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CHARACTERISTICS OF HEPATITIS B SURFACE ANTIGEN PRODUCED IN YEAST*

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We have constructed an expression plasmid for regulated expression of the hepatitis B surface antigen gene in yeast using promoter of the yeast *Pho5* gene. In the yeast transformants, the monomeric HBsAg (22K dalton) was estimated to constitute approximately 3% of the total proteins. On extraction, the HBsAg was found to have a bouyant density of 1.18 g/ml and an $S_{w,20}$ value of 54. Electron microscopy revealed particles of heterogeneous size ranging from 18-28 nm. When the yeast HBsAg was used to immunize guinea pigs, the anti-HBsAg antibodies produced could react with human serum HBsAg.

Several kinds of viral infection of human liver have been documented, the most significant class being infections due to hepatitis B virus (HBV). Liver injuries resulting from HBV infection are manifested in many forms. Recent epidemiological studies have associated HBV infection with cirrhosis and primary hepatocellular carcinoma (1-6). For example, HBV carriers who are positive for the surface antigen (HBsAg) are 220 times more prone to develop hepatoma than normal persons (2). Molecular studies have produced convincing evidence to indicate that the HBV genome integrates into the cellular DNA of hepatocytes of cirrhosis and hepatoma (4-6). In many developing nations in Asia and Africa, HBV infection is so prevalant that an effective vaccine is urgently

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen.

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needed. Such a vaccine may also reduce some forms of hepatoma which are etiologically linked to HBV infection.

A vaccine currently being used against HBV infection is prepared from 22 nm HBsAg particles purified from sera of HBsAg-positive carriers (7). As a first step towards preparation of a recombinant DNA vaccine, our laboratory has cloned the entire HBV genome (8). Subsequently, we have constructed a shuttle plasmid which allows regulated expression of the HBsAg gene in yeast (Choo *et al*, in preparation). In this communication, the characteristics of the yeast HBsAg are described.

MATERIALS AND METHODS

Strains and media: Yeast strain (*Saccharomyces cerevisiae*) YNN27 α *trp1-289, ura3-52 gal2* (9) was used in transformation experiments using the method of Ito *et al.* (10). For induction of the HBsAg gene expression, transformant cells were grown in mixtrue of low-phosphate YEPD (1% yeast extract, 2% peptone, 2% glucose) and minimal glucose medium (0.67% yeast nitrogen base, 2% glucose, 20 mg/ml uracil) in 1:4 ratio. The final phosphate concentration was 0.2 mM. Inorganic phosphate in the yeast extract and in peptone was removed as described (11).

Homogenization of yeast cells: Yeast cells grown to late log or early stationary phase were harvested and resuspended in 1/20 volume of PBS (20 mM phosphate pH 7.2, 140 mM NaCl). An approximately equal volume of glass beads (450-500 μm) was added and the suspension was vortexed at maximum speed at 4°C for 5 min. The supernatant obtained after low speed centrifugation was then assayed for HBsAg (Ausria^{R} immunoassay kit, Abbott Labs).

In vivo labelling of yeast total proteins: Yeast transformants were grown in minimal glucose medium with appropriate phosphate concentration to early log phase. Approximately 2×10^{7} cells were harvested, washed and resuspended in 1 ml of the same medium. 10 μCi of [^{35}S]methionine was added followed by incubation at 30°C for 1 h. After labelling, the cells were washed and resuspended in 25 μl of gel loading buffer (0.1 M Tris.HCl pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol and 0.1% bromophenol blue) and heated at 95°C for 5 min. Samples were electrophorezed in 12.5% polyacrylamide gel and autoradiographed.

RESULTS

We have constructed a yeast-*E.coli* shuttle plasmid p2μ-S11 (Fig. 1) for regulated expression of the hepatitis B surface antigen gene in yeast using the origin of replication of the yeast endogenous 2μm plasmid. Such recombinant plasmids are non-

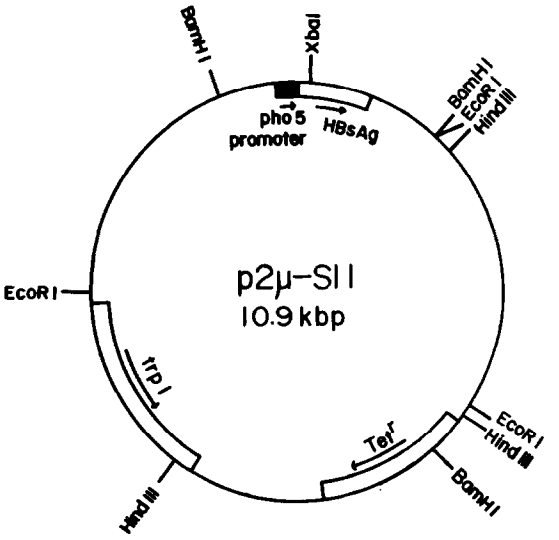

Fig. 1. Restriction map of expression plasmid p2μ-S11 for the hepatitis B surface antigen gene (Choo *et al.*, in preparation).

integrative, more stable and are present in host cells in high copy number (12). The yeast *trp1* gene was also inserted as a selection marker in *trp*$^-$ host strains. For the expression of the HBsAg gene, promoter sequences of the yeast *pho5* gene (coding for the repressible acid phosphatase) excised from plasmid pAP20 (courtesy of Dr. R. A. Kramer, Hoffmenn-La Roche Inc.) (13-14) were placed before the cloned HBsAg gene (subtype *adw*). The construction of plasmid p2μ-S11 will be described elsewhere (Choo *et al.*, in preparation).

