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Simultaneous synthesis of two polypeptides of hepatitis B surface antigen directed by the α-factor promoter in *Saccharomyces cerevisiae*

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Abstract. The yeast ***Saccharomyces cerevisiae*** was transformed by an expression vector YAMTS, in which the preS2 and S regions of human hepatitis B virus (HBV) were joined to the sequence encoding the promoter and leader region of the yeast mating pheromone a-factor. The HBsAg synthesized by yeast transformants contains two kinds of polypeptides: one encoded by the S region and the other encoded by the preS2–S region but with a higher molecular weight. The synthesized HBsAg also appears as spherical particles under the electron microscope and has biophysical properties similar to the 22 nm particles found in the sera of hepatitis B patients.

Key **word:** HBsAg; Recombinant DNA; ***Saccharomyces cerevisiae;*** Yeast a-factor promoter and leader sequence.

Introduction

The worldwide impact of hepatitis B infections has caused a serious problem in public health. In eastern Asia and tropical Africa more than 10% of the population are chronic carriers of HBV (Tiollais ***et al.,*** 1985). The ***HBV*** virion is a particle of 42 nm in diameter (namely the Dane particle), which consists of a surface envelope containing lipid and a core in which the viral DNA exists (Tiollais ***et al.,*** 1981). In the sera of hepatitis B patients, 22 nm spherical particles, containing mainly hepatitis B surface antigen (HBsAg), can also be found. It has been demonstrated that HBsAg from recombinant DNA technology can elicit anti-HBsAg antibody in humans and in chimpanzees, and prevent the vaccinated organisms from contracting HBV infection (McAleer ***et al.,*** 1984). Furthermore, it has been suggested that an additional 55 amino acids encoded by the preS2 region, if included, may increase the protective efficacy of vaccines. (Milich ***et al.,*** 1985; Neurath ***et*** al., 1985).

Abbreviations: ADH1, yeast alcohol dehydrogenase 1; ADH1-P, ADH1 promoter; Amp, ampicilin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; kb, kilobase(s); kDa, kilodalton(s); PB, phosphate buffer, pH 7.4; PMSF, phenylmethyl sulfonylfluoride; preS2 gene, HBV gene coding 55 amino acids preceeding of the S gene; R, resistant; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; S gene, HBV gene encoding the surface antigen; YNB, yeast nitrogen base w/o amino acids.

Several cloning systems, mainly *Escherichia coli* (Edman *et al.*, 1981; Fujisawa *et al.*, 1983), yeast (Valenzuela *et al.*, 1982; Bitter and Egan, 1984; Choo *et al.*, 1985) and mammalian cell cultures (Moriarty *et al.*, 1981; Nozaki *et al.*, 1985), have been used to produce HBsAg through recombinant DNA technology. Yeast is an especially promising system, since it is an eukaryote and has a long history of commercial fermentation. Indeed, the HBsAg product from yeast has recently been commercialized (Genetic Technology News, Vol. 6, No. 9, 1986; Genetic Engineering News, Vol. 7, No. 1, 1987). So far the studies concerning the production of HBsAg in yeast have adopted the strategy of using strong promoters to direct a high-level synthesis of HBsAg (Valenzuela *et al.*, 1982; Harford *et al.*, 1983; Hitzeman *et al.*, 1983; Miyanobara *et al.*. 1983; Bitter and Egan, 1984; Murray *et al.*, 1984; Valenzuela *et al.*, 1985; Dehoux *et al.*, 1986). The products of using these promoters were accumulated in the protoplasm of yeast cells. During the purification of HBsAg, both yield and immunogenicity are greatly decreased. If the products can be secreted into media by transformed yeast cells with a chimeric secretory vector containing signals for exocytosis, the purification processes of the products will be much simplified and the possibilities of contamination by other protein material will be significantly reduced.

a-Factor is a pheromone secreted by yeast cells of a-mating type during the process of conjugation with cells of a-mating type. It consists of a polypeptide of 12 to 13 amino acids. Its precursor is a protein of 165 amino acids, including a leader peptide of 83 amino acids and 4 copies of a-factor, each preceded by a spacer peptide of 6 to 8 amino acids (Kurjan and Herskowitz, 1982). Previous studies have shown that foreign genes, when joined to the α-factor promoter and leader encoding sequence, can be expressed efficiently and the produced protein molecules can be processed and secreted into the culture media (Bitter *et al.*, 1984; Miyajima *et al.*, 1985). We now report here the construction and expression in yeast of a chimeric plasmid containing the preS2 and S regions of HBV, which are joined to the yeast a-factor promoter and leader encoding sequence. The biophysical and biochemical charateristics of the produced HBsAg particles were also investigated.

