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EXPRESSION OF HEPATITIS B VIRUS SURFACE ANTIGEN P31 GENE IN YEAST

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SUMMARY The hepatitis B virus surface antigen (HBsAg) P31 gene has been expressed in yeast Saccharomyces cerevisiae. The gene products were shown to be glycoproteins with molecular sizes of 37,000 and 34,000 daltons (GP37 and GP34) containing polymerized albumin receptors. Successfully detecting these proteins depended on the extraction procedures. In the extract without protein denaturants and inhibitors, these products were degraded rapidly by proteases to yield smaller size derivatives lacking polymerized albumin receptors. As is the case in human serum-derived HBsAg, yeast HBsAg consisting of GP37 and GP34 was found to be particles or aggregates having a bouyant density of 1.2 g/cc; these particles bound to polymerized human serum albumin in species-specific manner.

Hepatitis B virus (HBV) infection is one of the most serious health problems in the world. Recombinant DNA techniques have been used to produce HBV vaccines in prokaryotic cells, mammalian cells, and, particularly important, yeast cells. A number of laboratories have developed their own yeast systems to achieve this goal (1-5). Most of the studies have focused on expressing the hepatitis B surface antigen (HBsAg) gene coding for 226 amino acid residues and producing a protein of 25-kD (P25) as the vaccine candidate.

Machida et al. (6) have indicated that the polymerized-albumin receptors (PAR) in HBV, and in 22 nm filamentous and spherical HBsAg particles derived from HBeAg-positive plasma are localized in a protein called P31. Their studies suggested that P31 is composed of P25 and an additional polypeptide of 55 amino acid residues encoded by the pre-S2 region in the HBV genome. The human serum-derived P31 protein has been glycosylated and referred to as GP33 and GP36 (7). It has been postulated that HBV attaches to the surface of the hepatocytes via PAR in HBV and polymerized-albumin on the hepatocytes. If such were the case, antibodies produced against PAR would efficiently interfere with the first step of HBV infection (6,8). Based on the idea that proteins bearing PAR may represent another candidate for HBV vaccine, we have focused our studies on producing P31 in E. coli (9) and in yeast. Here we report success in synthesizing, in yeast, glycosylated P31 (GP37 and GP34) that have PAR and HBsAg determinants.

Abbreviations used: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; PAR, polymerized-albumin receptors; SDS, sodium dodecyl sulfate; -kD, kilodalton; ELISA, enzyme-linked immunosorbent assay; poly-HSA, polymerized human serum albumin.

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MATERIALS AND METHODS

Materials : Reagents were obtained from the following sources: restriction endonucleases, T4 DNA ligase, E. coli DNA polymerase I (Klenow's fragment), Takara Shuzo Co. or Nippon Gene Co.; exonuclease Bal-31, Bethesda Research Laboratories Inc.; endo-β-N-acetylglucosaminidase H, Seikagaku Kogyo Co.; peroxidase-conjugated anti-mouse IgG, Bio-Rad Laboratories Inc.; human serum albumin, Nihon Seiyaku Co.; bovine, rat, rabbit and chicken serum albumins, Sigma Chemical Co.; monkey serum albumin, Cappel Laboratories Inc. Enzyme reactions were carried out under the conditions recommended by the suppliers. A murine monoclonal anti-HBsAg antibody HBs2-06 was supplied from Nihon Seiyaku Co. Polymerized-albumins were made by the method of Lenkei et al. (10).

Strains, plasmids and media : S. cerevisiae strain AH22R$^-$ (a leu2 his4 can1 cir pho80) (2), plasmid pJA 1 containing a yeast repressible acid phosphatase gene (PHO5) and a yeast-E. coli shuttle vector pSH 19 (11) were kindly provided by Dr. Y. Oshima in Osaka University. Plasmid pGLD 909 having a portable yeast glyceraldehyde-3-phosphate dehydrogenase gene (GLD) promoter was constructed from pGLD 9 containing the GLD gene isolated from S. cerevisiae strain Kyokai III. High-Pi medium (1.5 g of KH_2PO_4 per liter) and low-Pi medium (1.5 g of KCl per liter and 5 % glucose) were prepared from Burkholder minimal medium (12) supplemented with histidine (20 mg per liter).

