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Expression of hepatitis B virus surface antigen in yeast

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ABSTRACT

The structural gene of Hepatitis B virus surface protein (HBsAg) was introduced into a plasmid capable of autonomous replication and selection in both the yeast Saccharomyces cerevisiae and E. coli. In this plasmid transcription of the HBsAg is initiated by the 5'-flanking sequence of the yeast 3-phosphoglycerate kinase (PGK) gene and terminated by the 3'-flanking region of the yeast TRP1 gene. Yeast cells containing this plasmid produce a new major species of mRNA of 1200 nucleotides in length coding for HBsAg. Viral surface antigen is made in nonglycosylated form at a level of about 1-2 percent of total yeast protein. A small fraction of this polypeptide (2-5 percent) is found in aggregated form upon yeast cell disruption by glass beads. This material is similar in size, density, and shape to the 22nm particle, isolated from the plasma of human hepatitis carriers, and induced comparable levels of HBsAg antibodies in mice when compared with the natural particle.

INTRODUCTION

Hepatitis B is among the most serious human viral diseases now afflicting the world's population. In the U.S. alone there are an estimated 100,000 - 200,000 cases each year of which some 5 to 10 percent become chronic virus carriers. There are more than 170,000,000 Hepatitis B carriers in the world (1). The virus has also been shown to be a causative agent for liver cirrhosis and primary hepatocellular carcinoma (1). Therefore an economic vaccine for this disease has become a major focus of recombinant DNA technology (2).

The infectious Hepatitis B virus particle (Dane Particle), isolated from human plasma, measures 43 nm and contains a circular DNA genome of $2x10^6$ daltons (3). The major structural protein of the virus is the viral surface antigen (HBsAg) polypeptide or "Australia" antigen in glycosylated (P2; 27,000 daltons) and nonglycosylated (P1; 23,000 daltons) forms (4). Other proteins present in the virus are "core" antigen (HBcAg), viral DNA polymerase, minor viral proteins including the "e" antigen (HBeAg), as well

as host cell proteins (5). Several other types of viral derived particles have been isolated from the plasma of infected individuals including a non-infectious 22 nm particle (6). It is composed primarily of HBsAg and lacks HBcAg and viral DNA (7). This material, when extensively purified, is an effective vaccine against the disease (8).

The circular genome of the virus is a partially double stranded DNA about 3200 nucleotides in length (9). The structural genes coding for the HBsAg and HBcAg have been localized by DNA sequencing of the HBV gene cloned into E. coli (10). The HBsAg gene has 226 codons coding for a 25,400 dalton protein and can be readily aligned with the known amino-terminal and carboxy-terminal sequences of the HBsAg polypeptide (11). Until recently the exact ATG start of the gene was uncertain due to the presence of a large open reading frame 5' to the amino terminal codons of the gene. Additional methionine codons in this open reading frame have resulted in speculations about a larger HBsAg precursor polypeptide (2). However, recent experiments by Liu et al. (12) demonstrate that the nucleotide sequence coding for the HBsAg is sufficient for the synthesis and release of 22nm-like particles from tissue culture cells. This system as well as other tissue culture systems (13), including cultured primary human hepatoma cells (14), are thus attractive alternative sources for noninfectious HBsAg particles.

Several studies reported the production of Hepatitis B polypeptides in microorganisms including E. coli (15) and Bacillus subtilis (16). Although polypeptides produced in these organisms [or synthetic polypeptides (17)] appear to bind hepatitis antibodies (15), none has been shown to induce an immunogenic response with the same potency as the HBsAg 22 nm particle. Recently, plasmids have also been developed for expression of heterologous (non yeast) genes in yeast. This was first demonstrated by Hitzeman et al. (18) using a hybrid expression system composed of the 5'-flanking sequence of the yeast alcohol dehydrogenase I gene (19, 20), the human leukocyte interferon D gene (21), and the 3'-flanking sequence of the yeast TRP1 gene (22, 23). Transcription initiation and termination signals were supplied by these flanking sequences and interferon was expressed. Valenzuela et al. (24) have now demonstrated HBsAg expression in yeast mediated by the same yeast alcohol dehydrogenase promoter fragment. They have shown by analytical centrifugation and electron microscopy, that the HBsAg produced in yeast extracts is of similar size and density as natural serum-derived 22nm particle. Using a different yeast promoter system we have also demonstrated the presence of this HBsAg particle in yeast extracts and after

purification compared its antigenicity with the natural serum-derived 22 nm particle. However, although we have obtained similar or higher yields of particle as compared to Valenzuela et al. (24), we have found that the HBsAg monomer present in this particle form represents only 2 to 5 percent of the total monomer produced in yeast.

MATERIALS AND METHODS

Materials. Restriction endonucleases, T4 DNA ligase, and polynucleotide kinase were purchased from BRL or New England Biolabs and used as recommended by the manufacturers. E. coli DNA polymerase I (large fragment) was from Boehringer and S1 nuclease from Miles. ATP and deoxyribonucleotide triphosphates were obtained from PL Biochemicals while ^{32}P-labeled nucleotides were obtained from Amersham Radiochemicals. Glass beads (0.45-0.50 mm) were purchased from B. Braun Melsungen AG. HBsAg derived from plasma was obtained from North American Biologicals. HBsAg from tissue culture was obtained from LCW cells (14).

