyzed against 10mM phosphate buffer, pH 7.2, and allowed to bind to anti-HBsAg antibody coupled to Sepharose (in-house immunoaffinity matrix) for 20–30 min at 37°C. After binding, the immunoaffinity matrix was packed into a column (3 × 4.5 cm). The column was washed with 10 bed volumes of 10 mM phosphate buffer (pH 7.2) and eluted with 100 mM phosphate buffer, pH 11.5, at a flow-rate of 0.5 ml/minute. Ten fractions of 2 ml each were collected and analyzed by a sandwich-ELISA procedure as described above.

Gel Filtration

The Den2E–HBsAg hybrid protein after aerosil elution was dialyzed against 10 mM Tris-HCl (pH 9)/0.1% TritonX-100. Three milliliters of this protein, concentrated from 150 ml of dialyzed aerosil eluate was loaded on a Sepharose CL-4B column (4.8 × 60 cm) preequilibrated with the same buffer. The column was eluted at a flow rate of 1.5 ml/min. Fifty fractions of 12 ml each were collected and analyzed by HBsAg-particle specific ELISA.

Gradient Analysis

Gradient analyses were performed essentially as described before (24). An aliquot (50–100 μl) of the partially purified Den2E–HBsAg hybrid protein was layered on a 20% CsCl gradient (w/v) in 50mM Tris–HCl (pH 7.5) in an SW41 polyallomer tube and centrifuged at 35, 000 rpm for 48 h at 16–18°C in a Beckman ultracentrifuge. After the spin, the centrifuge tube was punctured at the bottom and 0.5 ml fractions of the gradient were collected and analyzed using the particle-specific ELISA kit described above.

Electrophoresis

Proteins were separated on 12.5% polyacrylamide gels under denaturing conditions (SDS–PAGE) as per the standard protocol (29). Nonradioactive proteins were visualized using Coomassie blue stain. ^{35}S-Labeled proteins were visualized by fluorography of dried gels.

Protein Estimation

Routine protein estimation was done according to Bradford (30), with bovine serum albumin (BSA) as the standard.

RESULTS

In Vitro Analysis of the Chimeric Gene Constructs

In order to verify if the two fusion genes did indeed code for the predicted protein chimeras, the pVAX1 based fusion constructs were transcribed with T7 RNA polymerase and translated in the presence of L-[^{35}S]methionine *in vitro* using a commercial coupled TNT kit. The radioactive proteins generated in the *in vitro* reaction were analyzed on a 12.5% denaturing gel. The results are shown in Fig. 2B. The TNT reactions were analyzed directly in lanes 1 and 2. In both these lanes a major ~68-kDa band can be easily discerned, consistent with the predicted sizes of HBsAg–Den2E and Den2E–HBsAg hybrid proteins. In the case of the latter, a doublet was seen at the predicted position. A second translational initiation event can be ruled out, as the fusion gene does not have a second in-frame ATG codon at its 5′ end. It is unlikely that the doublet is the result of proteolytic degradation. If this were to be the case, a similar doublet would likely have been evident in the HBsAg–Den2E lane (compare lanes 1 and 2). The slightly faster migrating band presumably represents the product of incomplete translation. While the TNT analysis demonstrated that proteins of the predicted sizes were made from the two gene fusions, it does not provide information regarding the identity of the *in vitro*-synthesized products. Therefore, the TNT products were analyzed by immunoprecipitation using an anti-HBsAg mAb. Examination of lanes 3 and 4 (Fig. 2B) shows that the anti-HBsAg mAb was able to recognize proteins of ~68 kDa. Further, it can be seen that the band pattern in lanes 3 and 4 exactly mirrors that seen in lanes 1 and 2, respectively. Thus, this analysis suggested that the two fusion genes did indeed encode the intended chimeric proteins.

Intracellular Expression of the Gene Fusions in P. pastoris

After confirming that the two fusion genes were capable of generating the predicted hybrid proteins *in vitro*, we next proceeded to express these genes in *P. pastoris*. To this end, we constructed yeast expression vectors by cloning the *HBsAg–Den2E* and *Den2E–HBsAg* fusion genes as *Eco*RI inserts into the unique *Eco*RI site of pAO815 (Fig. 3A) as described above. These vectors were then transformed into the histidine requiring auxotrophic *P. pastoris* host strain GS115. The transformation strategy adopted was designed to target integration of the hybrid protein-expression cassette plus the HIS4 marker into the *AOX1* locus of the *P. pastoris* genome. Transformants were selected on minimal plates lacking histidine and screened for the presence of the hybrid gene by PCR using appropriate primer