Materials and Methods

Strains

Plasmids were propagated in E. *coli* strain HB101 grown in LB medium. *E. coli* plasmid purification was carried out as described (Maniatis *et al.*, 1982). Yeast *Saccharomyces cereuisiae* strain TL154 (Mat, trpl, leu2, Cir^+) was developed in this laboratory and the transformation was carried out as described (Hinnen *et al.*, 1978).

Plasmids

Plasmid pMA56 was constructed from YRp7 (Tschumper and Carbon, 1980; Valenzuela *et al.*, 1982). Plasmid pYA41 was derived from pWR32, containing the yeast a-factor promoter and leader encoding sequence (Guo and Wu, 1982). Plasmid pUC8/HBV was derived from a plasmid with the entire HBV genome (adw_2 subtype) inserted into the *Eco*RI site of pUC8.

Yeast Cell Growth, Preparation of Extracts, and HBsAg Assay

Yeast cells were grown in yeast minimal medium (0.17% yeast nitrogen base w/o amino acid (YNB), 0.5% ammonium sulfate, 2% glucose) supplemented with leucine (0.04 g/l) at 28°C with aeration until the cell density reached about 7×10^6 cells/ml. Ten ml of cell culture was collected by centrifugation. It was

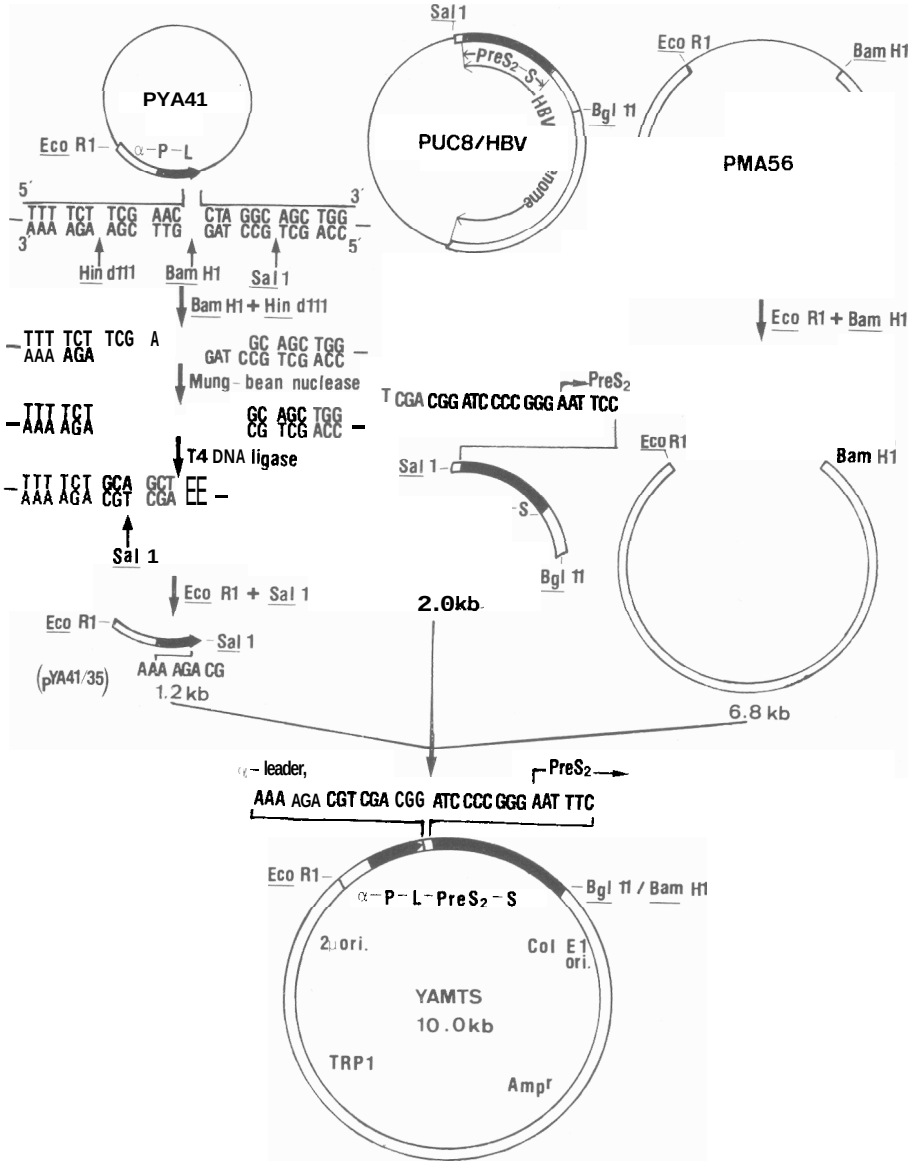

Fig. 1. The construction of yeast expression vector YAMTS, Plasmid pYA41 was first cut with *Bam*HI and *Hin*dIII, which were at the end of a-factor promoter and leader encoding sequence (a-P-L), digested with Mung Bean nuclease, and religated with T4 DNA ligase. By doing this, the 8 base pairs between the original *Bam*HI and *Hin*dIII cutting sites were eliminated. The resulting plasmid was named pYA41/35. When pYA41/35 is cut at the SalI site as shown in the figure, and ligated to the SalI site on plasmid pUC8/HBV, the following preS2-S gene encoding sequence on pUC8/HBV will be in frame.

Fragment I (1.2 kb) containing the a-factor promoter and leader encoding sequence was obtained by digestion of plasmid pYA41/35 with SalI and EcoRI. Fragment II (2.0 kb) containing the preS2-S gene encoding region of HBV genome was obtained by digestion of plasmid pUC8/HBV with SalI and *Bgl*II. The ADH1 promoter (ADH1-P) on plasmid pMA56 was deleted by digesting the plasmid with *Bam*HI and EcoRI, resulting in the fragment III of 6.8 kb. Fusion between fragments I, II, III was made by mixing these three fragments in the molar ratio of 3:3:1 (I:II:III) and then ligated with T4 DNA ligase. The resulting plasmid was named YAMTS, which contains in-frame a-P-L:: $preS_2$-S **Ag** gene fusion was used to transform yeast cells for the a-factor promoter-leader directed synthesis of HBsAg.