Strains, plasmids, and growth conditions. E. coli K-12 strain 294 (end A, thr$^-$, hsr$^-$, hsm$_K$+) (25) was used for all bacterial transformations. S. cerevisiae strains 20B-12 (α trp1 pep4-3)(26), XV610-8C (leu2 trp1 ade6 ade2 lys1 can1) and GM3C-2 (a leu2-3 leu2-112 trp 1-1 his4-519 cyc1-1 cyp3-1) (27) were used for yeast transformations. Five of the plasmids used have been described previously, pHS94 (12), pB1 (28), YEp13 (also called pCV13) (29), YRp7 (22), and pFRL4 (18). The plasmid pFRL4 is a derivative of YRp7 where one of the two EcoRI sites in this plasmid (that between the TRP1 gene and the ApR gene) has been removed (18). The plasmid pPGK-1600 (previously called pPGK-1500) is a derivative of pB1 and pFRL4 where the 5'-flanking sequence of the 3-phosphoglycerate kinase (PGK) gene, initially derived from pB1, was incorporated into the EcoR1 site of pFRL4 (see Fig. 2).

LB medium for E. coli growth was as described by Miller (30) with the addition of 20 μg/ml ampicillin (Sigma) for selection of plasmid transformants. Yeast were grown in these previously described (31) media: YEPD (nonselective), YNB+CAA (used for Trp$^+$ selection), and YNB-leu (leucine absent for Leu$^+$ selection). The transformed yeast were always grown in media for selective maintenance of the plasmid.

Yeast transformations, extracts, and HBsAg assays. The plasmids pYeHBs, pYeHBsd, and YEp13 were used to transform yeast strains XV610-8C, GM3C-2, or 20B-12 to leucine or tryptophan prototrophy. Strains were grown in YEPD and

prepared for transformation using standard methods (32). The transformant colonies were picked and grown in 10 ml of selective media at 30° with aeration to an absorbance of 660 mμ of 1.0. The cells were collected by centrifugation (5 min. at 5000 x g) and resuspended in 500 μl PBS (20 mM sodium phosphate pH 7 and 0.14 M NaCl). To this 1.5 g of sterile glass beads (.45 - .50 mm) were added and vortexed 5 min using an ice bath to keep the mixture cool. The mixture was centrifuged 5,000 x g for 1 min at 4°C and the supernatant (200 μl sample) assayed using the HBsAg radioimmune assay kit (Ausria^{R}) from Abbott Labs (33).

HBsAg particle purification from yeast. Yeast cell paste was homogenized with an equal volume of glass beads and 0.05M Tris HCl at pH 8.0 in a Bead-Beater (Biospec Products) at 25°C for six minutes. The complete homogenate was diluted 1:10 and made 5 percent (w/v) in PEG 8000 and 4 percent (w/v) in Dextran 500. The solution was stirred continuously for 30 minutes at 25°C, followed by centrifugation at 4000 rpm for 10 min. The top phase was recovered and DE-52 (Whatman), previously equilibrated to pH 8, was added to the suspension with stirring for 30 minutes at 25°C. The DE-52 was recovered by filtration on a coarse glass funnel and washed several times with 0.05M Tris HCl pH 8.0. The HBsAg was eluted with 0.5M NaCl then concentrated by ultrafiltration using a hollow fiber HP-100 cartridge (Amicon). Solid NaCl was added to 1M NaCl, and this material applied to a 5x81 cm column of A-5m agarose beads (Bio-Rad) equilibrated with 1M NaCl and 0.05M Tris HCl at pH 8.0. The aggregated HBsAg eluted near the void volume and was concentrated using the hollow fiber HP-100. After rechromatography on A-5m, the antigen was concentrated by ultrafiltration on a XM-300 membrane (Amicon). The material was further purified by ultracentrifugation using a CsCl gradient (0.3g of CsCl per ml of solution) in a Beckman SW-28 rotor at 25,000 rpm for 62 hrs at 8°C. The aggregated HBsAg material was then dialyzed against PBS.

Isotopic labeling of yeast proteins in vivo. Yeast cells growing in log phase were harvested between .5 and 2.0 A600 by centrifugation and washed once with 1X YNB (same concentration as used in yeast media). Samples of approximately $2x10^{7}$ cells were resuspended in .2 ml of YNB containing 2 percent glucose and "drop-out" amino acid mix without met or without cys. Ten μCi of ^{35}S met or ^{35}S cys were added followed by incubation at 30°C for 60 min with periodic shaking. To enable uptake by the yeast cells, cystine was reduced to cysteine with DTT. After labeling, the cells were pelleted at 12,000 xg for 30 seconds and resuspended in 50 μl

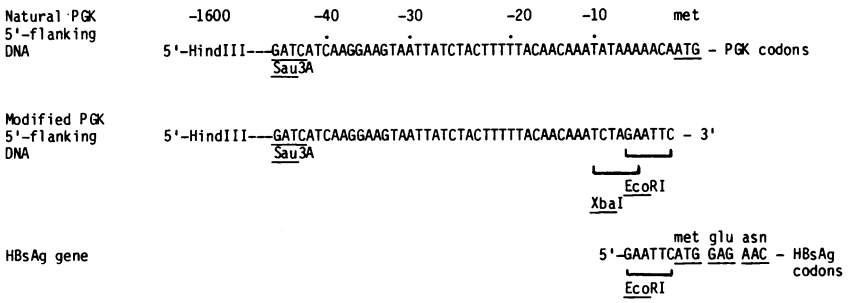

Fig. 1. Modification of PGK DNA sequence for expression of the HBsAg gene. The natural 5'-flanking DNA sequence for the PGK gene is shown from -46 through +3 with the HindIII site at -1600 upstream from the ATG. An oligonucleotide primer complementary to -10 through -21 of this sequence was used with this fragment in a primer repair reaction to obtain a Sau3A (-46) to blunt end (-10) double-stranded fragment. This primer repair reaction, addition of XbaI and EcoRI sites, and reconstruction of 5'-flanking sequence, from the Sau3A site to the HindIII site at -1600, have been described elsewhere (36). The modified PGK 5'-flanking sequence was then connected to the modified HBsAg gene via the EcoRI site.

electrophoresis sample buffer (.1M Tris HCl pH 6.8, 2 percent SDS, 10 percent glycerol, 5 percent mercaptoethanol, and 0.1 percent bromphenol blue) and heated to 95°C for 5 min. After centrifugation, the samples were analyzed by electophoresis on 12 percent SDS polyacrylamide gels (35) and autoradiography.