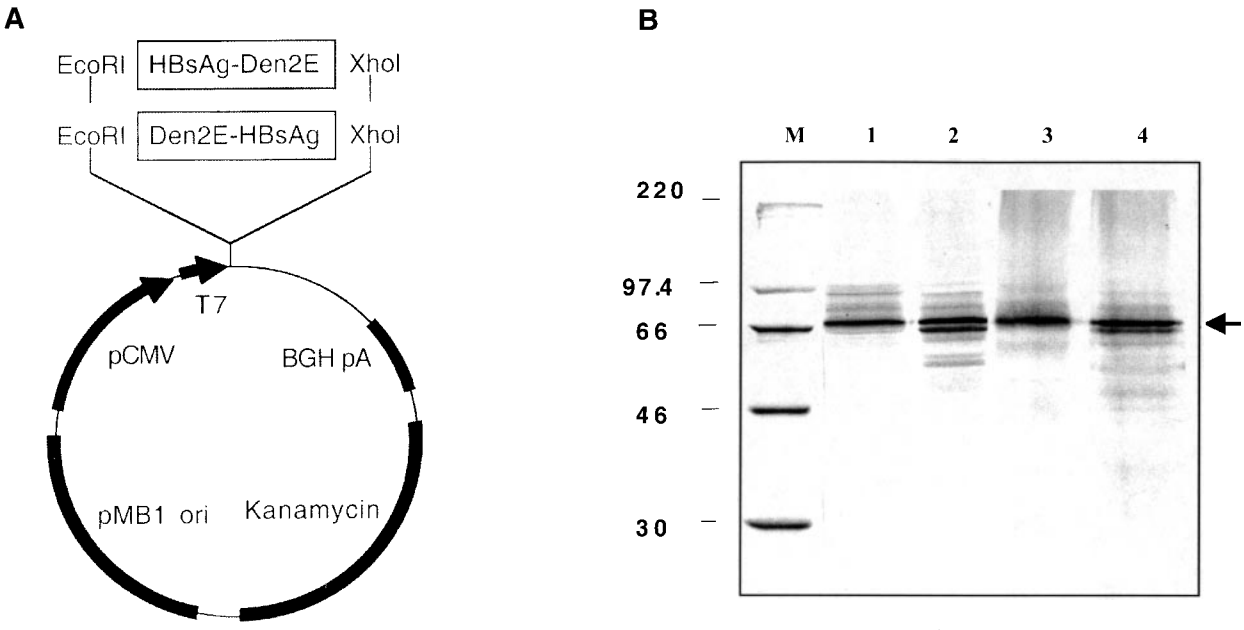

FIG. 2. *In vitro* expression analysis of the fusion constructs. (A) Plasmid pVAX1-based T7 expression vector. The fusion genes (described in the legend of Fig. 1) were digested with *Eco*RI and *Xho*I and inserted into pVAX1, immediately downstream of the phage T7 promoter to permit *in vitro* transcription and translation. (B) SDS–PAGE analysis of *in vitro*-synthesized, ^{35}S-radiolabeled HBsAg-Den2E (lanes 1 and 3) and Den2E-HBsAg (lanes 2 and 4) fusion proteins before (lanes 1 and 2) and after (lanes 3 and 4) immunoprecipitation with anti-HBsAg mAb. ^{14}C-labeled protein molecular weight markers were loaded in lane M. The molecular weights (in kDa) are shown on the left of the panel. The arrow to the right indicates the position of the fusion proteins.