Yeast transformation and growth conditions : Transformation was carried out as described by Hinnen et al. (13) and Leu$^+$ transformants were selected. Seed cultures of yeast were grown in high-Pi medium at 30°C for 24 hr. Aliquots (2 ml) of the culture were inoculated into 18 ml of low-Pi medium, grown at 30°C for 48 hr and the yeast cells were collected by centrifugation.

RESULTS

Construction of plasmids for the expression of HBsAg P31 gene in yeast

Two plasmids were constructed to direct the synthesis of the HBsAg P31 protein in yeast (Fig. 1). The PHO5 and the GLD promoters were introduced into the yeast-E. coli shuttle vector pSH 19 to obtain plasmids pPHO 17-1 and pGLD 906-1, respectively. The HBsAg P31 gene, which was used for the synthesis of P31 in E. coli (9), was isolated from pHBr P31 and inserted into pPHO 17-1 and pGLD 906-1 to yield plasmids pPHO P31-R and pGLD P31-R, respectively. The nucleotide sequences of the promoter-P31 gene junctions are also shown in Fig. 1.

Analysis of HBsAg P31 gene products

Plasmids pPHO P31-R and pGLD P31-R were introduced into S. cerevisiae AH22R$^-$ by the methods of Hinnen et al. (13). Cell extracts were prepared from mid-stationary phase culture as described in the legend to Fig. 2A . After being stored at 4°C for one week, aliquots of AH22R$^-$ / pPHO P31-R extracts were subjected to Western blotting. When the gene products were extracted with 0.1 % Triton X-100, a protein of about 28-kD was detected (Fig. 2A, lane 1). Urea and guanidine-hydrochloride extracts gave proteins of 37-kD and 30-kD, and a protein of 37-kD, respectively (lane 2 and lane 3). When the extract with 7 M urea wassubjected to Western blotting immediately after being extracted, the 30-kD protein was not detected (Fig. 2C). These observations suggested that the 30-kD and 28-kD proteins were degraded-forms of the 37-kD protein produced by proteolytic enzymes in the host cells during the extraction and storage period.

To determine if oligosaccharides exist in the gene products, the 7 M urea extract was treated with endo-β-N-acetylglucosaminidase H, which removes N-linked glycosyl

