

## EXPRESSION OF HEPATITIS B SURFACE ANTIGEN IN *SACCHAROMYCES CEREVISIAE*

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### ABSTRACT

The genome of HBV virus of serotype *ayw* cloned in pBR322 and expression shuttle vector pYES2 were used for construction of the HBsAg chimeric genes and their expression in *Saccharomyces cerevisiae*. Two recombinant plasmids were constructed. One of them contained the coding sequences for the major polypeptide of surface antigen. Another construct carried the major polypeptide with the pre-S2 antigenic determinant. These vectors were transferred into the yeast. Only pDF1 which contained the HBsAg gene was expressed. Some peculiar features of recombinant plasmid construction and expression of the HBsAg gene are discussed. *MJIRI, Vol. 13, No. 1, 67-73, 1999*

**Keywords:** HBV surface antigen, recombinant plasmids, *Saccharomyces cerevisiae* expression vector.

### INTRODUCTION

Hepatitis B virus infection is one of the most widespread human viral infections which causes acute and chronic hepatitis and hepatocellular carcinoma.<sup>1</sup> As infection by this virus is a worldwide problem, there is clearly a pressing need for well designed cost-benefit, cost-effective and cost-saving studies in different countries with high or medium (like the Islamic Republic of Iran) rates of hepatitis B endemicity in order to design more rational strategies for hepatitis B vaccination programs.<sup>2</sup>

The HBV genome consists of a single molecule of partially double-stranded, open-circular DNA. The complete long strand (the minus strand) has a fixed length of about 3200 bp, whereas the complementary short strand (the plus strand) is of variable length. All coding capacity is contained in the minus strand which contains four open reading frames without any introns termed S/Pre-S, C, P and X.

Three initiation sites within the S region result in the formation of three different HBV surface proteins. All of them have a common carboxyl terminus, but each has a unique amino terminus.<sup>3</sup>

The small or major surface protein (HBsAg), which is 226 amino acids long, is a product of the S domain. The middle protein is derived from the pre-S2 and S domains and the large one represents a product of pre-S1, pre-S2, and S domains. These proteins can be found on the outer surface of the infectious "Dane" particles and also in the noninfectious hepatitis B surface antigen (HBsAg) particles. The surface of HBsAg is antigenically complex, as at least five antigenic determinants have been found on these particles: a group specific *a* determinant with two pairs of subtype determinants (*d*, *y* and *w*, *r*). The immune response to the common *a* determinant is sufficient for human protection.<sup>4</sup>

The gene C codes for core antigen and the gene X codes for a 17KDa protein which can transactivate a wide range of cellular and viral genes.<sup>5</sup> The largest transcript from the gene P which extends from 1622 to 2309 bp codes for a

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## HBsAg Expression in *Saccharomyces cerevisiae*

basic protein which seems to have a polymerase activity. *In vitro*, this polymerase can elongate the 3'-end using the minus strand as a template to form fully double-stranded DNA (Fig. 1).

The first attempt of vaccine production was based on the purification of HBsAg particles from sera of chronic carriers. The risk of contamination with other blood-borne diseases made a new approach to vaccine production an absolute necessity. The availability of the techniques for direct expression of a foreign gene in different systems led to the production of a safer and a more potent vaccine using recombinant DNA technology. Furthermore, the HBsAg thus produced could be used for production of diagnostic kits such as ELISA, which is very important for blood screening.

DNA from various HBV serotypes has been successfully cloned and sequenced.<sup>6,7</sup> This permitted the design of new DNA molecules from which HBsAg and its derivatives should be expressed.<sup>8,9</sup>

The yeast cells are now widely used for production of recombinant variants of hepatitis B virus surface antigen (rHBsAg).<sup>9,10</sup> Such preparations of rHBsAg from transformed yeast strains have now been approved for routine use as a human vaccine.<sup>11</sup> A number of factors can control the level of gene expression in yeast. These are the copy number of the interested gene and the stability of that copy number, the nature of the promoter used and the sequence of the mRNA and the impact which this has on mRNA stability.