To characterize the yeast HBsAg, total yeast proteins were labelled *in vivo* in the presence of [^{35}S]methionine under induced and repressed conditions. The labelled proteins were then separated by SDS-polyacrylamide gel electrophoresis (Fig. 2). When grown under induced conditions (i.e. with low phosphate concentration), p2μ-S11/YNN27 produced a 22K dalton band which was absent from the same transformant grown under repressed

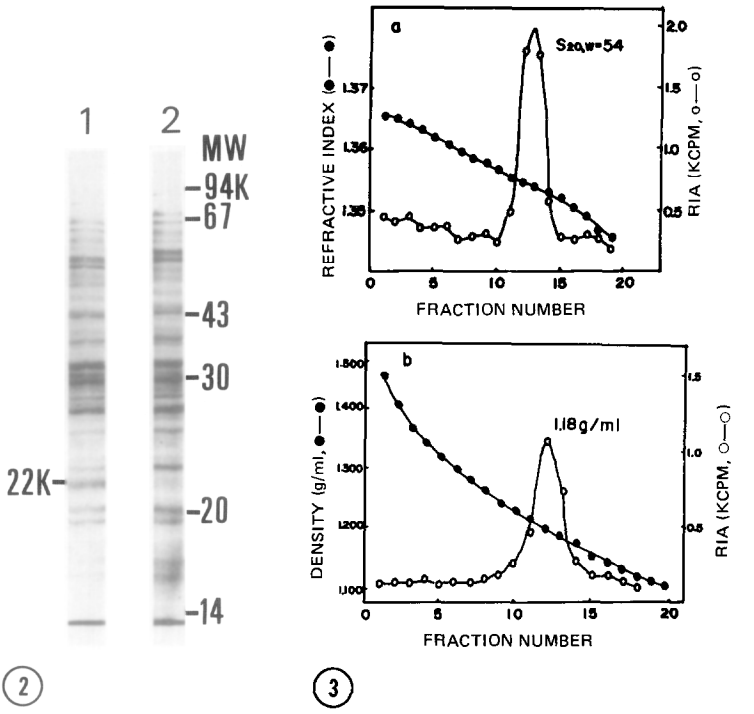

Fig. 2. SDS-polyacrylamide gel electrophoresis of [^{35}S]methionine-labelled proteins of YNN27 transformants of p2μ-S11. Cells were grown in media with 0.2 mM (lane 1) and 9 mM (lane 2) phosphate as described in Materials and Methods.

Fig. 3. (a) Sucrose gradient (5-20%, Kontron STS41.13 rotor, 33,000 rpm, 6 h, 4°C), and (b) CsCl gradient (1.1-1.4 g/ml, Kontron rotor TFT53.13, 45,000 rpm, 24 h, 4°C) centrifugation analysis of yeast HBsAg. After centrifugation, the fractions collected were assayed for HBsAg.

conditions (high phosphate concentration). Thus, the 22K dalton polypeptide was a gene product of p2μ-S11 induced in a low phosphate medium. HBsAg in the infectious Dane particles (15) is known to exist in 2 monomeric forms, P1 and P2。 P1 is glycosylated with a molecular weight of 27K dalton and P2 is non-glycosylated with a molecular weight of 22K dalton (16)。 The 22K dalton polypeptide of p2μ-S11/YNN27 therefore corresponds to the P2 form of the monomeric HBsAg. It can also be estimated from the autoradiograph in Fig. 2 that the 22K dalton polypeptide represents approximately 3% of the total cellular proteins in the tronsformants

The yeast HBsAg was subjected to CsCl and sucrose gradient analysis (Fig. 3)。 The buoyant density of the HBsAg was determined

