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Abstract

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KEYWORDS: hepatitis B surface antigen, preS2 region, plasmid, yeast, Saccharomyces cerevisiae

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Expression of the Hepatitis B Surface Antigen Gene Containing the PreS2 Region in *Saccharomyces cerevisiae*

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We constructed a plasmid, pBH103-ME5, in which the region encoding the 10 preS2 amino acid residues and the S domain of the hepatitis B surface antigen (HBsAg) were regulated by the promoter of the yeast repressible acid phosphatase gene. *Saccharomyces cerevisiae* carrying pBH103-ME5 produced the HBs antigen (yHBsAg), when it was cultured in a medium containing a low concentration of phosphate. The antigen was purified to homogeneity. Its molecular weight was determined by Western blotting to be 24,000, and its amino acid composition agreed well with that deduced from the nucleotide sequence. The C-terminal amino acid sequence of yHBsAg was exactly the same as that predicted from the nucleotide sequence, while the N-terminal amino acid acetylserine, which was followed by 8 amino acid residues coded by the preS2 region. These results indicate that the recombinant yeast produced a single polypeptide consisting of the preS2 region and the subsequent S domain after being processed at the N-terminus.

Key words : **hepatitis B surface antigen, preS2 region, plasmid, yeast, *Saccharomyces cerevisiae***

The hepatitis B virus (HBV) causes not only acute and chronic hepatitis but also liver cirrhosis and primary hepatocellular carcinomas. It is a major health problem throughout the world (1). The hepatitis B surface antigen present in HBsAg carrier plasma has been used as a source of hepatitis B vaccine. The antigen can also be produced by a recombinant yeast which carries the HBsAg coding gene on a plasmid. The yeast-derived HBsAg (yHBsAg) is biologically and morphologically similar to the human plasma-derived antigen (hHBsAg) (2, 3), and has proven safe and efficacious for human use (4, 5, 6). The advantages of the utilization of a recombinant yeast system are that preparation of the antigen from yeast cells is much safer and less expensive than from human plasma, and that a more immunopotent antigen might be obtained through gene manipulation.

The HBV genome contains one large open reading frame coding for HBsAg, which consists of three domains, preS1, preS2 and conventional HBsAg (7). The preS2 sequence in HBV has been suggested to play an important role in HBV infection (8). In addition, an antibody to the preS2 sequence has been shown to exhibit

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neutralizing activity against HBV (9). This led us to expect that the preS2 sequence may somehow exert an additive or stimulatory effect on the immunogenicity of HBsAg, if the two antigens were used as a single polypeptide. We attempted to clone the gene encoding for the HBs gene and the preS2 region into a yeast. The present report describes the construction of a plasmid in which the HBs gene preceded by the preS2 region was inserted downstream of a repressible promoter of the yeast acid phosphatase gene (*pho5*). We also present evidence that a yeast clone carrying this plasmid produces yHBsAg with 9 preceding amino acid residues of the preS2 region.

Materials and Methods

Materials. HBsAg (subtype, adr) positive human plasma was kindly provided by Dr. M. Takahashi (Department of Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) and used as a source of HBs DNA. The microorganisms and plasmids used in this study were as follows: yeast strain, *Saccharomyces cerevisiae* SHY4 (ATTC44772, a *ste-vc9 ura3-52 trp1-289 leu2-3 leu2-112 his3-1*) (10); *Escherichia coli* strains, *E. coli* χ1776 (11) and JM109 (12); plasmids, pPHO5 (13), pBR322 (14), pBR325 (15), YEp13 (16) and pUC13 (17). Goat anti-hHBsAg serum was purchased from Dako Japan Co., Ltd. (Kyoto, Japan). Enzymes and chemicals were purchased from the following sources: restriction enzymes, T4 DNA polymerase, T4 DNA ligase, a M-13 sequencing kit and a kilo-sequence deletion kit from Takara Shuzo Co., Ltd. (Kyoto, Japan); nuclease Bal31 from Bethesda Research Laboratories (Gaithersburg, USA); a DNA *in vitro* packaging kit from Toyobo Co., Ltd. (Osaka, Japan); [α-^{32}P]dCPT (> 400 Ci/mmol) from Amersham International plc (Buckinghamshire, England); carboxypeptidase Y from Oriental Yeast Co., Ltd. (Tokyo, Japan); a HBs detection kit, Ausria II, from Abbott Lab. (Chicago, USA); a silver staining kit from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan); and peroxidase-conjugated anti-goat or anti-guinea pig immunoglobulin G, for Western blot analysis, from Cappel, Organon Teknika Co. (Westchester, USA).