Many different yeast promoters have been cloned and sequenced. Some of them are constitutive and some regulatable, the latter being very useful in efficient heterologous gene expression. Vectors which contain promoters that function continuously are not ideal for every foreign protein, because the yeast cell must synthesize it throughout the culture period. In some cases this excessive production of foreign proteins increases the doubling time by several hours, which is undesirable in large-scale processes and also some proteins are toxic to yeast.

Following a national program for producing rHBV vaccine, we constructed two recombinant plasmids containing the S and pre-S2+S regions of the HBV genome. Several HBV virus subtypes and yeast vectors were used for expression of HBsAg sequences in yeast and this is a first report of using Gal promoter in expressing HBsAg. Although it was shown that the S domain is sufficient to induce highly protective immunity, experiments in animals have highlighted the potential benefits which might result from the inclusion of the pre-S domain in vaccines.<sup>9</sup>

Following these recommendations two chimeric plasmids were constructed, coding for HBsAg protein and pre-S2 antigenic determinants. These constructs were transferred into the *S. cerevisiae* cells and the expression of them was evaluated.

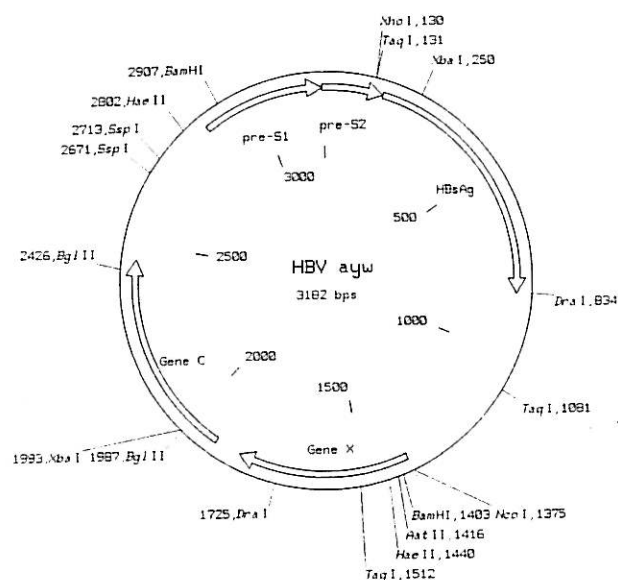


Fig. 1. Restriction map of the HBV DNA subtype ayw.

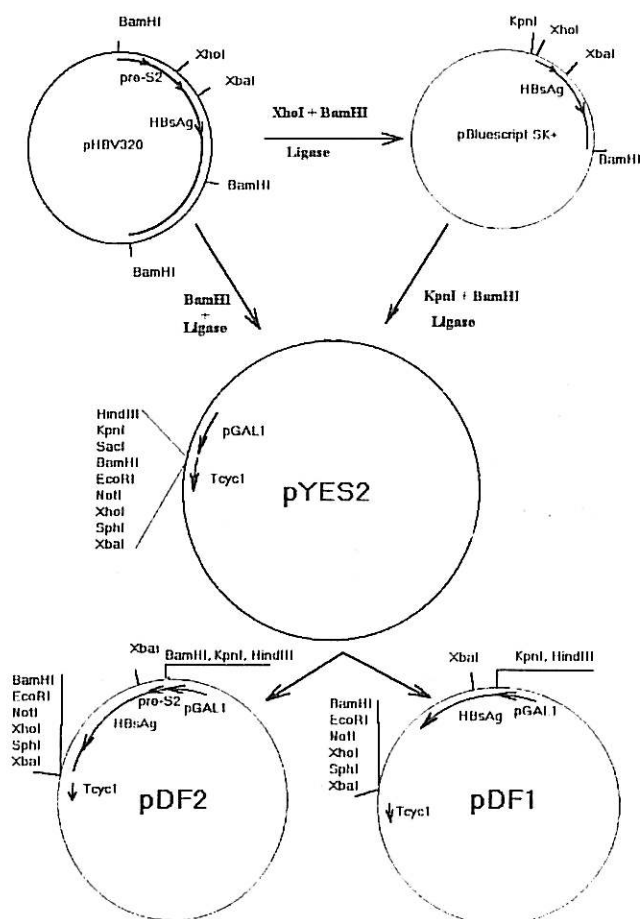


Fig. 2. Schematic representation of the pDF1 recombinant plasmid construction. The details of the work are described in the text.