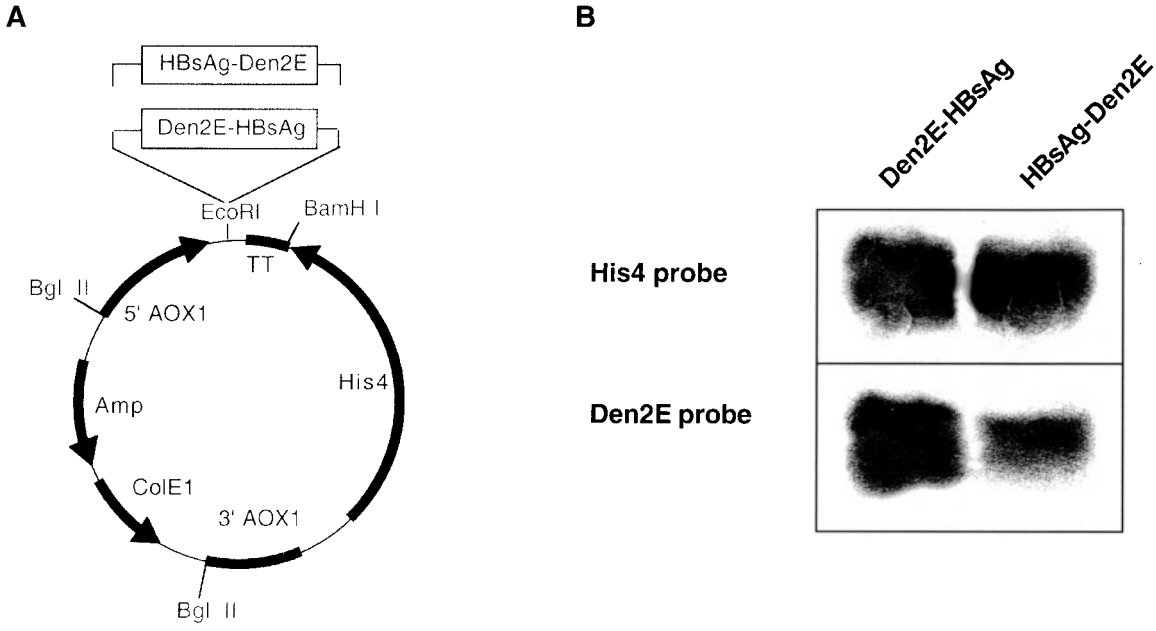

FIG. 3. *In vivo* expression of the fusion constructs in *Pichia pastoris*. (A) Plasmid pAO815-based *Pichia* integrative vector. The fusion genes (described in the legend of Figure 1) were digested with *Eco*RI and inserted into the unique *Eco*RI site of pAO815, under the control of the methanol-inducible, *AOX1* promoter. (B) Northern analysis of *Pichia* clones harboring either the *Den2E-HBsAg* or *HBsAg-Den2E* fusion genes. The ^{32}P-labeled probes used to identify the fusion gene-specific messages are indicated on the left of the panel.

pairs as described under Materials and Methods. Test-tube cultures of selected *P. pastoris* transformants were grown overnight and induced with 1% methanol to check for expression of the recombinant fusion proteins by ELISA (using Hepanostika kit). We consistently noticed relatively greater levels of recombinant protein expressed by *P. pastoris* clones harboring the *Den2–HBsAg* fusion gene as compared to the clones harboring the reverse fusion gene, *HBsAg–Den2E* (data not shown). This prompted us to examine the steady-state levels of the corresponding mRNAs. To this end, total RNA was isolated from two representative *P. pastoris* clones (carrying the two different fusion genes) after methanol induction and subjected to Northern analysis using a *Den2E*-specific probe. *HIS4* mRNA was analyzed in the same blot as an endogenous control in order to normalize the RNA levels. The results are shown in Fig. 3B. It is evident that the steady-state level of *Den2E–HBsAg* mRNA is two- to three-fold greater than that of *HBsAg–Den2E* mRNA. Since these data showed that Den2E–HBsAg could be expressed comparatively more efficiently than HBsAg–Den2E, subsequent studies focused on the former hybrid protein.

Optimization of Den2E-HBsAg Expression in Shake-Flask Cultures

We next proceeded to investigate the conditions necessary to achieve optimal induction of Den2E-HBsAg expression in *P. pastoris*. First, the expression of Den2–HBsAg was measured at varying inducer concentrations ranging from 0.5–3% methanol, keeping the duration of induction constant at 48 h (Fig. 4A). At 0.5% (v/v) methanol, the level of induction was quite modest. Increasing the methanol concentration to 1% (v/v) was accompanied by a significant enhancement (~4.5-fold) in the magnitude of induction. Increasing the methanol concentration thereafter resulted in diminished induction. At methanol concentrations between 1.5–3%, the level of induction was diminished by ~20–45% with respect to induction obtained at 1% (v/v) methanol. At concentrations exceeding 1% (v/v), the decrease in recombinant protein accumulation could perhaps be attributed to the toxic effects of increased formaldehyde production from methanol as well as decreased cell growth. It has been shown that growth of *P. pastoris* is markedly inhibited at high methanol concentrations (31).

Next, the expression of the recombinant protein was monitored as a function of duration of induction (Fig. 4B). In this experiment, a logarithmically growing *P. pastoris* culture was induced with 1% (v/v) methanol. Induction was maintained for a total period of five days by the addition of methanol to 1% (v/v) final concentration at 24-h intervals. Aliquots of the culture were withdrawn at daily intervals and analyzed by ELISA (Hepanostika kit). From the data in Fig. 4B, it can be seen that maximal expression was attained 72 h postinduction. Beyond three days, it is likely that toxic metabolites