RESULTS

Modification of a PGK 5'-flanking DNA sequence for use as a transcriptional start. A new yeast promoter fragment was isolated from the PGK1 gene, which encodes the highly expressed glycolytic enzyme, 3-phosphoglycerate kinase (PGK). The PGK structural gene is located on a 3.1 kbp HindIII fragment (28) and the entire nucleotide sequence of the structural gene has been obtained (31). Fig. 1 shows a portion of the 5'-flanking sequence preceding the ATG translational start. Using this sequence information, a series of recombinant DNA techniques were used to replace residues -9 through -1 with XbaI and EcoRI restriction sites as previously described (36). The modified portion of this 1600 bp PGK

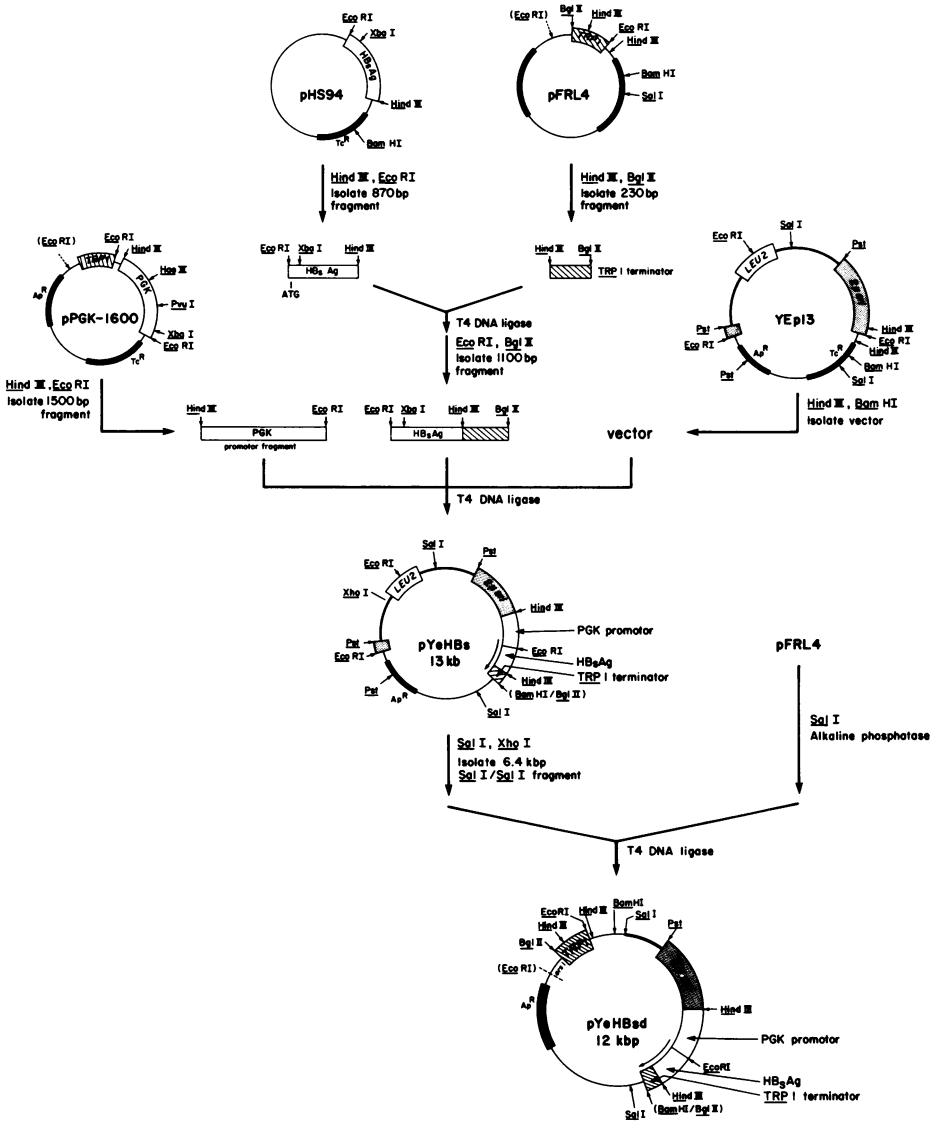

Fig. 2. Construction of HBsAg expression plasmids pYeHBs and pYeHBsd.

promoter fragment is shown in Fig. 1 and the plasmid in Fig. 2 called pPGK-1600 contains this fragment from HindIII to EcoRI. This promoter fragment was then connected to the HBsAg gene by means of the EcoRI site as shown in Fig. 1.

Construction of plasmids for expression of HBsAg in yeast. YEp13 (29) is a yeast vector (Fig. 2) which can be selected for and replicated in both yeast and E. coli due to the presence of the replication origin and ampicillin resistance gene from E. coli vector pBR322, the yeast LEU2 gene (37), and a portion of the yeast 2μ plasmid containing a yeast origin of replication. Fig. 2 shows how a HindIII to BamHI region of this vector was eliminated to allow its use as a component of a 3-factor ligation.

The HBsAg gene was obtained from plasmid pHS94, which has previously been described (12). This plasmid contains the gene and a portion of its 3'-untranslated region from Hepatitis viral DNA on an EcoRI to HindIII fragment. The EcoRI end or 5'-end of the gene is shown in Fig. 1 and the translation stop of the gene occurs approximately 130 nucleotides from the HindIII end of the fragment.