washed one time with 10 mM phosphate buffer, pH 7.4 (PB), treated with 8 ml of a solution containing zymolyase (100 μg/ml)/1 M sorbitol/ 10 mM PB and 0.1% 2-mercaptoethanol and then incubated at 30℃ for 30 min. The resulting spheroplasts were collected by centrifugation, washed one time with 10 mM PB/1 M sorbitol and then lysed on ice by vortexing with glass beads in 3ml of 0.1% Triton X-100/10 mM PB/2 mM phenylmethyl sulfonylfluoride (PMSF). The extracts were clarified by centrifugation at 6,000 rpm for 5 min. The HBsAg activity was measured quantitatively and qualitatively by using the Abbott Ausria II radioimmunoassay kit.

Isotopic Labelling of Yeast Proteins and Immunoprecipitation of HBsAg

Yeast cell were harvested, labelled and extracted as described in the legend of Fig. 6. Preparation of protein A of *Staphylococcus aureus* (Staph A protein) and immunoprecipitation were carried out mainly according to Perbal (1984).

Results

Construction of Plasrnid and Expression in Yeast Cells

For the in-frame joining of the a-leader-promoter sequence and the HBV preS2-S gene, plasmid pYA41 was first deleted of 8 base pairs as described in Fig. 1. Plasmid YAMTS (Fig. 1) was derived from plasmid pMA56, pUC8/HBV and pYA41, in which the preS2-S gene of the HBV genome from pUC8/HBV was fused in-frame to the a-factor promoter-leader encoding sequence from pYA41. The remainder of YAMTS was derived from plasmid pMA56, which contributed to its autonomous replication in yeast (2μ ori) and in *E. coli* (Col El ori), and the auxotrophic and antibiotic resistance selection markers (TRP1 and Amp^R). *S. cerevisiae* strain TL154 was transformed with YAMTS. Samples of cell culture were taken out at different growth stages for detection of HBsAg. After pelleting cells by centrifugation, HBsAg activity is not detectable in the culture medium at any growth stage. When samples from culture medium were concentrated into 5 times, a detectable amount of HBsAg activity was observed. However, the cell pellets were found to contain a substantial amount of HBsAg proteins. Production reached its highest level (29 μg/l of cell culture) when the cells were in a mid-log phase and dropped gradually when the cells grew further (Fig. 2).