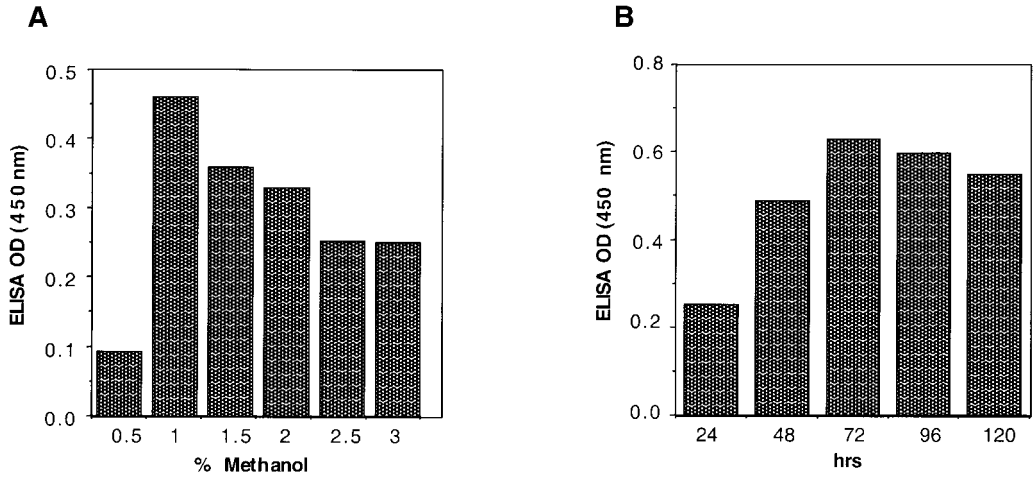

FIG. 4. Optimization of methanol induction of Den2E-HBsAg expression in *Pichia pastoris*. (A) Effect of methanol concentration on the expression of recombinant fusion protein. Small-scale cultures, growing in logarithmic phase, were induced in parallel with varying concentrations of methanol (ranging from 0.5 to 3%) for 48 h. (B) Effect of duration of methanol induction on the expression of recombinant fusion protein. Aliquots were withdrawn from a culture at the indicated time points after initiating induction with 1% (v/v) methanol. In both experiments, the levels of recombinant protein in the samples were determined using the Hepanostika ELISA kit.

accumulate in the culture medium to growth-inhibitory levels and this could in turn adversely affect recombinant protein expression levels. Another possibility is that dissolved oxygen, which can be a limiting factor in shake-flask cultures, could be responsible for decreased cell growth and the resultant decline in the levels of recombinant protein production. The data in Figs. 4A and 4B indicate that induction times beyond three days and methanol concentrations higher than 1% (v/v) can result in a reduction in recombinant protein accumulation. Accordingly, in subsequent experiments, induction was carried out in the presence of 1% (v/v) methanol for 72 h.

Purification of Recombinant Den2-HBsAg Hybrid Protein

Based on the results above, ~50 g cells (wet weight, from a 2-liter culture of the appropriate *P. pastoris* clone) were induced with 1% (v/v) methanol for 3 days and used as the starting material for the purification of recombinant Den2E–HBsAg hybrid protein. The major purification steps are summarized in Table 1. Essentially, the purification strategy involved clarification of the initial lysate by acid precipitation followed by hydrophobic interaction chromatography on aerosil. Final purification was achieved by immunoaffinity chromatography on immobilized anti-HBsAg antibody column. Column fractions from this immunoaffinity step were assayed using a sandwich ELISA procedure designed to specifically detect the hybrid alone. This assay utilized two neutralizing antibodies, one specific for the Den2E component (3H5 mAb) and the other specific for the HBsAg component of the hybrid protein. In this sandwich assay, neither the component proteins (separate Den2E and HBsAg proteins) nor extracts prepared from untransformed *P. pastoris* GS115 cells elicit any significant positive signals. The immunoaffinity elution profile of Den2E–HBsAg obtained using this sandwich assay is depicted in Fig. 5A. The peak fractions from the immunoaffinity column were pooled together and dialyzed against 10 mM phosphate buffer, pH 7.2. An aliquot of this immunopurified preparation was analyzed on a denaturing polyacrylamide gel and visualized by Coomassie stain as shown in Fig. 5B. In this gel the purified Den2E–HBsAg fusion protein was run alongside bovine serum albumin (BSA) as a reference in addition to the regular low molecular weight protein markers. One major protein species migrating at ~67–

TABLE 1

Summary of the Purification of rDen2-HBsAg Hybrid Protein

Step	Total Protein[a] (mg)	Total ELISA ODs[b]	Yield[c] (%)	Fold[d] purification
Cell lysate	1252	21.3×10^3	100	1
Acid Precipitation	740	11.6×10^3	54	1.7
Aerosil eluate	145	7.9×10^3	37	8.6
Immunoaffinity eluate	0.9	5.8×10^3	26	1391

[a] Total protein was estimated with Bradford reagent using BSA as standard.

[b] ELISA ODs in appropriately diluted samples were measured at 450 nm using Hepanostika Sandwich assay with developing time of 30 min.

[c] Yields reported are based on ELISA ODs.

[d] Fold purificaton is based on total protein recovered.