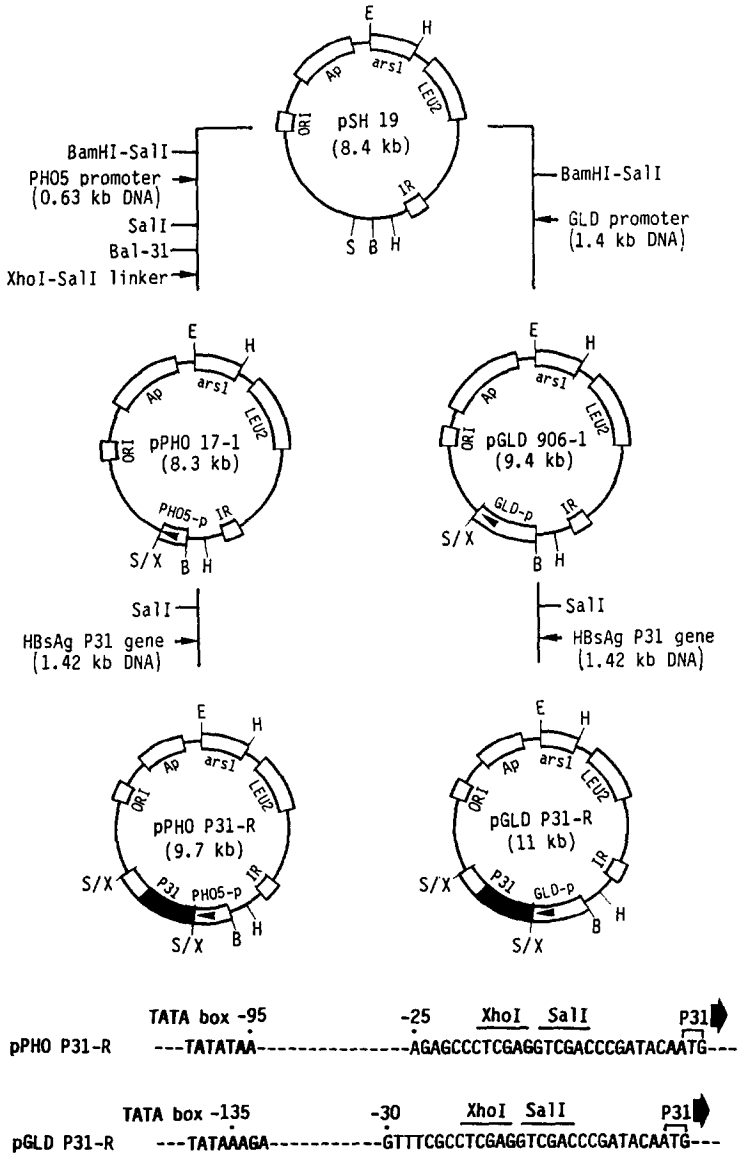


Figure 1. Construction of expression plasmids. A 0.63 kb fragment containing the PHO5 promoter region and 83 bp of the structural gene was isolated by BamHI and SalI digestion of plasmid pJA 1. This fragment was ligated to pSH 19, which was previously linearized by BamHI and SalI, with T4 DNA ligase. The resulting plasmid was digested with SalI, and treated with exonuclease Bal-31 to eliminate the PHO5 structural gene, and then XhoI and SalI sites were introduced to form a plasmid pPHO 17-1. Another expression plasmid with yeast GLD promoter was constructed. pGLD 909 was digested with BamHI and SalI to isolate a 1.4 kb DNA fragment containing the GLD promoter region. The 1.4 kb fragment was ligated to pSH 19, which was previously treated with BamHI and SalI, with T4 DNA ligase to form a plasmid pGLD 906-1.

Separately, pHBr P31 (9) was cleaved with ClaI and BamHI to isolate a 1.42 kb fragment containing the HBsAg P31 gene. The fragment was filled in with DNA polymerase I (Klenow's fragment), SalI site was added, and the fragment was inserted into pPHO 17-1 and pGLD 906-1, which were previously linearized by SalI. As a result, we constucted expression plasmids pPHO P31-R and pGLD P31-R, in which the HBsAg P31 gene was in line with the promoters.

B, BamHI; E, EcoRI; H, HindIII; S, SalI; X, XhoI

Nucleotide sequences of the promoter-HBsAg P31 gene junctions are shown at the bottom.

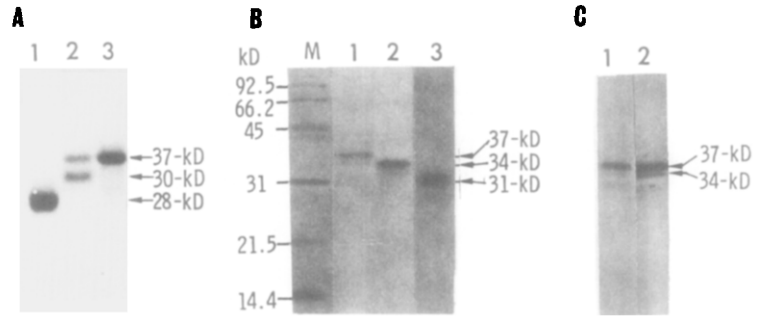
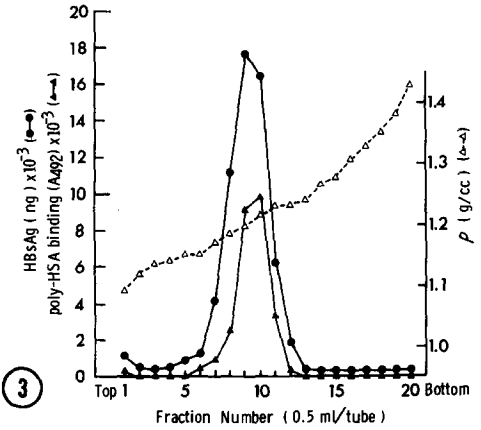