To obtain efficient expression of a eukaryotic gene in a yeast plasmid, it is necessary to provide DNA sequence at the end of the gene which allows for proper processing of the mRNA including termination of transcription and polyadenylation (38, C.C. and R.H. unpublished results). A DNA fragment possessing a termination sequence was obtained from the 3' region of the yeast TRP1 gene, which codes for N-5' phosphoribosyl anthranilate isomerase (39). The HindIII to BglII fragment was used previously for termination of transcription in a yeast plasmid producing interferon D (18). This TRP1-terminator fragment was ligated with the structural gene for HBsAg (EcoRI to HindIII) and the 1.1 kbp combination fragment was isolated. The HindIII to EcoRI PGK promoter fragment was then isolated from pPGK-1600. Promoter fragment, HBsAg gene-terminator, and vector were then ligated to give the plasmid pYeHBs. Another plasmid, pYeHBsd, was constructed by inserting the 6.4 kb SalI fragment from pYeHBs containing the expression elements and the yeast 2μ origin of replication into the SalI site of pFRL4 (18) as shown in Fig. 2. It allows for Trp^+ complementation of yeast and contains an ars1 chromosomal origin of replication (22, 23) in addition to the 2μ origin of replication (29). This plasmid was constructed in an attempt to improve upon yields of HBsAg produced by pYeHBs. By the combination of two origins of replication in pYeHBsd, it was hoped that the high copy number characteristics of the ars1 origin (22) and the comparatively better segregation characteristics of the 2μ origin (29) would improve upon the copy number of pYeHBs, which contains only the 2μ origin.

Expression of HBsAg in Yeast. Plasmid pYeHBsd was introduced into yeast strain GM3C-2 by transformation and the HBsAg mRNA was identified by

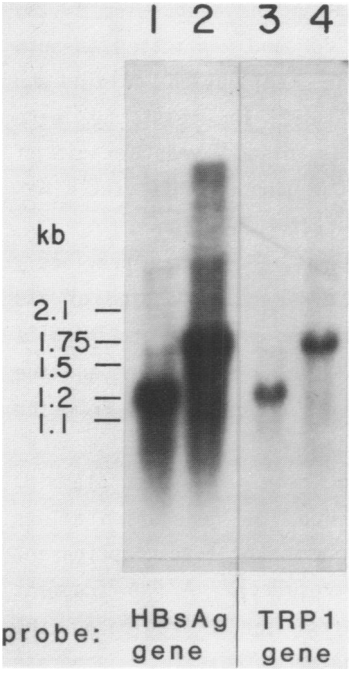

Fig. 3. Analysis of HBsAg specific mRNA from pYeHBsd. Total RNA isolation, gel electrophoresis, transfer of mRNA to nitrocellulose paper, hybridization with DNA probes, and autoradiography were done as previously described (31). Lanes 1 and 3 on the gel contained RNA from pYeHBsd/GM3C-2 yeast; while lanes 2 and 4 contained RNA from pYeHBsd-LeIFD/GM3C-2 yeast, in which the plasmid contained a 560 bp DNA fragment between the promoter and the HBsAg gene. The nitrocellulose paper containing lane 1 and 2 was hybridized with ^{32}P-labeled EcoRI to HindIII HBsAg DNA; while the paper for lanes 3 and 4 was hybridized with ^{32}P-labeled HindIII to BglII Trp1 DNA.

Northern analysis (34). As Fig. 3 demonstrates, a novel species of mRNA 1200 nucleotides in length was found upon hybridization with either the HBsAg gene (lane 1) or that portion of the TRP1 gene (lane 3), which flanks the HBsAg gene as transcriptional terminator. To further prove that this mRNA species is in fact transcribed on the plasmid from the HBsAg gene, we inserted a 560bp EcoRI fragment between the promoter and HBsAg gene. This fragment contains the leukocyte interferon D gene, which has no transcription termination signals recognized by yeast (18). Thus a new mRNA was detected which is proportionally larger (Fig. 3, lanes 2 and 4) confirming the identity of the transcript. The role of the TRP1 region in terminating the HBsAg transcripts is confirmed by its hybridization with both of these mRNA species.

Detection of HBsAg by labeling with ^{35}S methionine or cysteine. When yeast cells containing pYeHBsd were grown in ^{35}S methionine, the analysis of whole cell extracts by SDS gel electrophoresis and autoradiography revealed a prominent band at about 23K (Fig. 4, lane 2) (representing 1-2 percent of total cellular protein based on band intensity with respect to other yeast proteins). This band is absent

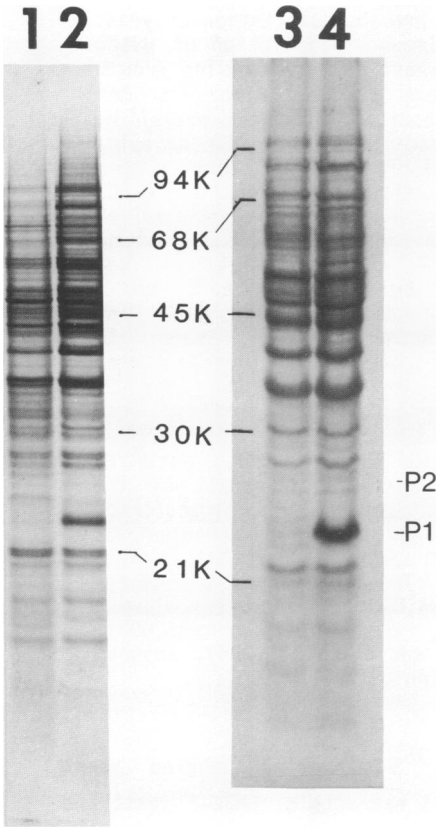

Fig. 4. Detection of HBsAg in total yeast extracts labeled with ^{35}S methionine or ^{35}S cysteine. Yeast strains 20B-12 and 20B-12 transformed with pYHBsd in log phase were labeled with 30 μCi ^{35}S methionine or cysteine for one hour. Labeled proteins were extracted by heating in sample buffer and resolved on a 12 percent polyacrylamide gel. Autoradiography was for ~2 hrs. Molecular weight markers are as in Fig. 5 and the lanes are as follows: Lane 1, 20B-12 untransformed, methionine label; Lane 2, 20B-12 with pYHBsd, methionine label; Lane 3, 20B-12 untransformed, cysteine label; Lane 4, 20B-12 with pYHBsd, cysteine label.