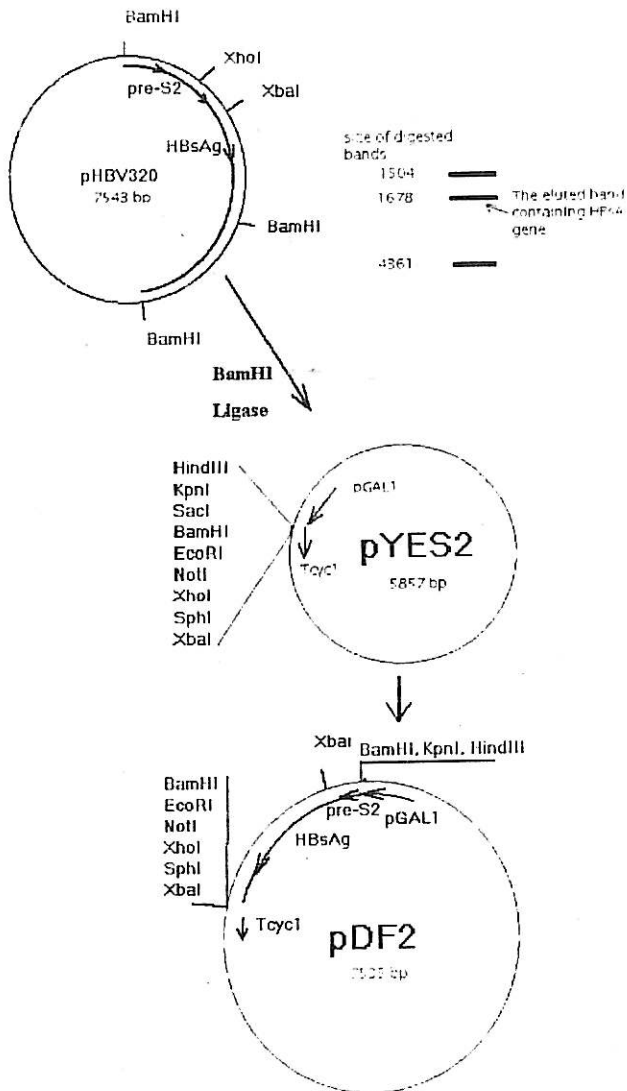


Fig. 3. Schematic representation of the pDF2 recombinant plasmid construction. The details of the work are described in the text.

## MATERIALS AND METHODS

### Strains and plasmids

*E. coli* strains TG-1 {sup E1, hsdΔ5, thi, Δ(lac-proAB) F'[traD36, proAB<sup>+</sup>, lacI<sup>q</sup>lacZΔM15]} and Top10F {mcrA, Δ(mrr-hsdRMS-mcrBC), F80ΔlacZDM15, DlacX74, deoR, recA1, araD139 Δ(ara, leu) 7697, gulU, galK, F, rpsL, endA1, nupG, F'} and yeast strain INVSC 1 (MATα, his3-Δ1, leu2, trp1-289, ura3-52) were used in the experiments. Top 10 F' and yeast strain were from Invitrogen.