Construction of plasmids. HBV DNA was prepared from HBeAg positive human plasma as described by Sattler and Robinson (18) and then digested with *Xho*I. A 3.2-kb fragment containing the HBs gene was ligated to the *Xho*I site of the Charon28 phage vector (19). The ligation products were packaged *in vitro* to generate intact phage particles. A 3.2-kb *Xho*I fragment of the HBV gene was isolated from the recombinant and ligated into the *Bam*HI site of pBR322, the resultant plasmid being named pM1B11. From pM1B11 was constructed a plasmid, pBH103-ME5, in which the 1.2-kb HBV gene was located downstream of a yeast repressible acid phosphatase gene (*pho5*) promoter, as shown in Fig. 1. pBH103-ME5 was introduced into *S. cerevisiae* SHY4 by the alkali cation method of Ito *et al.* (20). A recombinant yeast was selected after incubation at 37°C for 3 days on a leucine-free SD agar plate consisting of 6.7 g Bacto-yeast nitrogen base w/o amino acids (Difco Laboratories, Detroit, USA), 20 g glucose, 20 mg uracil, 200 mg L-trypophan, 20 mg L-histidine HCl and 20 g agar per liter. The HBV positive clone, thus obtained, was termed SHY4/pBH103-ME5.

Purification of yHBsAg. SHY4/pBH103-ME4 was cultured in 10 liters of Burkholder medium (21) supplemented with tryptophan, histidine, uracil and potassium dihydrogen phosphate (200 mg of each). Cultures were grown at 30°C for 28 h. Afer centrifugation at 12,000 × g for 10 min, the cell pellets were suspended in 500 ml of 0.01 M phosphate buffer, pH 9.0, and then disrupted 5 times with a Gaulin homogenizer (Gaulin Co., Ltd., USA) at 8,000 psi while cooling in ice-water. Then the cellular debris was removed by centrifugation at 12,000 × g for 20 min. The supernatants were adsorbed to 2 % silica gel and then eluted with 0.25 M carbonate buffer, pH 10. The eluates were treated with 1 % active carbon, and the pH was adjusted to 6 with 0.4 N HCl. The yHBsAg fraction was further purified by 25-to-50 % sucrose density gradient centrifugation at 59,000 × g at 4 °C for 20 h. The yHBsAg fraction was collected and dialyzed aginst 5 mM phosphate buffer, pH 9.0. To 10 ml of the fraction was added 40 ml of a potassium bromide solution (40 %, w/w), followed by banding on an equilibrium density gradient by centrifugation at 210,000 × g at 4 °C for 48 h. The purified yHBsAg was dialyzed against 5 mM phosphate buffer, pH 9.0.

Nucleotide sequencing. A 1.2-kb *Xho*I fragment of the HBs gene was obtained from pBH103-ME5 and ligated into the *Xho*I site of pUC13. Clones to be sequenced were generated by exonuclease Bal31 deletion. DNA sequencing was carried out by the dideoxy chain termination method (22) using 2'-7'-deazaguanosine triphosphate instead of dGTP (23).