from untransformed cells (Fig. 4, lane 1) and cells containing the same plasmid without the HBsAg gene (data not shown). The corresponding migration of P1 (23K) and P2 (27K, glycosylated) monomers of HBsAg from serum-derived 22 nm particles is indicated (data not shown). A polypeptide corresponding to the glycosylated monomer form of HBsAg (P2) was not observed in the yeast extracts.

When ^{35}S-cysteine was used for the labeling the 23K dalton band was much enhanced (lanes 3 and 4 of Fig. 4). This is consistent with the unusually high content of 13 cysteines as opposed to 6 methionines within the 226 amino acids of HBsAg.

Serological identification of the 23K dalton protein as HBsAg. Further evidence for the identity of the 23K protein was obtained by its reaction with rabbit antiserum raised against 22nm particles from human carrier serum. Fig. 5 shows an autoradiograph of an SDS polyacrylamide

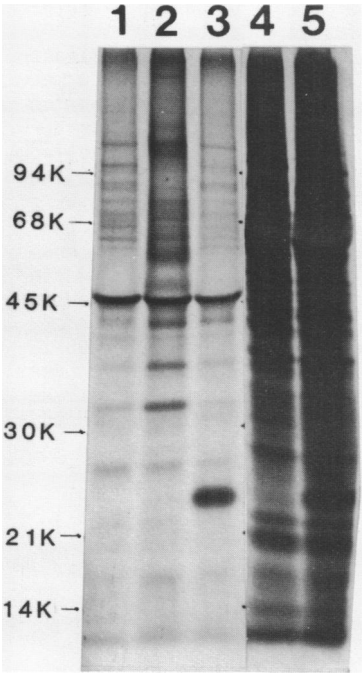

Fig. 5. Immunoprecipitation of yeast HBsAg. Immunoprecipitation of HBsAg made in yeast cultures in log growth phase were labeled with ^{35}S met for one hour. Cells were broken by glass bead treatment and proteins were solubilized in SDS sample buffer. After addition of NP40, immunoprecipitation was performed as described under methods. Immune complexes were dissociated and resolved by electrophoresis on a 12 percent polyacrylamide gel. The autoradiograph shows: Lane 1, strain GM3C-2 (untransformed control), anti HB serum; Lane 2, GM3C-2 transformed by pYeHBsd, non immune serum; Lane 3, GM3C-2 transformed by pYeHBsd, anti HB serum. Lane 4, 10 percent of the total extract from untransformed control; and Lane 5, 10 percent of the total extract from GM3C-2 with plasmid. MW markers are phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (14,500).

gel of immunoprecipitated HBsAg from ^{35}S-methionine labeled yeast extracts. Lanes 4 and 5 again show the SDS extracts of GM3C-2 yeast and pYeHBsd/GM3C-2 yeast, respectively, as shown in Fig. 4. However, lanes 1, 2, and 3 were obtained by immunoprecipitation of SDS extracts of strains GM3C-2 (lane 1) and pYeHBsd/GM3C-2 (lanes 2 and 3). Lanes 1 and 3 were obtained by precipitation with HBsAg antiserum; while lane 2 was treated with preimmune antiserum. It is evident that the 23K daltons protein in lane 5 is specifically immunoprecipitated in lane 3 confirming the identity of this yeast derived protein as HBsAg. However only about 10 percent of the total amount of monomer was reactive with the antiserum. Presumably this is due to the solubilization with strong ionic detergent (SDS) and the nature of the antiserum, which was raised against native 22nm serum-derived particles.

Detection of 22nm-like particles in yeast. We also detected the HBsAg by a radioimmune assay (RIA) (33) in yeast cell extracts prepared by vortexing with glass beads in PBS buffer. However, the quantity of HBsAg from pYeHBsd as determined by this assay was only 50µg of HBsAg per liter of cells at A660 of 1.0 (.05 percent of total yeast protein). This

level is 20-40 times less than the level of monomer detected. Since the RIA is specific for the detection of particles, the yeast-derived HBsAg was further analyzed by sedimentation on sucrose and CsCl gradients. As shown in Fig. 6A, the HBsAg in these extracts behaves like serum-derived 22nm particle in its sedimentation rate, suggesting a size and shape similar to this particle. In Fig. 6B the buoyant density of the yeast-derived particle is about 1.18 g/cc, similar to that obtained for the natural serum-derived 22 nm particle (7). Since this density is due to the combined presence of the HBsAg protein and phospholipid (7), we conclude that both components are present in the yeast particle.

The yeast-derived particles were purified and then compared with serum-derived 22nm particles by electronmicroscopy. Figs. 6C (from yeast) and 6D (from serum) show that by negative staining both types of particles appear very similar in size and shape. SDS gel analysis of purified particles from yeast (data not shown) demonstrate that, besides the HBsAg P1, the yeast derived particles contain several additional proteins presumably of yeast origin. Again, the glycosylated form P2 was not seen.