Biophysical Characters of Synthesized HBsAg

To further characterize the HBsAg proteins synthesized in yeasts with plasmid

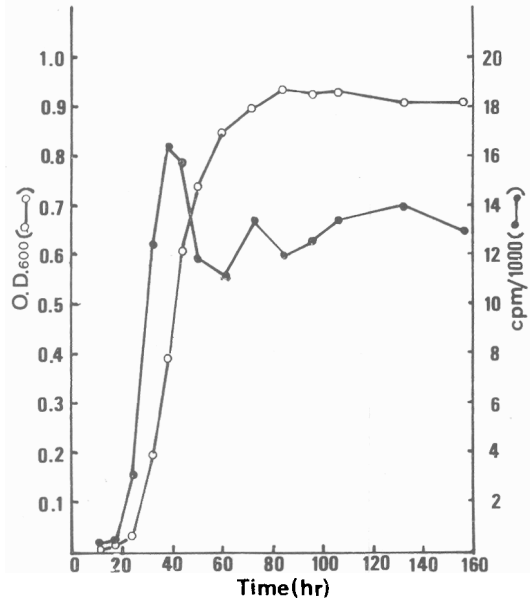

Fig. 2. The relation between yeast cell growth and HBsAg production. The growth of yeast cells and the measurement of HBAg were described in Materials and Methods, CPM measured by Abbott Ausria II radioimmunoassay kit; optical density of yeast cell culture measured at A_{600nm}.

YAMTS, extracts of transformed yeast cells were subjected to analysis by equilibrium sedimentation in CsCl gradients and velocity sedimentation in sucrose gradients as described in the figure legend. A control tube of HBsAg purified from human serum was run parallelly (data not shown). In both cases, single peak of the HBsAg was detected, the buoyant density (1.2 g/cm³) of the HBsAg synthesized this way correspond exactly to the control HBsAg purified from human serum (Fig. **3).** These data clearly suggest that the HBsAg synthesized by using plasmid YAMTS, like those in human serum, is in the form of large particles. The morphology of the particles was further characterized by electron microscopic observation (Fig. **4).** These particles appeared spherical or oval in shape and the diameters varied from 20 to 25 nm.

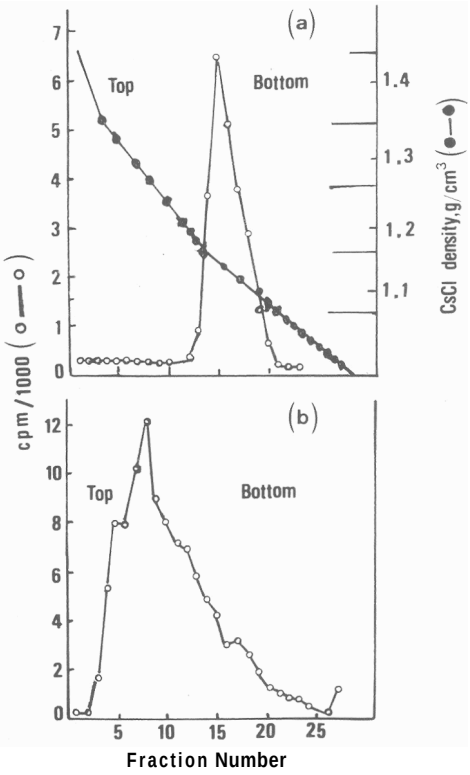

Fig. 3. CsCl and sucrose gradient centrifugation analyses of HBsAg produced via plasmid YAMTS. (a) A 0.5 ml clarified cell extract was layered onto a 10.6 ml two-steps gradient (1.1 and 1.4 g/cm³) of CsCl in 0.85% NaCl/10 mM PB and centrifuged at 41,000 rpm for 38 h in a Beckman SW41 rotor. Fractions (0.5 ml each) were collected by Buchler Auto Densi-Flow II C pump and LKB fraction collector from the top of the tube. (b) Velocity sedimentation in sucrose gradient. A 0.5 ml clarified cell extract was layered onto an 11 ml 10–30% liner sucrose gradient in 0.85% NaCl/1 mM PB and run at 33,000 rpm for 6 h at 5°C in a Beckman SW41 rotor. Fractions were collected as described above.