The pBluescript SK+ (Stratagen cloning systems, USA) was used for subcloning and the *S. cerevisiae*-*E. coli* high copy shuttle vector pYES2 (Invitrogen Corp.) designed for inducible high level expression of recombinant proteins in *S. cerevisiae* was used for construction of the expression

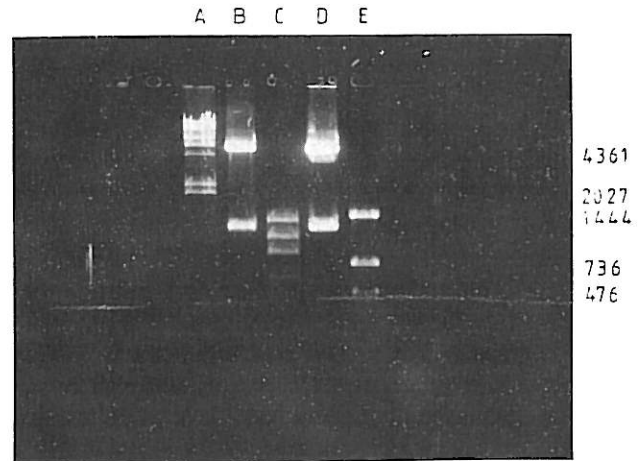


Fig. 4. Agarose gel electrophoretic analysis of fragments generated after restriction endonuclease digestion of pDF1 and pDF2 constructs showing the correct orientation of the HBsAg gene. A-λ HindIII digest; B-pDF2 digested with XbaI; C-φX-174 digested with HaeIII; D-pDF1 digested with XbaI; E-pUC19 digested with TaqI. The sizes of the fragments in bp are indicated by numbers.

cassettes. It contains the Gal1 portion of divergents GAL1/GAL10 promoter and CYC1 transcription termination signal from *S. cerevisiae*, the 2μ origin of replication and partitioning element from YEp24 and the URA3 gene for selection in yeast *ura3*-mutants. The ampicillin gene and *E. coli* origin from pUC19 as well as the T7 RNA polymerase promoter and F1 origin are present for maintenance and selection, transcription and single strand rescue in *E. coli*. It contains a polyclonal site between GAL1/GAL10 promoter and CYC1 terminator which can be used for insertion of the coding sequences of the needed genes.

The pHBV320 plasmid, containing the full genome of hepatitis B virus serotype *ayw* cloned in pBR322, was used as a source for isolating the HBsAg gene.<sup>6</sup>

### Biochemicals and enzymes

Restriction and modification enzymes used in this study were purchased from Gibco-BRL. Biochemicals of the highest quality were used in all experiments.

### Recombinant DNA techniques

Standard techniques for recombinant DNA construction were used.<sup>12</sup> Competent *E. coli* cells were prepared using the FB-buffer according to Hanahan and stored in liquid nitrogen.<sup>13</sup>

The DNA fragments used for cloning were purified by electrophoresis in agarose gel, immobilized onto DEAE-membranes NA-45 (Schleicher & Schuell, Germany) and eluted by 1M NaCl, 5mMEDTA, pH 8.0. After precipitation with ethanol, fragments were ligated with pYES2 vector

## HBsAg Expression in *Saccharomyces cerevisiae*

dephosphorilated by calf intestine phosphatase.

### Computer analysis

"DNASIS" (Hitachi software engineering) and "Clone" (Clone manager, Scientific & Educational Software) software packages were used to perform sequence analysis, GeneBank database searches, finding the restriction sites and construction of the restriction maps.