Figure 2. **A.** Analysis of the gene products synthesized in the yeast. Wet cells (AH22R-/pPHO P31-R, 0.2 g) were disrupted by glass beads in 1 ml of PBS containing 0.1 % Triton X-100, 7 M urea or 7 M guanidine-hydrochloride. After centrifugation, proteins in 100 μl of the supernatants were collected with ethanol precipitation and boiled for 5 min in 50 μl of Laemmlie's sample buffer (18). The samples were electrophoresed on a 15 % polyacrylamide gel, and transferred to a nitrocellulose filter (0.2 μm pore); the gene products were detected using murine monoclonal anti-HBsAg antibody (HBs 2-06) and the peroxidase-conjugated anti-mouse IgG antibody. lane 1, 0.1 % Triton X-100; lane 2, 7 M urea; lane 3, 7 M guanidine-hydrochloride.
B. Endo-β-N-acetylglucosaminidase H and alkali treatments of the P31 gene products. Cell extracts were prepared as described in the legend to A with 7 M urea. Aliquots of the extract were boiled for 3 min in 0.1 M 2-mercaptoethanol, 0.2 % SDS to inactivate the proteases in the extract. Then an equal volume of cold ethanol was added and the proteins were collected by centrifugation. The pellet was resuspended in 50 mM sodium-citrate buffer (pH 5.5) and incubated at 37°C for 3 hr with or without 0.05 units of endo-β-N-acetylglucosaminidase H. Alkali treatment was carried out by adding 5 N NaOH to final 0.1 N and incubated at 4°C for 16 hr.
lane 1, incubated without endo-β-N-acetylglucosaminidase H; lane 2, incubated with endo-β-N-acetylglucosaminidase H; lane 3, incubated with endo-β-N-acetylglucosaminidase H followed by alkali treatment
C. Detection of the initial gene products of the yeast. Cell extracts were prepared as described in the legend to A with 7 M urea. Immediately after the extraction, aliquots of the extracts were subjected to Western blotting. lane 1, pPHO P31-R; lane 2, pGLD P31-R.

groups (14), and then by an alkali, which cleaves O-linked glycosylation bond (15), and subjected to Western blotting (Fig. 2B). In the untreated extract, the main protein of 37-kD and a small amounts of a 34-kD protein were observed (Fig. 2B, lane 1 and Fig. 2C). After the extract was digested for 2 hr with endo-β-N-acetylglucosaminidase H, the 34-kD protein was mainly detected (Fig. 2B, lane 2). Further treatment of the digest with 0.1 N NaOH formed the protein migrating as the 31-kD species (lane 3). Since the smallest value, 31-kD, agrees with the result obtained earlier by the E. coli system, this value may represent the carbohydrate-free protein. So we conclude that the initial products of the P31 gene in yeast are 37-kD and 34-kD glycoproteins (GP37 and GP34).

Formation of HBsAg particles having PAR

As the P31 gene products were considerably protease-labile, we used extraction buffers containing 5 - 7 M urea to prevent proteolysis. Yeast harboring pPHO P31-R or pGLD P31-R produced plenty of HBsAg having PAR activity (see below). The amounts of HBsAg in yeast were 0.55 mg per liter broth in the case of pPHO P31-R and 1.4 mg per liter broth in the case of pGLD P31-R. To examine the density of the HBsAg, we