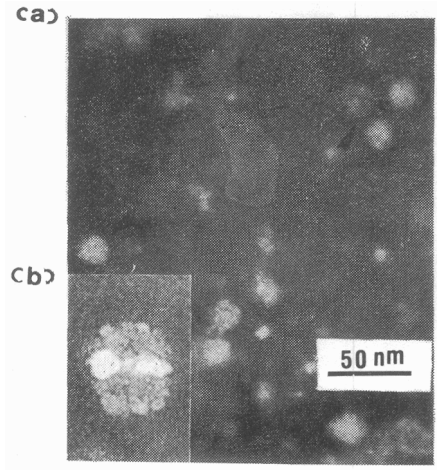

Fig. 4. Electron micrographs of HBsAg particles. The positive fractions from the sucrose gradient centrifugation analysis shown in Fig. 3 were pooled, dialyzed against 10 mM PB and then concentrated. (a) Particles (arrowheads) were visualized after negative staining with 2% phosphotungstic acid. × 200,000. (b) A higher magnification of the particle.

Immunoprecipition Analysis of Protein Composition of HBsAg

The chemical composition of the HBsAg proteins synthesized in yeast transformants was determined by immunoprecipitation of ^{35}S-labelled yeast extracts. Two kinds of antibodies were used: the anti-HBsAg antibody was raised in guinea pig against HBsAg particles from humans and the anti-preS2 antibody was raised against a chemically synthesized polypeptide contained 1st to 18th amino acid of preS2 region of HBV.

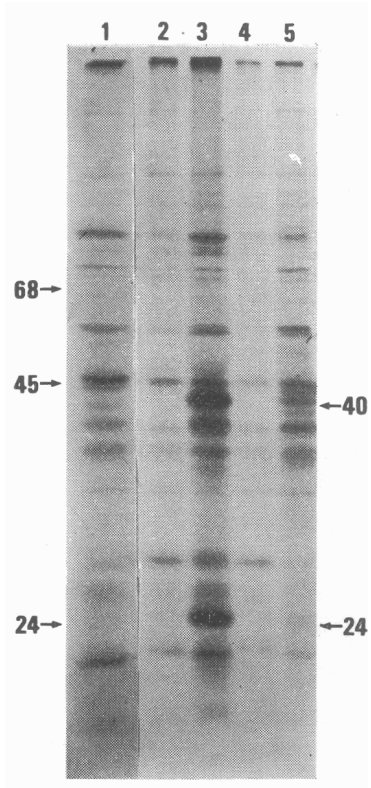

Fig. 5. SDS-polyacrylamide gel electrophoresis analysis of HBsAg polypeptides produced in yeast transformants. Yeast cells at mid-log phase were harvested by centrifugation and washed once with 0.17% YNB. Every 4ml of original culture was resuspended in 1ml YNB supplemented with amino acids except methionine. 150 μCi of ^{35}S-labelled methionine was added to each sample and incubated at 30℃ for 2h with shaking. After labelling, the cells were washed with 1 M sorbitol and then treated with 1% glusulase/1M sorbital at 30℃ for 20 min. The resulted spheroplasts were lysed in 0.5 ml 1% NP40/10 mM PB/2 mM PMSF/0.1% Triton containing glass beads. The extracts were clarified by centrifugation. Antibodies were added to the extracts and incubated at 0℃ for 2h. Staph A proteins were then added and further incubated for 30 min.
The precipitates were collected by centrifugation and washed three times with 0.1 M Tris HCI, pH 8.0/0.5 M NaCl/1% NP40/1% sodium desoxycholate and one time with 10 mM Tris-HCl, pH 8.0. The pellet was resuspended in 15 μl eletrophoresis sample buffer (0.1 M Tris-HCI, pH 6.8/2% SDS/10% glycerol/5% 2-mercaptoethanol/1% bromphenol blue) and boiled at 100℃ for 5 min. After centrifugation, the samples were analyzed on 12% SDS-polyacrylamide gels (Laemmli, 1970) and by autoradiography. Lane 1, strain TL154 transformed by plasmid YAMTS, non-immue serum; Lanes 2 and 4, strain TL 154 (untransformed control), anti-HBsAg serum and anti-preS2 serum, respectively; Lanes 3 and 5, strain TL154 transformed by plasmid YAMTS, anti-HBsAg serum and anti-preS2 serum, respectively. MW markers are α-chymotrypsinogen (24,000), ovalbumin (45,000), bovine serum albumin (68,000).