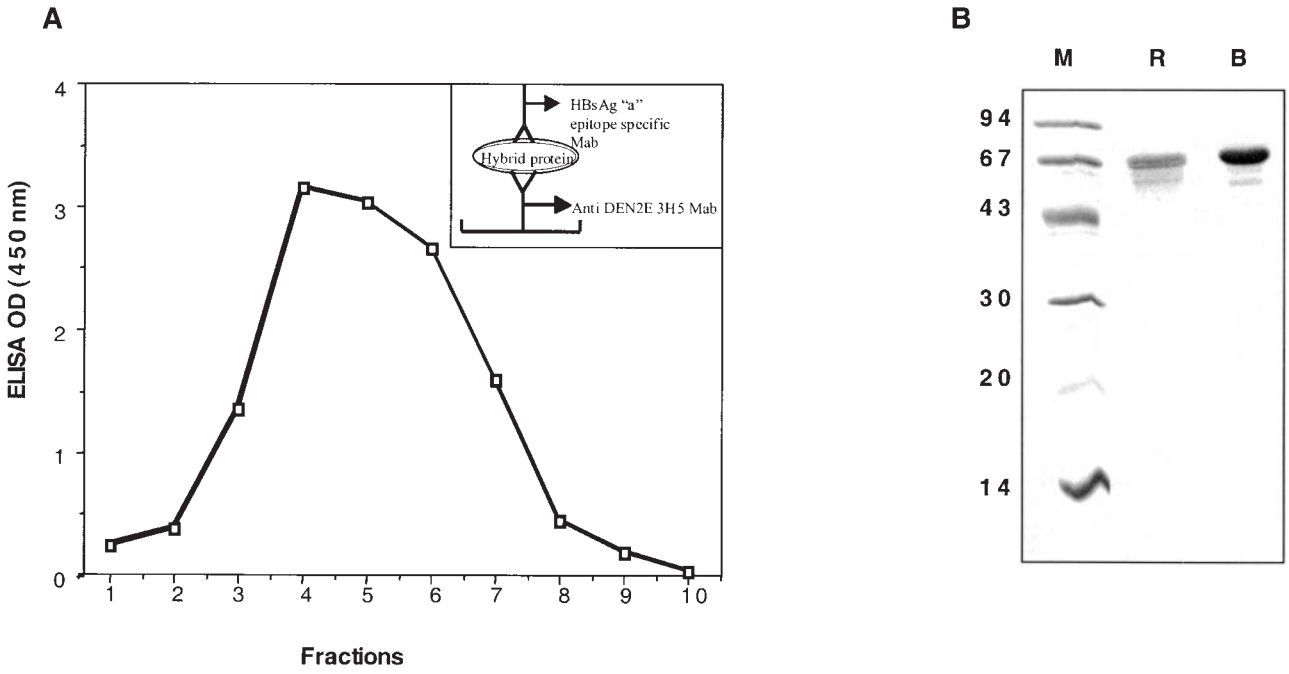

FIG. 5. Immunoaffinity purification and SDS-PAGE analysis of the recombinant hybrid protein. (A) Immunoaffinity chromatographic elution profile of recombinant Den2E-HBsAg hybrid protein. Column fractions were analyzed by a customized sandwich ELISA procedure, designed to score for both components of the hybrid protein (inset). (B) SDS–PAGE analysis of the immunoaffinity purified recombinant hybrid protein (lane R) run along with BSA (lane B) for comparison. Low molecular weight protein markers were loaded in lane M (their sizes, in kDa, are shown on the left of the panel).

68 kDa was present in the purified preparation. This electrophoretic mobility is consistent with the predicted molecular weight of the Den2E–HBsAg fusion protein (624 aa protein with a predicted molecular weight of ~68 kDa). Taken together, the data presented in Figs. 5A and 5B demonstrate that indeed the *P. pastoris* system is able to generate the intended hybrid protein consisting of Den2E and HBsAg components (based on sandwich ELISA data) of the predicted molecular weight (based on SDS–PAGE analysis). Further, the data presented in Table 1 taken in conjunction with SDS–PAGE analysis (Fig. 5B) show that the strategy we adopted to purify the recombinant hybrid protein resulted in approximately 1400-fold purification with a recovery of 26% (corresponding to ~1 mg pure protein) with respect to the initial crude lysate. The purity of the final preparation was judged to be ~95%.