Amino acid analysis. The purified yHBsAg was carboxymethylated with iodoacetic acid and hydrolyzed as follows. For the analysis of serine and threonine, hydrolysis in 6N HCl containing 1% phenol was carried out at 110°C for 24 and 48h. For tryptophan, the hydrolysis was performed in 4N methanesulfonic acid at 110°C for 24, 48 and 96h (24). The rest of the amino acids were analyzed after hydrolysis in 9.3N HCl containing 10% trifluoroacetic acid (TFA) and 0.1% phenol at 150°C for 1, 2, 4 and 24h (25). The hydrolysates were subjected to analysis with an amino acid autoanalyzer, Model 835 (Hitachi Ltd., Tokyo, Japan). Cystine and cystein were quantitated as carboxymethylcysteine (26).

Amino acid sequencing. The N-terminal amino acid sequence of the yHBsAg was determined with a gas phase amino acid sequencer, Model 4704A (Applied Biosystems, Foster, USA). Prior to analysis with the sequencer, the purified yHBsAg was treated with cyanogen bromide (BrCN) and trypsin as follows. A 400-μl sample containing 3.66mg of yHBsAg/ml was added to 933μl of formic acid containing 4.3mg/ml of BrCN. Incubation was carried out at room temperature for 24h. After the addition of 12ml of distilled water and lyophilization, a sample was dissolved in 300μl of 0.1% TFA and then separated by reverse phase high-performance liquid chromatography (HPLC). HPLC was carried out on an ODP-50 column (Asahikasei, Tokyo, Japan) in a Model 655 HPLC apparatus (Hitachi). Elution was performed with 0.1% TFA (0.1%) in a linear gradient from water to acetonitrile at 1ml/min. The peak corresponding to the N-terminal peptide was identified by analyzing the amino acid composition. The N-terminal peptide (approximately 5nmol) was further digested with 5μg of trypsin at 37°C for 5h and then separated by HPLC under the same conditions as described above except for the column (VYDAC 218TP54, 4.6 × 250mm). The C-terminal amino acid sequence was determined by digestion with carboxypeptidase Y, followed by analysis of the amino acid serially released.

Idetification of the N-terminal modified amino acid. The N-terminal peptide liberated on BrCN cleavage was subjected to FAB-mass spectrometric analysis, which was performed with a ZAB-HF apparatus equipped with a 11/25 computer system (VG Analytical, Manchester, England). After mixing with glycerol, a sample was applied and ionized by means of the FAB technique. The operating conditions were: acceleration potential of primary ion Xe^+, 8kV; FAB emission current, 1mA; acceleration potential of secondary ions, 8kV; scanning speed, 30sec/decade; scanning interval, 5sec; scanning range, m/z = 10 to 1,500.

SDS-Polyacrylamide gel electrophoresis and Western blot analysis. The yHBsAg was treated with the sample buffer containing 1% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol at 95°C for 2min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% polyacrylamide gel by the method of Laemmli (27). Each gel was silver-stained. PAS staining was also carried out for detection of carbohydrates as described by Glossmann and Neville (28). Proteins in unstained gel were transferred to a nitrocellulose filter in a transfer apparatus (Trans-blot cell; Bio-Rad Lab., Richmond, USA). Western blotting was performed by the procedure of Towbin *et al.* (29). The antibody bound to yHBsAg was detected on a nitrocellulose filter by using peroxidase-conjugated rabbit anti-goat or anti-guinea pig immunoglobulin G.

Other procedures. The protein content was determined by the method of Lowry *et al.* (30). The yHBsAg content was determined by radioimmunoassay according to the instruction manual published by the company (Abbott Lab., Chicago, USA). Guinea pig anti-yeast cell serum was obtained by subcutaneous injection of a yeast extract in Freund incomplete adjuvant (Difco) into Hartley guinea pigs.