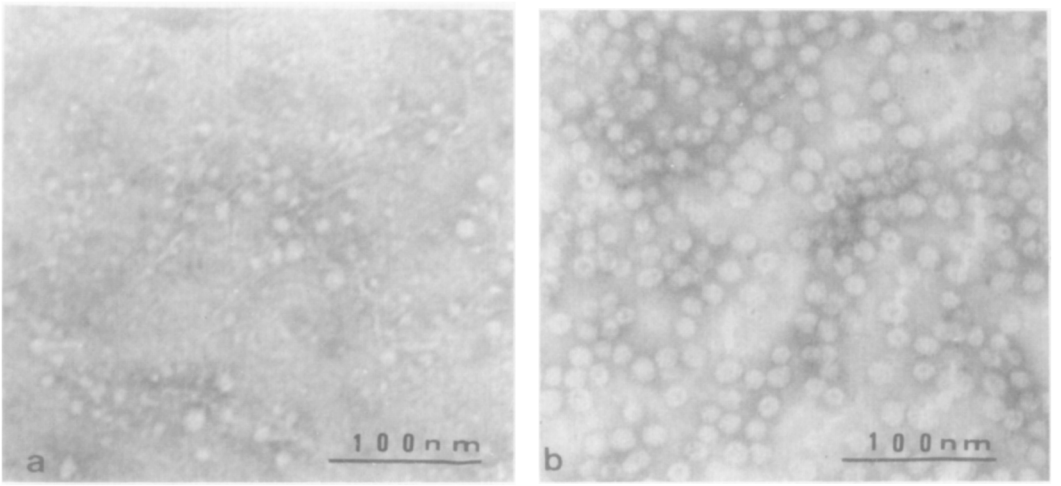

Fig. 4. Electron microscopic morphology of (a) yeast and (b) serum HBsAg. The HBsAg was loaded on Formvar-coated grids and negative stained with 5% uranyl acetate. Samples were examined with a Joel 100 CX electron microscope at 60 kV.

to be 1.18 g/ml and the $S_{w,20}$ value was 54. Thus, the yeast HBsAg produced by p2μ-S11/YNN27 was very similar to the serum HBsAg particles from HBV carriers (17). Examination by electron microscopy showed that the yeast HBsAg particles were heterogeneous in size with a range of 18-28 nm without a central pit, in contrast to the homogeneous size of 22 nm of the serum HBsAg (Fig. 4). A few larger particles of 32 nm size were also observed.

The yeast HBsAg was used to inoculate guinea pigs to test for immunogenicity (Table 1). When cell extracts from YNN27 transformant of plasmid II6-5 which has HBsAg gene sequences in the incorrect reading frame (Choo *et al*, in preparation) were used, no anti-HBsAg antibodies were detected in the serum of the guinea pig. However, when the inocula contained crude yeast extracts with less than 10ng of HBsAg or about 25ng of partially purified HBsAg, anti-HBsAg antibodies against the human HBsAg used in the assay kit (AUSAB^{R}, Abbott Labs) were detected.

Table 1. Immunization of guinea pigs with yeast HBsAg

Animal	Inoculum[a]		Titer (P/N ratio)		
	clone	HBsAg(ng)	Day 1	Day 27	Day 37
1	II6-5[b] (crude)	0	1.27	0.82	1.52
2	p2μ-S11 (crude)	<10	0.58	0.88	3.42
3	p2μ-S11 (partially purified)	ca.25	0.82	6.84	5.04

Immunization schedule: Day 1 (in complete Freund's adjuvant), days 8 and 15 (in incomplete adjuvant); bleeding schedule for RIA test for anti-HBsAg antibodies: [a]Days 1, 27 and 37. P/N ratio >2 was considered positive. [a]Crude extract was supernatant obtained by homogenization of cells with glass beads and centrifugation to remove cell debris. Partially purified samples were obtained by preparative centrifugation of crude homogenate in CsCl gradients. [b]Plasmid of clone II6-5 carried the HBsAg gene in an incorrect translational reading frame (Choo *et al.*, in preparation).

DISCUSSION

Expression vectors for the hepatitis B surface antigen gene in yeast using promoters of the 3-phosphoglycerate kinase and alcohol dehydrogenase genes have been reported (18, 19). Using promoter of the repressible acid phosphatase gene, plasmid p2μ-S11 described in this communication can regulate expression of the HBsAg gene (20). The production of HBsAg could be induced by low levels of inorganic phosphate in the media to a yield of 3% of the total proteins. Since the antibodies used for the immunoassays of HBsAg were directed against human 22nm HBsAg particles, the yeast HBsAg must have been assembled into 22nm-like particles as confirmed by buoyant density, sedimentation coefficient and electron microscopy determinations. However, in contrast with the serum HBsAg particles, the yeast product contains only the 22K dalton (P2) monomeric form and is therefore not glycosylated as has also been reported by other laboratories (18-10). Nonetheless, antibodies produced in guinea pigs after immunization with the yeast HBsAg were reactive with human HBsAg particles. Murray *et al.* (21) have reported that yeast HBsAg could produce

neutralizing antibodies in immunized chimpanzees against HBV infection.

Despite the fact that HBsAg represents 3% of the cellular proteins in p2μ-S11/YNN27, only a very low amount of HBsAg could be extracted in particle forms (ca. 30μg/l culture) using the glass-bead homogenization procedure (data not shown) (see also 18). Since HBsAg particles are about 1000 times more immunogenic than the monomeric HBsAg (21), and since the yeast HBsAg is not secreted into the media, extraction procedure must therefore be developed for efficient recovery of immunogenic HBsAg particles from cell homogenate.

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