### Yeast transformation and selection

Yeast cells (INVSC I) were transformed according to the standard procedure using lithium acetate (LiAc).<sup>14</sup> Briefly, cells were grown overnight in YPD broth (Yeast extract 10 g/L, Peptone 20 g/L, Dextrose 20 g/L) at 30°C. After overnight growth the culture was diluted to  $4 \times 10^6$  cells/mL in fresh, warm (30°C) YPD and was regrown to  $1-2 \times 10^8$  cells/mL. Cells were harvested and suspended in sterile water and transferred into 1.5 mL microfuge tubes. After spinning, the cell pellet was washed in TE (Tris-EDTA)/LiAc (1/0.1M), made fresh from 10X filter sterile stocks: 10X TE (0.1M Tris-HCl, 0.01M EDTA, pH 7.5) and 10X LiAc (1M LiAc, pH 7.5). The cells were suspended at  $2 \times 10^9$  cells/mL in 1X TE/LiAc. The transforming DNA (~100 ng) and 50 µg of single-stranded salmon sperm DNA (carrier DNA) were mixed in a microfuge tube to which 50 µL of yeast cells suspension was added (all steps were carried on ice). 300 µL 1X TE/LiAc/40% polyethylene glycol 4000 (PEG) was added to each mixture and after mixing throughly, it was incubated at 30°C with agitation for 30 min and heat shocked in a 42°C waterbath for 15 min. After spinning the cell pellet was resuspended in 1X TE and plated onto selective medium, YNB (0.67% yeast nitrogen base, 2% glucose 0.1 mM histidine, 20 mg/L tryptophan, 30 mg/L leucine). The plates were incubated at 30°C and checked for a week.

### Yeast cell growth and cell lysis

Fermentation of yeast cells was carried out in a fed batch culture. Fifty mL of overnight culture (YNB media) of the transformed cells was inoculated into one liter of YNB media (supplemented with required amino acids and 1% glucose). After 48h incubation at 30°C with aeration, the amount of glucose in the medium was checked and after its exhaustion one liter of new YNB medium which contained 1% galactose as a carbon source was added. The pH of the medium was raised, using sterile 5N NaOH and further incubated for 48 hours. The culture was then centrifuged and the cell pellet washed and dissolved in the disruption buffer containing 50 mM sodium phosphate buffer, pH 7.2, 1mM phenylmethylsulfonyl fluoride, 1mM EDTA and 1% Triton X-100. The glass beads (212300 microns, Sigma, USA) were added and the cells were ground, using liquid nitrogen to keep the mixture cool and solid enough. The mixture was centrifuged at 12000g, 4°C

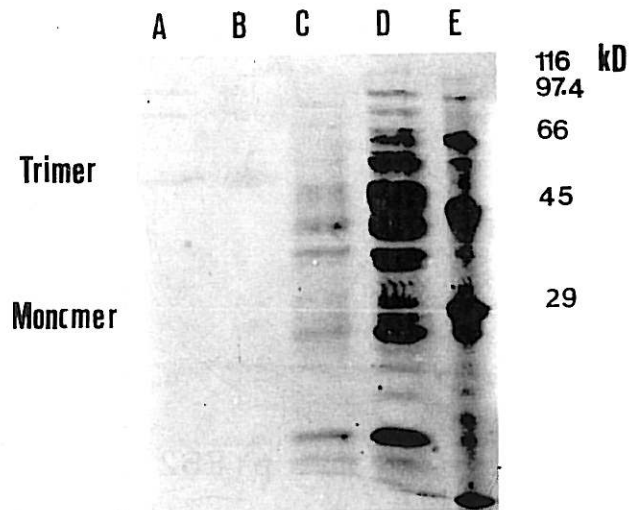


Fig. 5. SDS-page analysis of the recombinant HBsAg.

Lane A: rHBsAg isolated from yeast.

Lane B: HBsAg isolated from sera.

Lane C and D: Total cell soluble extract from a negative and positive yeast respectively. Lane E: MW markers (kDa).

for 30 min. and the supernatant collected and assayed for total protein using Lowry's method,<sup>15</sup> and HBsAg by ELISA kit (Hepanostika®, microelisa system, Organon Teknika BV, Holland). The cell extract was also used for further purification, using immunoaffinity chromatography. HBsAg purification and characterization were performed according to the previously published procedure.<sup>16</sup>

### pDF1 construction

The coding sequences for the major part of the HBsAg was excised from pHBV320 plasmid as a XhoI-BamHI fragment and recloned in pBluescript SK+ plasmid. Then it was excised by KpnI and BamHI and cloned in pYES2 vector. According to the HBV subtype ayw sequence found in GeneBank (see also ref. 2) the cloned fragment does not contain any ATG-codon in any frames in front of the HBsAg coding sequence. So, the start codon for HBsAg sequence would be the first ATG-codon after GAL1/GAL10 promoter and could be used for translation.