Recombinant Den2E-HBsAg Assembles into High Molecular Weight Aggregates

One of the reasons for using HBsAg as the fusion partner for Den2E expression was the expectation that the HBsAg component would confer on the hybrid protein its inherent capacity to self-aggregate and give rise to high moleular weight VLPs. The ability of HBsAg expressed in *P. pastoris* to assemble into virus-like particles is well documented (24, 25). We therefore investigated whether the Den2E-HBsAg fusion protein also possesses this ability to generate VLPs. Figure 6A displays the elution profile of a partially purified preparation of Den2E–HBsAg on a Sepharose CL-4B gel filtration column (4.8 × 60 cm). The column fractions were analyzed using a HBsAg particle-specific ELISA (Hepanostika). The recombinant fusion protein eluted close to the void volume of the column indicating its multimeric nature. The sedimentation behavior of the recombinant fusion protein, centrifuged through a 20% CsCl density gradient, was investigated. The sedimentation analysis was performed with two different samples of the recombinant fusion protein. One was the crude lysate and the other was a partially purified preparation. This was done to estimate the relative proportion of monomers in the recombinant fusion protein preparation. Fractions were collected from the bottom of the gradient and analyzed by ELISA using the same particle-specific anti-HBsAg antibody described above. The resultant data profiled in Fig. 6B show a major peak sedimenting in the bottom half of the gradient for both samples of the recombinant fusion protein (fraction 8). In a parallel gradient, HBsAg VLPs displayed a very similar sedimentation profile (data not shown). The peak fraction containing Den2E–HBsAg particles (fraction 8) and the top fraction (fraction 20) expected to contain monomeric forms if any, from both samples, were analyzed using a linear epitope specific anti-HBsAg mAb (capable of recognizing HBsAg irrespective of its conformation).

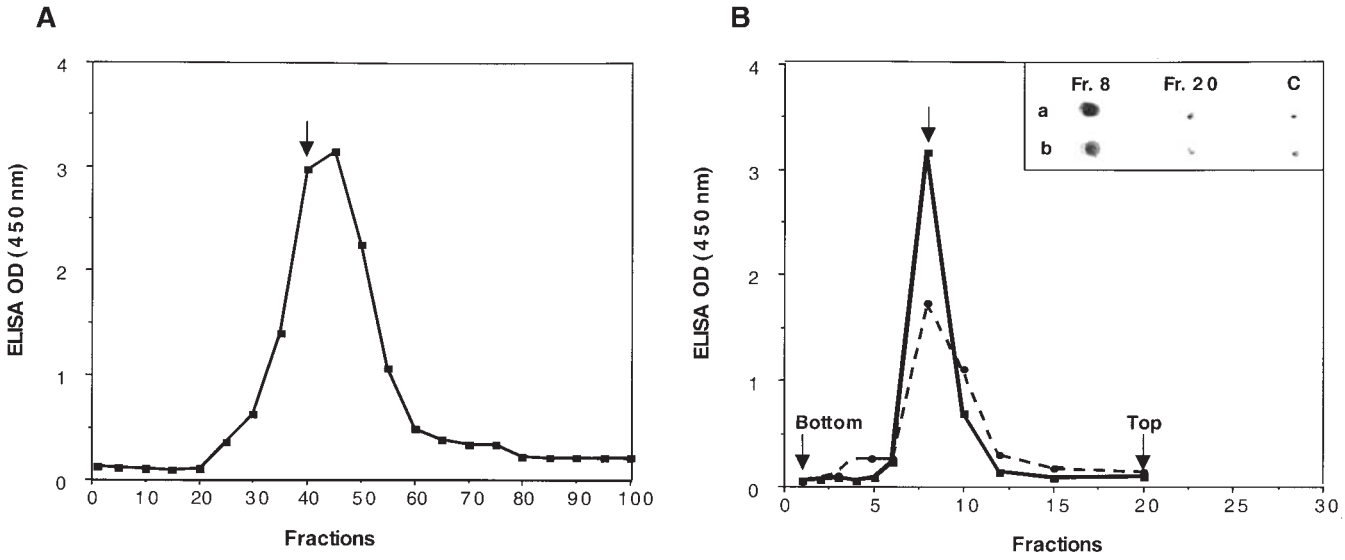

FIG. 6. Characterization of the particle-like behavior of recombinant hybrid protein. (A) Gel exclusion chromatographic elution profile of recombinant Den2E-HBsAg hybrid protein on a Sepharose CL-4B column (4.6 × 60 cm). The arrow above the peak indicates the void volume peak. (B) Sedimentation profile of the recombinant hybrid protein, through a 20% CsCl density gradient. The arrow above the peak represents the position of sedimentation of HBsAg particles run in a parallel gradient. Both Sepharose CL-4B as well as density gradient fractions were analyzed using a particle-specific anti-HBsAg antibody (Hepanostika). The inset in B shows a Western blot in dot-blot format (performed with a linear epitope-specific anti-HBsAg mAb) of the peak fraction (Fraction 8) and the top fraction (fraction 20) obtained from crude (dashed curve) and partially pure (solid curve) recombinant fusion protein. C represents a control dot-blot developed in parallel using an aliquot of 20% CsCl.