As an alternative to the cell fragmentation by glassbead treatment we also prepared cell extracts by a gentle lysis procedure. When yeast cell walls were removed enzymatically by treatment with zymolyase (40) and the resulting spheroplasts were lysed by osmotic shock (gentle lysis), the RIA failed to detect significant levels of HBsAg. However, when the extract pellet was treated with glass beads, particles were again detected in the cleared supernate. Since the RIA is specific for the detection of particles as opposed to monomer and since these particles were not significantly detected after this gentle lysis procedure, we suspect that the particles are formed from monomers embedded in membranes by the glass bead process itself. Alternatively the particles might not be released from some cellular compartment by the gentle lysis procedure or they may be present in large aggregated forms.

The level of particles was about the same for pYeHBsd in each of two yeast strains, GM3C-2 and 20B-12, which are both $\underline{trp1}^{-}$. However, in a third strain XV610-8C, which was also used by others for expression of HBsAg (24), pYeHBsd produced only 2-5 µg of HBsAg per liter at the same absorbance. Thus a strain effect may result in differences in yield of up to 10-25 fold. This same strain effect also occurs for the expression of leukocyte interferon D in XV610-8C versus GM3C-2 (C.C. and R.H.,

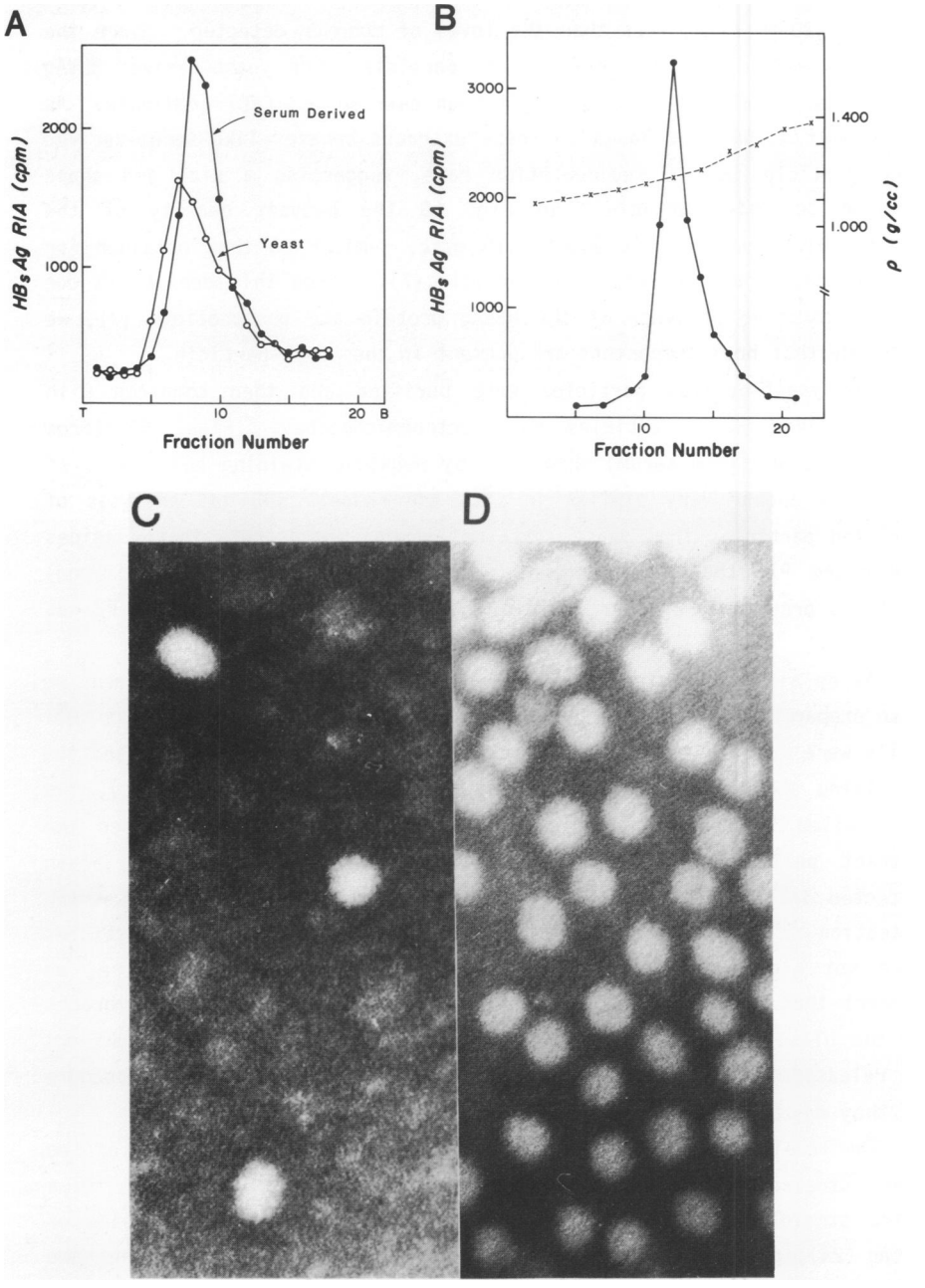

Fig. 6. Characterization of HBsAg from yeast extracts. Yeast cell extracts were made vortexing cells with glass beads in PBS buffer. Panel A shows a sucrose gradient of this material compared with one for

serum-derived 22 nm particles. Two hundred µl of yeast extract (pYeHBs/XV610-8C) was layered on top of a 5 ml linear gradient consisting of 5-20 percent sucrose (w/v) in 20 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, and 0.5 M NaCl followed by centrifugation in a SW50.1 rotor at 45,000 rpm for 2 hours. Fractions were collected and assayed for HBsAg by the radioimmune assay. The open circles are from a gradient with yeast extract. The closed circles are from an identical gradient with plasma-derived material.

Panel B shows an equilibrium CsCl gradient centrifugation of the same yeast extract. Two hundred µl of extract was layered on top of a 5 ml solution containing 20mM Tris-HCl (pH 7.4), 0.5mM EDTA, and CsCl (density 1.2g/cc) then centrifuged in a SW50.1 rotor at 45,000 rpm for 70 hours. HBsAg radioimmune activity was assayed and the density of CsCl was determined by recording the refractive index of each gradient fraction.