Results

Construction of pBH103-ME5. A plasmid, pBH103-ME5, that expresses the HBs gene in *S. cerevisiae* was constructed from pM1B11, as shown in Fig. 1. In this plasmid the HBs gene is located downstream of the promoter of the yeast acid phosphatase gene (*pho5*) derived from pPHO5. Furthermore, the *bla* and *leu2* genes in this plasmid allow the selection of *Escherichia coli* and *S. cerevisiae* transformants, respectively. The nucleotide sequences of the *pho5* promoter and its downstream inserts of the pBH103-ME5 were determined (Fig. 2). The methionine codon present 82bp downstream of the Hogness box (TATATAA) could be the translational initiation codon. The open reading frame starting at this position encodes 10 amino acid residues (aa) of the preS2 region and the subsequent 226 aa of

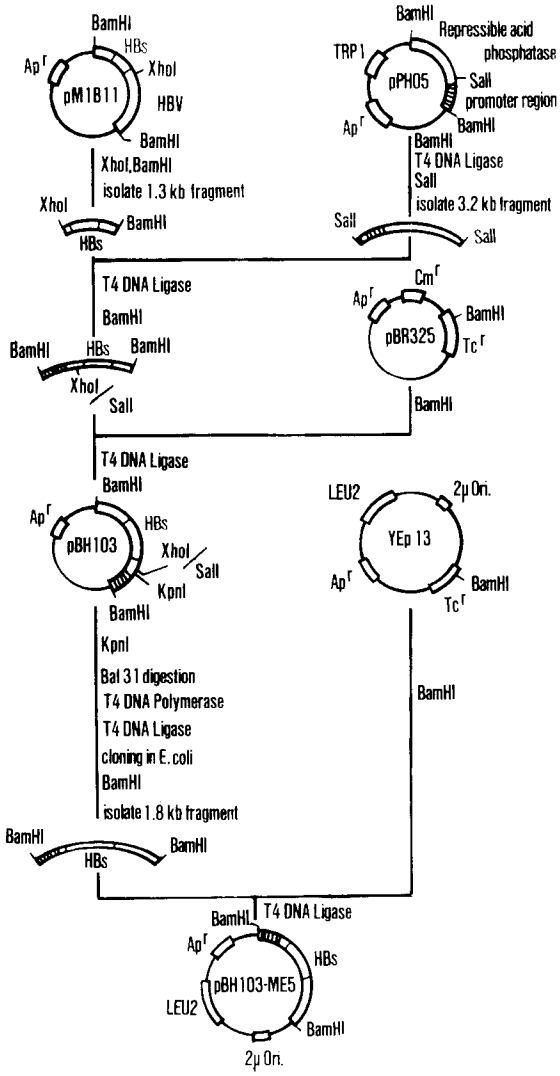

Fig. 1 Construction of pB103-ME5. A 1.3-kb fragment (*XhoI*/*Bam*HI) containing the HBs gene was isolated from pM1B11. A 3.2-kb *SalI* fragment containing a promoter region of the yeast repressible acid phosphatase gene was obtained by digesting pPHO5 with *Bam*HI, followed by ligation and digestion with *SalI*. This fragment was ligated to the 1.3-kb (*XhoI*/*Bam*HI) fragment, followed by digestion with *Bam*HI. The resulting 4.5-kb fragment was ligated to the *Bam*HI site of pBR325. The resultant plasmid, named pB103, was digested with *KpnI*, followed by exonuclease Bal31 deletion to eliminate the translational codon of the *pho5* gene. After filling up with T4 DNA polymerase, both ends were ligated and pME5 was obtained. A 1.8-kb *Bam*HI fragment derived from pME5 was inserted into the *Bam*HI site of YEp13. The resultant plasmid, named pBH103-ME5, was cloned into *E. coli* χ1776.

HBsAg. Therefore, the recombinant yeast, SHY4/pBH103-ME5, would presumably synthesize yHBs consisting of 236 aa. The nucleotide sequence of the 226 aa coding region was compared with that reported for the same subtype (adr) HBs gene, which was cloned into pBRHBadr4 (31) and pHBr330 (32). Although there are nucleotide substitutions at 4 positions (Fig. 2),

Fig. 2 Nucleotide sequence of the *pho5* gene promoter and HBs gene. The nucleotide sequence is numbered at the left, and the location of the Hogness box is indicated by a line above the sequence. The open reading frame coding the preS2 region (underlined) and conventional HBsAg is boxed. Nucleotides different from those reported for other HBs genes cloned into pBRHBadr4 (31) and pHBr330 (32) are indicated by arrowheads.

there is a different amino acid only at position 165, where pBH103-ME5 and pHBr330 encode for tryptophan, but pBRHBadr4 encodes for glycine. In order to determine whether or not the yHBsAg gene can be stably maintained in the yeast, its nucleotide sequence was determined after SHY4/pBH103-ME5 had been repeatedly subcultured in the leucine-free Burkholder medium. Neither nucleotide substitution nor deletion was observed even after 38 subcultures.