The results from this experiment, shown in Fig. 6B, inset, did not reveal the presence of any detectable monomeric form of the recombinant fusion protein both in crude as well as partially purified samples. The above data indicate that the recombinant Den2E–HBsAg fusion protein indeed assembles into high molecular weight aggregates akin to HBsAg VLPs when expressed in *P. pastoris*. The failure to detect monomers of the recombinant protein even in the crude lysate may be attributable to the slow methanol feed rate during an induction phase spanning three days. Under such conditions, it is likely that there is adequate opportunity for practically all of the recombinant protein monomers expressed in *P. pastoris* to assemble into particles. We have observed similar HBsAg particle assembly using a recombinant *P. pastoris* clone harboring as many as eight copies of the *HBsAg* gene (24).

DISCUSSION

We have explored the possibility of utilizing *P. pastoris* as a host system for the expression of the envelope proteins of dengue viruses. To begin with, we have focused on the E protein of dengue virus type 2 (Den2E). Several lines of evidence indicate that dengue E protein is a potential vaccine candidate. In experimental animals, passive immunization with anti-E-monoclonal antibodies has been shown to protect against dengue infection (9). Purified E protein can induce the production of neutralizing antibodies and confer protection against lethal dengue virus challenge (10, 11). Further, the E protein is responsible for eliciting the first and longest lasting antibody response (8). As a result, considerable effort has been focused on the expression of recombinant E protein in an attempt to create a subunit vaccine. The E protein has been expressed using both prokaryotic (12, 13) as well as eukaryotic systems (11, 12, 14–17). Although, the *E. coli*-based system has been used successfully to express defined domains of the E protein for the purpose of antigenic structure analysis (32), this system with its reducing intracellular environment does not appear to be appropriate for the expression of properly folded E protein. Therefore, an eukaryotic expression system is preferable as it can ensure proper folding of the recombinant E protein molecule. Of the eukaryotic systems available for the heterologous expression of the dengue E protein, the tissue culture based baculovirus- (11, 15) and vaccinia virus-expression (16, 17) systems can be very expensive. From the perspective of cost-effective large-scale expression of heterologous proteins, the methylotrophic yeast, *P. pastoris*, is an ideal host. However, information available in the literature regarding the use of *P. pastoris* for the expression of dengue viral antigens is very limited. Sugrue *et al.* (12) found that expression of full-length

E protein of dengue virus type 1 as a glutathione S-transferase fusion, in *P. pastoris*, was accompanied by extensive proteolysis of the recombinant protein. Deleting the carboxy terminal hydrophobic domain of the E protein could minimize this problem (12). Accordingly, we decided to express a similarly truncated version of Den2E. In order to minimize the potential for proteolysis even further, we chose to use as fusion partner the HBsAg protein, known to be quite stable in the *P. pastoris* host. Further, HBsAg has been used as a carrier to express antigens of poliovirus (33), human immunodeficiency virus 1 (34), and *Plasmodium falciparum* (26), because it is a good immunogen by virtue of its ability to generate high molecular weight polymers. HBsAg has been successfully used as a fusion partner to augment the immunogenic potential of the circumsporozoite protein of *P. falciparum* to develop a malarial vaccine candidate, called RTS, S, which spontaneously assembles into particles (26). Thus, the choice of HBsAg has two obvious advantages. First, it may confer protection against proteolytic cleavage of the resultant fusion protein. Second, the inherent ability of HBsAg to spontaneously assemble into highly immunogenic virus-like particles (VLPs) may be retained in the fusion protein.