Panel C shows an electron micrograph of purified yeast particles (see Methods). Panel D shows an electron micrograph of 22 nm particles from the serum of Hepatitis B virus carriers.

unpublished results). The nature of this effect has not been thoroughly examined but appears to be related to a decrease in plasmid stability in XV610-8C compared to other strains. Plasmids pYeHBs versus pYeHBsd were also compared as to particle production and expressed about the same levels in GM3C-2, suggesting that the presence of both the arsl and 2µ origins of replication on the same plasmid is not advantageous in obtaining a higher level of expression in comparison to a 2µ origin containing plasmid.

Recovery of HBsAg from yeast extracts by mild detergent. From the data already described, it is clear that the quantitation of HBsAg by RIA varies considerably depending on the method of extract preparation. Moreover, even under our best conditions, the levels detected by RIA were 20-40 times below that seen by SDS gel analysis after labeling with ^{35}S-methionine. To account for these apparent losses of HBsAg under different conditions, we performed the following extraction experiments in combination with SDS gel analysis (Fig. 7). Cells containing pYeHBsd (even lanes) or no plasmid (odd lanes) were broken with glassbeads in PBS and after short centrifugation yielded a soluble supernatant which contains the 22nm yeast-derived particles (lanes 1 and 2). After reextraction with PBS, lanes 3 and 4 were obtained. The pellet was then extracted with nonionic detergent (NP40) in PBS buffer (lanes 5 and 6) and then reextracted (lanes 7 and 8). The insoluble residue from the NP40 extractions was finally boiled in 2 percent SDS with mercaptoethanol to obtain lanes 9 and 10. It is seen now that the fraction containing the 22nm particles revealed almost undetectable amounts of monomer.

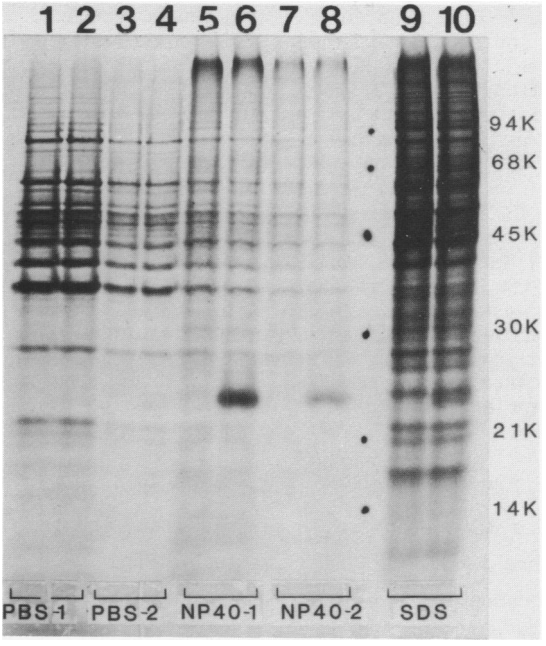

Fig. 7. Detergent extraction of HBsAg from yeast cells. Cells were labeled with methionine for one hour and fragmented by glass bead treatment in PBS buffer. Insoluble material was recovered by centrifugation. This extraction was repeated once. The pellet was then vortexed in presence of 1 percent NP40 in PBS. A second supernatant was obtained after centrifugation. The NP40 extraction was repeated again. The insoluble material after NP40 extraction was heated in sample buffer. Lanes 1,2 are PBS extracts; Lanes 3,4 are second PBS extracts; Lanes 5,6 are PBS with NP40 extracts; Lanes 7,8 are second PBS with NP40 extracts; and Lanes 9,10 are SDS sample buffer extracts. MW markers are as in Fig. 5. Lanes 1, 3, 5, 7, and 9 are controls without plasmid, while lanes 2, 4, 6, 8 and 10 are 20B-12 with pYeHBsd.

However, the bulk of the monomer was extracted by the nonionic detergent rather specifically, with only moderate contamination of yeast proteins. The final solubilization by strong ionic detergent (SDS) and reducing agent yielded the remainder of HBsAg along with the bulk of cellular material. The solubilization of HBsAg in yeast extracts by mild detergent would indicate an association with lipids, i.e. cell membranes. Also, a significant enrichment of the monomer was achieved by such an extraction with mild detergent.

Immunogenicity of the yeast HBsAg particle. Serum-derived 22nm particles can induce high titers of neutralizing antibodies upon injection into mice. We therefore compared the antibody response of mice to natural 22nm particles and partially purified yeast derived particles (see Methods). As Fig. 8 demonstrates, the yeast-derived particles induced a comparable if not better response. They were effective even without complete Freunds adjuvant which may be a nonspecific effect of yeast impurities.