### pDF2 construction

The expression vector containing the sequence coding for HBsAg with pre-S2 antigenic determinant was constructed by cloning the BamHI fragment (which contains the desired gene) of pHBV320 plasmid in pYES2. This fragment contains the open reading frames for HBsAg and pre-S2 determinant. As a result of this cloning two constructs could be obtained, one with correct and another with the

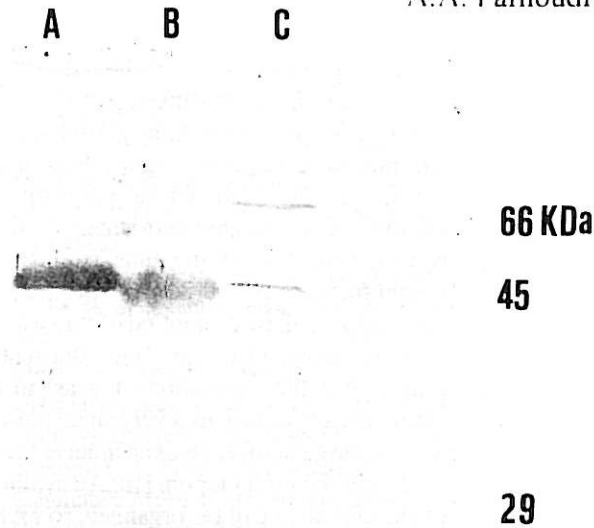


Fig. 6. Western blot analysis of the purified HBsAg expressed in yeast.

Lane A: HBsAg isolated from the sera.

Lane B: Recombinant HBsAg expressed in yeast.

Lane C: Total cell soluble protein of yeast expressing HBsAg.

reverse orientation of the coding sequence. The orientation of the coding sequence was checked by XbaI digestion. In this case the plasmid that contained the cloned fragment in correct orientation produced two fragments 1700 bp and 6335 bp in length (Fig. 4). Plasmids with the reverse orientation of the cloned fragment produced XbaI fragments 527 bp and 6963 bp in length.

Construction of both recombinant plasmids (pDF1 and pDF2) for expression of HBsAg gene in yeast cells is schematically presented in Fig. 2 and 3, respectively.

#### Yeast transformation efficiency

During this study by using LiAc procedure for

transforming the yeast strain we received  $10^2$  transformants per  $\mu\text{g}$  of DNA which was good enough for our purpose.

#### Expression of HBsAg

The yeast transformants were induced for expression of the HBsAg by adding the inducer, galactose. The cell extract was assayed for HBsAg by ELISA and also the confirmatory test was done for each positive result by micro ELISA (Tables I and II). Our data showed that the HBsAg was expressed in the yeast which was transferred by pDF1. About 0.01% of the total soluble protein of the yeast extract was HBsAg. Comparison of the purified HBsAg from the yeast and the HBsAg isolated from human sera showed that both of them could be seen in the trimer and monomer forms (Fig. 5) (the serum HBsAg was kindly provided by Mr. Khabiri, Immunology Dept., Pasteur Institute). Western blot analysis also confirmed that the purified protein was HbsAg which could be detected in monomer and dimer forms (Fig. 6). The strains which were transformed by pDF2 did not show any sign of expression of the antigen.

#### DISCUSSION

Expression of hepatitis B surface antigen coding sequences in yeast leads to the production of particles immunoreactive with anti-HBsAg antibodies. These particles are similar to those made by human carrier patients and are widely used for human immunization.<sup>8,11</sup>

The HBV virus is highly specific for replication in the liver. Specificity is suggested to be due to the receptor recognition on the surface of the liver cells and the presence of transcription factors that are either specific or abundant in the liver cells,<sup>17,18</sup> which could explain the difficulties in propagation of the virus using cell culture.

The first approach to produce the HBV vaccine was

Table I: ELISA test results.