Production of yHBsAg by the recombinant yeast. The production of yHBsAg by the recombinant yeast, SHY4/pBH103-ME5, was examined after various growth times in a phosphate-limiting medium (Fig. 3). The phosphate concentration in the culture medium decreased as the yeast cells grew. When phosphate was depleted, the production of both yHBsAg and chromosomally encoded acid phosphatase were induced, and thereafter continued to increase into the stationary phase, indicating that yHBsAg was synthesized under the control of the repress-

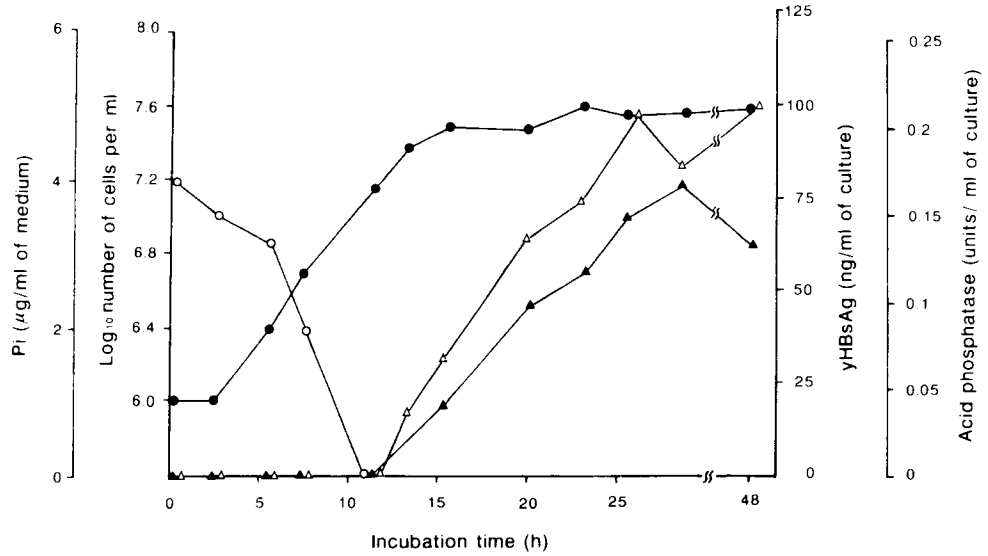

Fig. 3 Production of the yHBsAg by the recombinant yeast at various growth phases. *S. cerevisiae* SHY4 carrying pBH103-ME5 was grown in Burkholder medium supplemented with tryptophan, histidine, uracil and KH_2PO_4 (20 mg/liter of each). The Pi content of the medium (○) was determined by the phosphomolybdic acid method (38). The cell number (●) was determined under a light microscope. Acid phosphatase activity and the amount of yHBsAg were measured after cells had been disrupted as described in the text. Acid phosphatase activity (△) was determined by the method of Toh-e *et al.* (39) and yHBsAg (▲) by radioimmunoassay.