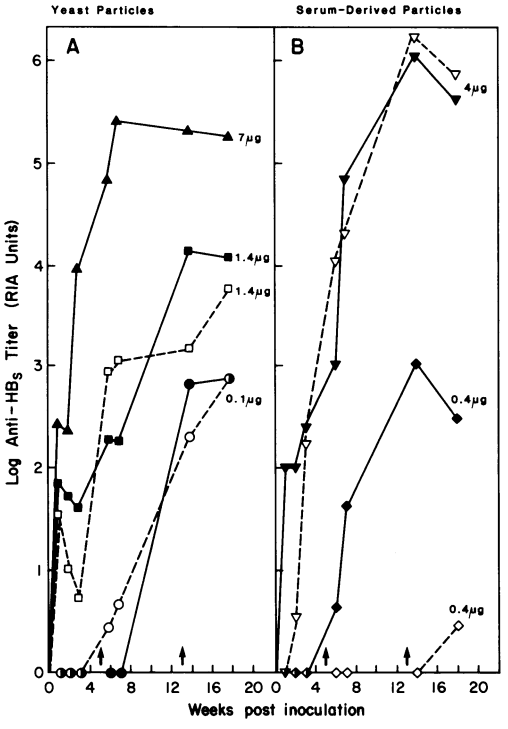

Fig. 8. Kinetics of antibody response to purified HBsAg particles. Particles were obtained from yeast (A) or from human serum (B) (North American Biologicals). Six week old CD-1 mice (Charles River) were injected intraperitoneally with HBsAg in 0.2 ml of a 1:1 (v/v) mixture of 16 mM $NaPO_4$ pH 7.2 0.14 M NaCl (PBS) with Freund's adjuvant (Grand Island Biological Co.) (filled symbols), or in 0.2 ml of PBS alone (open symbols). Initial injections contained Freund's complete adjuvant and subsequent injections (indicated by the arrows) contained Freund's incomplete adjuvant. The injections contained a dose of 0.1 μg (○,●), 1.4 μg (□,■), or 7.0 μg (▲) of yeast HBsAg, or 0.4 μg (◇,◆) or 4.0 μg (▽,▼) of HBsAg from human serum. The amount of HBsAg in all cases was determined using the Ausria radioimmunoassay kit (Abbott Laboratories). Mice were bled from the orbital route and the blood was allowed to clot on ice from 4 to 16 h. The anti-HBs titers of the serum fraction (expressed as $\log_{10}$ and representing the mean titers of five mice) were calculated from a standard curve generated using the positive control serum in the Ausria radioimmunoassay kit (Abbott Laboratories).

DISCUSSION

Yeast cells containing plasmid pYeHBs or pYeHBsd produce significant quantities of HBsAg (1-2 percent of the total cellular protein) indicating that the hybrid plasmid construction containing the HBsAg structural gene and yeast 5'- and 3'-flanking DNA is functional in transcription and translation. The observed mRNA length of 1200 nucleotides is consistent with the size of the gene and with termination occurring in a region of yeast DNA which normally results in proper termination and polyadenylation of the yeast TRP1 gene.

Two forms of HBsAg were identified in yeast extracts depending on the method of extract preparation. Cell fragmentation by glassbeads in PBS

buffer yielded HBsAg particles in the clarified extract which are similar in size and density to the natural serum-derived 22nm particles. Electron micrographs of the purified particles from yeast and serum revealed essentially similar appearance. The yeast derived particle contains HBsAg monomer of 23K daltons known as P1 and lacks the extensively glycosylated P2 monomer of 27K daltons. These results are in agreement with the results published by Valenzuela et al. (24) using the alcohol dehydrogenase promoter. Their yields were reported as 10 to 25 μg per liter of culture using strain XV610-8C grown to mid-log phase. Assuming that mid-log phase means at the least an absorbance of 1.0, this is between our yield of 2 to 5 μg per liter (A_{660} = 1) for pYeHBsd in XV610-8C and 50 μg per liter for the same plasmid in GM3C-2 or 20B-12. This difference between expression levels in XV610-8C does not reflect differences in promoter strength since plasmid difference, promoter-structural gene junction, and transcription terminator differences are also present in their system versus ours. In fact, keeping all other variables constant, recent comparisons of the ADH1 and PGK promoter fragments have demonstrated that both result in essentially identical expression levels of IFN-α1 (36). However, by comparison of the same HBsAg expression plasmid in XV610-8C versus two other yeast strains, we have found that the yeast XV610-8C showed grossly lower levels of particle production than two other yeast strains. In view of the known potency of serum-derived 22nm particles as vaccine (8), the yeast-derived particles may represent an economical and safe vaccine material; however, contaminating yeast proteins in the particle may pose purity problems in making such a vaccine for human use.

In contrast, when whole yeast cellular protein was extracted by strong detergent in the presence of reducing agent, HBsAg was solubilized as monomer representing a much higher proportion of total yeast protein (1 to 2 percent or 1 to 2 mg per liter of culture at A_{660} of 1) than the particle yield discussed above (50 μg per liter of culture at A_{660} of 1). This polypeptide was enriched for cysteine as expected and its identity as HBsAg was substantiated by specific immune precipitation. The bulk of HBsAg monomer which is not extracted by saline solutions can easily be solubilized by even mild detergent. This observation suggests an association with lipid membranes which is in agreement with the transmembrane nature (contains internal hydrophobic region) of HBsAg (2).

Previous observations show that viral particles bud off the membranes of infected animal cells. In the yeast expression system we have not observed

any HBsAg particles in the media of producing cells, even though yeast cells secrete a number of their own proteins (40) and even foreign proteins (36,41). If the natural mechanism of secretion for 22nm particles is a budding process, then one might not expect secretion of HBsAg from yeast cells in which the cell membrane is surrounded by a cell wall. Furthermore, our experiments suggest that the particle may result from the glass bead extraction procedure and yeast may thus be defective in initiating a budding process. Indeed when cell walls were removed, no particles were observed. It is less obvious why (detectable) glycosylation of HBsAg did not seem to occur in yeast cells which glycosylate some of their own proteins (40,42). Although the particles isolated from yeast cells have shown strong immune responses in mice and may provide a suitable vaccine material, their yield has so far been limited. Furthermore they are associated with a number of contaminating yeast proteins. The 20-40 fold higher yield of monomer and its potential for extensive purification may well make the monomer the preferred form for development of a vaccine.

After submitting this manuscript for publication, Miyanohara et al. (43) reported a high level production of HBsAg 22nm-like particles from yeast using the repressible acid phosphatase promoter of yeast. Since their extracts were prepared using 0.1 percent Triton X-100, it is possible that these particles are formed in vitro. A comparison of this particle form with that obtained using glass beads without detergent would certainly be interesting.

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