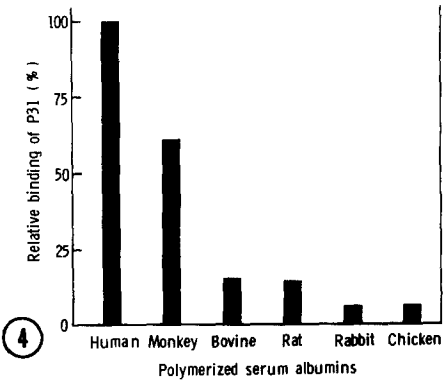


Figure 3. Isopycnic centrifugation in CsCl. Wet cells (AH22R⁻ / pPHO P31-R, 0.2 g) were disrupted with glass beads as described in the legend to Fig. 2B. Aliquot of the clear supernatant was layered on a 11 ml of discontinuous CsCl gradient (10 % - 40 %) containing 5 M urea - 2 mM EDTA - 2 mM PMSF - 10 mM potassium-phosphate buffer (pH 7) and run in a SW41 rotor at 40,000 rpm at 4°C for 16 hr. Fractions were collected and assayed for HBsAg with Auszyme II. The human serum-derived HBsAg was used as a standard sample. PAR activity was measured by ELISA (9) and the results were expressed as the total A_{492} units per fractions (0.5 ml).

Figure 4. Relative binding of P31 to polymerized-albumins from various species. Cell extract was prepared from AH22R⁻ / pPHO P31-R as described in the legend to Fig. 2B. Binding assay was carried out according to the method of Fujisawa et al. (9).

subjected the extract to CsCl equilibrium centrifugation (Fig. 3). HBsAg and PAR activity banded at 1.2 g/cc, which is same as human plasma-derived HBsAg particles (8). From these data, P31 expressed in yeast seems to be particles or aggregates. Electron microscopy of the purified P31 gene products revealed that they form particles of about 20 nm consisting of GP37 and GP34 (personal communication).

Species specificity of the PAR on the P31 gene products

The species specificity of the PAR on the yeast P31 gene products was studied by ELISA. Polymerized-albumins from six species were coated onto wells of the plate and tested for binding with the gene products. The yeast P31 gene products effectively bound to polymerized human and monkey serum albumins, but not to polymerized-albumins from other species (Fig. 4).

DISCUSSION

Fujisawa et al. reported success in synthesizing HBsAg P31, in E. coli, that specifically reacted with poly-HSA and antibody against HBsAg. The molecular weight of the HBsAg P31 in E. coli, about 31-kD, agreed with the theoritical one (9). In parallel with the E. coli system, we have constructed expression plasmids containing the HBsAg P31 gene and have tried to express it in yeast. The P31 gene products had a similar density as the natural plasma-derived HBsAg particles and contained PAR

activity. The results differed from those of the E. coli system in that the initial P31 gene products in yeast were GP37 and GP34. Successful detecting GP37 and GP34 depended on the extraction methods. In the absence of protein denaturants in the extraction buffers, 30-kD and 28-kD proteins were formed. This observation suggests that the 30-kD and 28-kD proteins are proteolytically degraded-products of GP37 and GP34. The proteolytic cleavage of GP37 and GP34 probably occurs at the pre-S2-encoded portion, because the appearance of 30-kD or 28-kD proteins was associated with the loss of the PAR activity (data not shown). This finding agrees with the observation that the PAR on the human serum-derived HBsAg particles is also lost by the action of proteolytic enzymes (8). Our results indicated that the 7 M guanidine-hydrochloride completely protected GP37 and GP34 from the proteolysis. However, this protein denaturant is not acceptable for HBsAg extraction, since it irreversibly dissociated particle structure (data not shown). Recently, Valenzuela et al. (5) constructed a plasmid that was designed to express HBsAg P31 gene in yeast and indicated that the purified HBsAg was composed of a 28-kD protein. Since they used 0.1 % Triton X-100 to extract HBsAg, their result does not contradict ours.

In this paper, we demonstrated the glycosylations of yeast P31 gene products. Because endo-β-N-acetylglucosaminidase H digestion and alkali treatment of the gene products shifted the molecular weight lower, both N-linked and O-linked glycosylation should occur during the biosynthesis of HBsAg P31 protein in yeast. GP37 and GP34 would have a different level of glycosylation on the backbone of the P31 protein.

Recently, Milich et al. showed that the 55 amino acid residues of the pre-S2 region elicit greater antibody production in the mouse (16) and is more immunogenic at the T-cell level than P25 (17). This suggests that HBsAg carrying the pre-S2-encoded region may be particularly valuable as a HBV vaccine. To obtain the yeast P31 particles, we have constructed modified P31 genes encoding protease-resistant P31. These studies will be reported elsewhere.

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