Construct	Total cell soluble protein	Reading of ELISA	Negative control
pDF1	1980 $\mu\text{g}$	0.411	0.177

The negative control was the yeast harboring the designated plasmids and cultivated in an uninduced condition.

Table II: Confirmatory micro ELISA test result.

ELISA reading	After neutralization	Positive control of the kit	Negative Control
0.411	0.121	1.213	0.129

Positive control is HBsAg supplemented in each kit and the negative control is this HBsAg after neutralizing with the anti-HBsAg available in the kit.

## HBsAg Expression in *Saccharomyces cerevisiae*

from plasma containing purified 22 nm HBsAg particles obtained from asymptomatic carriers,<sup>19,20</sup> but the origin of the vaccine and the fear of transmission of blood-borne diseases and the limitation in the availability of human serum made a different approach which was the production of HBsAg using recombinant DNA techniques desirable. Attempts to produce HBsAg in *E. coli* were less successful; yields were poor, and since HBsAg is a conformational antigen, its full immunogenicity is highly dependent on its tertiary structure which could not be achieved in the bacteria.<sup>10,21</sup> Therefore a eukaryotic system such as yeast or mammalian cells was needed. Yeast is a highly suitable system for large scale fermentation products and also the lower price of the production has made it a suitable choice for the production of recombinant HBV vaccine.

The factor that must be considered in using regulatable promoter, such as that used in this study, is the induction rate which is the difference between the lowest and highest level of expression. The most widely used regulated vectors are based on the Gal promoter. The Gal 1 and Gal 10 genes are regulated by galactose with an induction ratio of about 1000 and maximum levels of expression are moderately high with each transcript comprising about 0.25% of total mRNA. The levels of protein are still lower than predicted from the strength of the promoter and the copy number of the plasmid. The basis for the failure to achieve maximum theoretical efficiency of heterologous gene expression in any yeast vector to date is not known. It may in some cases be due to inefficient transcription.

It should be noted that both gene constructions described in this paper contain some extra sequences preceding and following the coding region. It is not known whether these extra sequences influence the expression efficiency of the constructed chimeric genes, e.g. by altering the mRNA stability, but similar sequences also present in various constructs have been used for rHBsAg production.<sup>8,21</sup> Data also reported by other workers showed that longer untranslated sequences had a remarkable effect on the synthesis of desired protein.<sup>22</sup> The HBsAg which was expressed was 0.01% of the total soluble protein which, in comparison to the result that has been reported, was much lower.<sup>23</sup> Furthermore the pDF2 construct which harbors the gene for middle protein (pre-S2+S) could not be expressed in the yeast or the amount of product was too low to be detected by microelisa technique. It must be mentioned that the pre-S region is much more susceptible to the protease,<sup>24</sup> so the product may be degraded during the extraction procedure. After cell lysis, the clarified extract contained HBsAg particles which migrate as a 25 kDa monomer in SDS-PAGE under nonreducing conditions. During the early phases of purification, some interchain disulfide bonds form so that the antigen migrates as a mixture of monomer and dimer under nonreducing conditions. After treatment with concentrated ammonium or potassium

thiocyanate, additional interchain disulfide bonds formed between dimers, and the fully cross-linked particles were obtained, which could not enter the running gel of the SDS-PAGE system, unless a reducing agent was used. Immunoblotting detects all of the HBsAg polypeptides, regardless of conformation or aggregation state.<sup>25,26</sup> In our study even by increasing the boiling time in Laemmli buffer, the different forms of HBsAg in SDS-PAGE were detectable which could result by promotion of thiocyanate in the formation of interchain disulfide bonds. During this study, we observed that the cultivation of yeast in the medium containing galactose gave us a very small amount of cell mass, because the galactose acts as an inducer for the Gal promoter and turns the promoter on. HBsAg synthesis had an effect on the growth rate of the organism, so we had to cultivate it in the medium with glucose to reach higher cell mass and then change the carbon source of the medium to galactose.

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