We successfully expressed the first 395 aa residues of the envelope protein of dengue type 2 virus (Den2E) fused in frame to the 226 aa major surface antigen protein of hepatitis B virus (HBsAg). The fusion protein, Den2E–HBsAg, was purified using a simple strategy that involved a preliminary acid precipitation step, followed by hydrophobic interaction chromatography on an aerosil (silica) matrix, and finally affinity chromatography on an anti-HBsAg antibody column. Fold purification obtained at the acid precipitation step (Table 1) is relatively insignificant. However, this step is essential as it greatly facilitates in clarifying the initial lysate, by getting rid of lipids that can interfere in the subsequent aerosil-binding step (35). The aerosil step, which resulted in ~9-fold enrichment was incorporated in the purification strategy to take advantage of the relatively strong hydrophobic property of the HBsAg component of the recombinant fusion protein. The final immunoaffinity step resulted in a further ~160-fold purification. In our experience, it was necessary to include the prior acid precipitation and aerosil binding steps to achieve maximal purification in the final immunoaffinity step. The purification strategy per se has worked satisfactorily considering that we have been able to achieve an overall purification of nearly 1400-fold with a recovery of 26%. However, in terms of absolute quantities, the yield is quite low. Starting from ~50 g (wet weight) of *P. pastoris* cells, ~1 mg purified recombinant protein could be obtained (Table 1). Proteolytic degradation contributing to low yields can be ruled out based on SDS–PAGE analysis of the purified hybrid protein, which revealed a single major band of the predicted electrophoretic mobility. No other bands, that may be indicative of the presence of degradation products, are evident. Inefficient and or improper particle assembly (in conjunction with the elimination of unassembled/improperly assembled Den2E-HBsAg during purification) contributing to the recovery of low levels of particulate Den2E-HBsAg can be ruled out based on the immunoblot data obtained with CsCl gradient fractions of crude and purified recombinant fusion protein. Apparently, low expression levels are responsible for the low yield of recombinant protein obtained. One obvious reason for low expression is that only one copy of the fusion gene has been integrated into the genome of *P. pastoris*. It should be possible to enhance expression levels using higher gene dosage as reported for HBsAg by Vassileva *et al.* recently (24). This was done by an *in vitro* sequential multimerization approach. The strategy relies on inserting additional copies of the recombinant gene in the form of 5′ *Bgl*II–*Bam*HI 3′ fragments into the *Bam*HI site of the *P. pastoris* integrative vector (see Fig. 3A). Using this strategy, we have cloned DNA inserts as large as 16 kb into genome of *P. pastoris* (24). The presence of an internal *Bam*HI site in the *Den2E* gene is not compatible with this *in vitro* multimerization strategy. In order to circumvent this potential problem, we are currently deleting the internal *Bam*HI site of the *Den2E* gene without altering the amino acid sequence of the encoded protein. We believe that increasing the copy number can contribute significantly to the enhancement of overall expression levels. Another factor that could be responsible for the low recombinant protein yield may pertain to the efficiency of induction. In shake flask cultures, we maintain the induction phase by periodic methanol addition to a final concentration of 1% (v/v). It is quite conceivable that induction is maximal immediately following the methanol "pulse," and tapers off with time as the methanol is utilized. Thus, lack of maintenance of uniform and sustained induction phase could be partly responsible for low recombinant protein yields. It is, therefore, very likely that scale-up of the shake-flask to fermentor cultures can greatly improve recombinant protein yields, since critical parameters such as dissolved oxygen and methanol feeding strategy that influence cell growth and induction can be very precisely controlled. Extraction of soluble protein from methanol induced *P. pastoris* using glass beads and vortex mixer is inefficient and highly variable. More efficient and reproducible extraction methods have to be devised to improve overall yields of recombinant protein from *P. pastoris* cells.

We designed a sandwich ELISA protocol to specifically detect the hybrid protein. In this assay, the hybrid protein was first captured on the ELISA plate using

3H5 mAb, a Den2E-specific neutralizing antibody, followed by detection with a particle-specific anti-HBsAg polyclonal antibody coupled to HRPO. The identity of the purified recombinant hybrid protein was confirmed in terms of its constituent components using this customized sandwich ELISA method. Successful capture of the hybrid protein indicates that the accessibility of the neutralizing epitope (on the Den2E component) to 3H5 mAb has not been compromised as a result of fusion to HBsAg. It has been shown that HBsAg expressed in *P. pastoris* assembles into particulate structures (24, 25). In the light of this, it was important to know if HBsAg expressed in *P. pastoris* as a fusion protein retains its ability to form particles. Preliminary indication, that the HBsAg component of the Den2E-HBsAg hybrid protein exists in particle form, came from the initial experiments, wherein putative *P. pastoris* clones were screened for recombinant fusion protein expression using particle-specific ELISA (Hepanostika). This was reconfirmed using purified Den2E–HBsAg protein using the sandwich ELISA discussed above, in which the revealing antibody was particle-specific. This is a neutralizing antibody that recognizes the "a" determinant, which is a conformational epitope on particulate HBsAg. It does not recognize monomeric HBsAg. Further evidence, that Den2E–HBsAg assembles into higher order structures akin to HBsAg VLPs, is based on molecular exclusion chromatography and sedimentation analysis. The recombinant fusion protein eluted close to the void volume of a Sepharose CL-4B column, indicating its macromolecular nature (Fig. 6A). On a CsCl density gradient, the recombinant fusion protein migrated to a position very similar to that of HBsAg VLPs (Fig. 6B). Our observation, that Den2E–HBsAg is produced in particulate form, is consistent with earlier reports that describe hybrid particles formed between HBsAg and other viral (33, 34) or protozoan antigens (26). Sugrue and coworkers reported the formation of low levels of VLPs when they coexpressed all three structural proteins (C, prM, and E) of dengue type 1 virus in *P. pastoris* (14). An interesting observation in this study is that dengue E protein can be incorporated into VLPs in *P. pastoris* in the absence of other dengue virus structural proteins.

In conclusion, this work has demonstrated the expression, in *P. pastoris*, of a novel chimeric protein derived by fusion of the surface protein of Hepatitis B virus with the envelope protein of type 2 dengue virus. A significant finding is that this hybrid protein, Den2E-HBsAg, retains the inherent property of the HBsAg molecule to self-associate and generates high molecular weight VLPs. Further, neutralizing antibodies specific to both components recognize it. This has important implications for multivalent vaccine development.

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