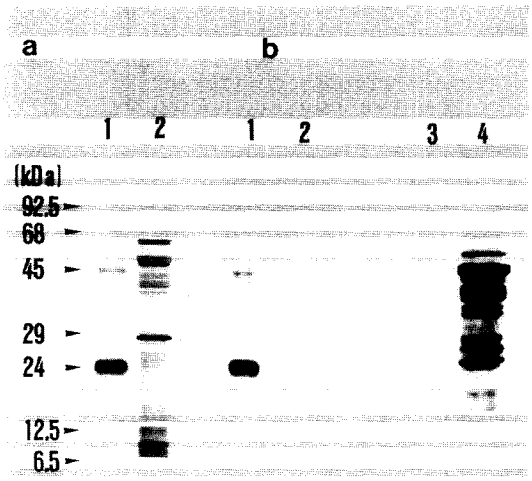

Fig. 4 Analysis of the purified yHBsAg by SDS-PAGE and Western blotting. (a) A gel was silver-stained after SDS-PAGE. Lane 1, yHBsAg; lane 2, extract from *S. cerevisiae*. (b) Polypeptides separated by SDS-PAGE were transferred to a nitrocellulose membrane (BA-85; S & S, West Germany) using a Trans-blot cell and then allowed to react with goat anti-HBs serum (lane 1 and 2) or guinea pig anti-yeast serum (lanes 3 and 4) at room temperature for 180 min, and then with peroxidase-labeled anti-goat IgG or anti-guinea pig IgG. The reactive polypeptides were visualized with peroxidase substrate H_2O_2 and 4-chloro-1-naphthol. Lanes 1 and 3, yHBsAg; lanes 2 and 4, extracts from *S. cerevisiae*. The amounts of protein of yHBsAg and the yeast extract applied were 2.6 and 40 μg, respectively.

ible promoter of the acid phosphatase gene.

Purification of yHBsAg. The yHBsAg was prepared from 10 liters of SHY4/pBH103-ME5 culture and then purified as described under Materials and Methods. The purified yHBsAg was analyzed by SDS-PAGE and Western blotting (Fig. 4). Only two bands were detected for the purified yHBsAg on silver staining. Both the minor upper band and the major lower one were reactive with the anti-hHBs antibody but not with

the anti-*S. serevisiae* antibody. Furthermore, no band was detected with the anti-hHBs antibody for a sample prepared from *S. cerevisiae* alone. The molecular weight of the major antigen was estimated to be 24,000, which is similar to the molecular weight, 26,300, calculated from the deduced amino acid sequence. The upper band corresponding to a 45kDa polypeptide can be regarded as a dimer of a 24kDa monometer (see Discussion). These results clearly indicate that the recombinant yeast, SHY4/pBH103-ME5, produces the yHBs antigen, as would be expected, and that the yHBsAg was highly purified. When a gel was stained with PAS-staining, no positive band was observed for the purified yHBsAg, indicating that it is not glycosylated (data not shown).

Amino acid composition and sequence of yHBsAg. In order to determine whether or not the yHBsAg has the same amino acid sequence as that deduced from the nucleotide sequence, the amino acid composition of the yHBsAg, and the amino acid sequences of its N- and C-termini were analyzed. The amino acid composition determined essestially coincided with that predicted from the nucleotide sequence (Table 1). When determination of the N-terminal amino acid sequence was carried out under the conditions routinely used, no amino acid residue was liberated from yHBsAg, indicating that the amino group of the N-terminal residue should be blocked. Therefore, the N-terminal amino acid sequence was determined as follows. Based on the assumption that cleavage by BrCN occurs between the 10th aa residue, asparagine, and the 11th one, methionine, and trypsin cuts between the 3rd aa residue, arginine, and the 4th one, threonine, in the amino acid alignment shown in Table 2, yHBsAg was subjected to BrCN treatment and trypsin digestion. After treatment with BrCN, an N-terminal small fragment was purified by HPLC. This fragment was further digested with trypsin.

The amino acid sequence of the tryptic digest was determined to be Thr-Gly-Asp-Pro-Ala-Pro-

Table 1 Amino acid composition of yHBsAg.