The SDS-gel electrophoresis of immunoprecipitated materials by anti-HBsAg antibody revealed two bands of Mr 24 K and 40 K, which were not found in the non-transformed cells (Fig. 5). When anti-preS2 antibody was used, only the 40 kDa band was found. The 24 kDa polypeptide, which cannot be detected by anti-preS2 antibody and has the same size as the nonglycosylated human HBsAg polypeptide, is likely to be the S gene product. Because the normal product of the preS2-S gene is only 28 kDa (Valenzuela *et al.*, 1985), the 40 kDa polypeptide, which obviously contains the oligopeptide encoded by the preS2 region, might be the translation product initiated at the AUG of the a-leader encoding sequence. The a-leader peptide, however, was not cleaved after translation. This may, in part, account for the failure of secretion of the protein.

Discussion

In this communication, we report the use of the promoter and the leader sequence of a-factor to express HBsAg. We detected the synthesis and assembly of HBsAg particles. These particles have the same density as the HBsAg 22 nm particles from human serum. Immunoprecipitation analysis of the products with antibodies against human HBsAg and chemically synthesized preS2 region-encoded polypeptide revealed two polypeptides. One has a molecular weight of 24K and reacted only with the anti-HBsAg antibody. It is likely to be the S gene product. The other one has a molecular weight of about **40K.** This protein reacted with antibodies against derived from preS2-encoded polypeptide and human HBsAg, and this is a protein that

contains the products both the preS2 and S regions. From this result we postulate that both AUGs on the cloned sequence, i.e., the AUG of the a-leader and the AUG of the S gene itself (the preS2 encoding region did not contain its own AUG in this construction, see Fig. 1), are used as translation start sites, so the leader peptide might be part of the composition of the 40 kDa protein. (The a-leader peptide contains 83 amino acids and its molecular weight might be approximately 11 K). This is different from what has been previously reported (Valenzuela *et al.,* 1685; Dehoux ***et*** *al.,* 1986). When more than one AUG exist in the cloned HBV genes, usually only the 5'-proximal one was used, and only a single protein product was made. The molecular basis of this difference is not clear, but it might be related to the different distances between the translation starting codon and the 5' end of the respective messages or the different secondary structure associated with the different construction (Kozak, 1986; Mark Cigan and Donahue, 1987). From the autoradiographic result, the 5′ AUG is still much prefered over the internal one. The 40 kDa band can be readily detected by silver staining whereas the 24 kDa band cannot. Also we cannot exclude the possibility that these proteins are made from two separate massages.

Secretion of foreign proteins from S. *cerevzsiae* directed by a-factor promoter and leader sequence has been reported for human epidermal growth factor (Brake *et al.,* 1984), β-endorphin and α-inteferon (Bitter *et al.,* 1984) and mouse interleukin-2 (Miyajima *et al.,* 1985). So far, we can not detect meaning amount of secreted HBsAg in the medium. The molecular weight of HBAg plus the preS2 encoding region is about 28 K. It is unlikely that molecules of this size would be too large to affect secretion, since wheat a-amylase (44 kDa) and the amylo-a-1,4 glucosidase of S. *diastaticus* (60 kDa) have been efficiently secreted by yeast cells (Kingsman *et al.,* 1985). It is possible, however, that the configuration of a-leader peptide when joined to HBsAg might be significantly altered to affect the proper cleavage by signal peptidase. This might account for the failure of secretion of HBsAg in this investigation.

In summary we have employed a-factor promoter and leader to synthesize HBsAg. We were able to simutaneously synthesize higher leve of preS2-S and S proteins, the secretion of these proteins was undetectable, however, further studies will be necessary to characterize the nature of the secretion system.

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利用酵母菌 α-因子的啓動子製造兩種 B 型肝炎表面抗原單白

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本論文是欲構築一酵母菌分泌性載體系統，試圖將B型肝炎前表面抗原及表面抗原（preS2+SAg）經由此分泌性載體系統，在酵母菌之分泌途徑表現。所用的起動子是酵母菌之費洛蒙，α-因子所有，在此起動子後連接前表面抗原及表面抗原之基因。然後將此段 **DNA** 接在酵母菌載體 2-μ 質體中，經由此載體將此段 **DNA** 移入酵母菌中，由轉殖株中可以找到能製造B型肝炎表面抗原的菌株，其所製造者仍是 22 nm 的顆粒，可經由電子顯微鏡證明之。其所產製之表面抗原蛋白，經由 **SDS** 膠體電泳法及免疫學證明：十爲表面抗原，另外一種分子量較大者爲前表面抗原加表面抗原所接連的產物。