Amino acid	Number of residues	
	Determined	Deduced from DNA sequence
Asx	12.0	11
Thr	18.0[a]	19
Ser	24.1[a]	24
Glx	10.5	9
Pro	24.5	26
Gly	15.0	15
Ala	7.5	7
1/2 Cys	11.2[b]	14
Val	9.6	10
Met	5.5	7
Ile	10.9	14
Leu	29.1	35
Tyr	5.4	5
Phe	14.2	17
Lys	3.2	2
His	1.5	1
Trp	12.5[c]	13
Arg	6.9	7

Asx and Glx represent total asparagine plus asparate and total glutamine plus glutamine, respectively.
a: Determined by hydrolysis with 6N HCl.
b: Determined as S-carboxymethyl-cystein.
c: Determined by hydrolysis with 4N methanesulfonic acid.

Table 2 Amino- and carboxy-terminal sequences of yHBsAg

	1	2	3	4	5	6	7	8	9	10	11	.....	232	233	234	235	236	
DNA sequence	ATG	TCG	AGG	ACT	GGG	GAC	CCT	GCA	CCG	AAC	ATG	.....	CTT	TGG	GTA	TAC	ATT	TAG
Amino acid sequence																		
Deduced	Met	Ser	Arg	Thr	Gly	Asp	Pro	Ala	Pro	Asn	Met	.....	Leu	Trp	Val	Tyr	Ile	Amb
Determined		acetyl-Ser	Arg	Thr	Gly	Asp	Pro	Ala	Pro	Asn	Met	.....	Leu	Trp	Val	Tyr	Ile	

The predicted amino acid sequence is numbered at the top of the table.
Amb denotes amber stop codon.

Asn, as expected. The amino acid composition of the N-terminal fragment liberated on BrCN treatment was also determined. The results suggest that the above-cited sequence is preceded by Ser-Arg and followed by homoserinelactone. To identify the N-terminal residue, the N-terminal fragment was analyzed by FAB-mass spectrometry. $(M+H)^+$ was detected at 997 of m/z. If the N-terminal serine residue were not modified, it would have been detected at 1015. The difference, 42 of w/z, clealy indicates that the N-terminal serine residue is acetylated. Although such processing occurred at the N-terminus, the C-terminal amino acid sequence was completely the same as that deduced from the nucleotide sequence. N- and C-terminal aa sequences of yHBsAg were found to be as shown in Table 2.

Discussion

We have demonstrated that *S. cerevisiae* carring pBH103-ME5 produced HBsAg preceded by the 9 amino acid sequence of the preS2 region. Amino acid sequence analysis of the purified yHBsAg showed that the first methionine at the N-terminus was removed and the resultant N-terminal residue, serine, was acetylated. Similar processing at the N-terminus has been demonstrated in *S. cerevisiae* by Tsunasawa *et al.* (33). The N-terminal methionine is cleaved by yeast aminopeptidase when it is followed by a small residue like serine but not when it is followed by a large residue like a glutamic acid. They also suggested that the yeast contained an acetyltransferase that acetylated the N-terminal residue. The N-terminal methionine of our yHBsAg is followed by serine, while that of conventional HBsAg is followed by glutamic acid. Therefore, only the first methionine of our yHBsAg can be removed. Our results are consistent with the finding of Tsunasawa *et al.* (33).

SDS-PAGE showed that two polypeptides were present in the purified yHBsAg, which were both reactive with anti-hHBsAg antiserum. When the minor 45 kDa polypeptide was extracted from a gel and electrophoresed after being re-reduced, it migrated to a position corresponding to that of a 24 kDa polypeptide (data not shown). This may imply that some, if not all, of the 24 kDa monomer polypeptide is present in the form of a dimer, of which monomerization requires strict reducing conditions. The yHBsAg contains 14 cysteine residues per molecule. Such an unusually high cysteine content should allow the formation of not only intra- but also intermolecular disulfide bridges as was suggested elsewhere (34).

The final goal of our research is to develop a HBs-antigen of high immunopotency. Our approach has focused on the application of genetic engineering, which allows the synthesis of a polypeptide consisting of a part of the preS2 region and conventional HBsAg. The preS2 sequence may alter the antigenic profile of HBsAg or may lead to the production of a specific antibody to it, which could increase the immunopotency of yHBsAg (9, 35, 36). We are currently concentrating on characterization of its immunological properties (37).

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