Expression of hepatitis B surface antigen gene in yeast
(recombinant DNA/serology/gene engineering/vaccine/acid phosphatase promoter)

ATSUHI MIYANOHARA*, AKIO THO-E†, CHIKATERU NOZAKI‡, FUKUSABURO HAMADA‡, NOBUYA OHTOMO‡, AND KENICHI MATSUBARA‡

*Institute for Molecular and Cellular Biology, Osaka University, Kita-ku, Osaka, 530 Japan; †Faculty of Engineering, Hiroshima University, Saijo, Higashihiroshima City, 724 Japan; and ‡The Chemo-Sero-Therapeutic Research Institute, Shimizu, Kumamoto City, 860 Japan

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ABSTRACT The DNA sequence coding for hepatitis B virus surface antigen (HBsAg) was placed under control of the repressible acid phosphatase promoter of the yeast Saccharomyces cerevisiae in a plasmid capable of autonomous replication in both yeast and Escherichia coli. Yeast transformed by this plasmid synthesized HBsAg polypeptide in phosphate-free medium. The HBsAg polypeptides produced in the yeast cells were assembled into 50- to 22-nm spherical or oval particles and were immunogenic.

Hepatitis B virus (HBV) causes serious human liver disease, including hepatoma. Infection by this virus is a worldwide health problem, and a considerable number of people, particularly in Southeast Asia, the Middle East, and some areas of Africa, suffer from transient or chronic HBV infection. The infectious agent has been identified as a 40- to 50-nm spherical particle, called the Dane particle, that is detected in patients' blood. The Dane particle contains a 3.2-kilobase (kb) circular DNA that has a single-stranded gap (1, 2).

Intensive efforts have been made to understand the structural and behavioral characteristics of this virus in order to control the disease and to produce vaccines. However, such efforts have been hampered seriously by the fact that HBV replicates only in human and chimpanzee livers.

The viral genome has been converted to double-stranded form by filling-in and has been cloned and propagated in Escherichia coli (3–6). Analyses of the cloned HBV genome have brought new insights into the structure and function of this viral genome. At the same time, it was expected that vaccines might be produced in E. coli cells by allowing expression of the cloned surface antigen (HBsAg) gene. Despite many efforts, however, production of vaccines in E. coli has not succeeded because, in E. coli cells, the HBsAg gene product seems to be either unstable or to cause effects deleterious to the host, or both. In the present study, we report a yeast system that allows expression of the HBsAg gene.

One of the yeast acid phosphatases [APase; orthophosphoric-monooester phosphohydrolase (acid optimum), EC 3.1.3.2] is an exocellular 60-kilodalton polypeptide designated P-60; the expression of the gene for P-60 is controlled by the level of inorganic phosphate (7–12). We took this gene, freed its promoter from the coding sequence of the polypeptide, and joined the promoter with the HBsAg gene in such a way that free, nonfusion polypeptides of HBsAg were made. The promoter–HBsAg gene complex was inserted into a shuttle vector that replicates in both yeast and E. coli and then was used to transform yeast. The transformants produce a large quantity of HBsAg polypeptide in low P i medium, and the HBsAg is assembled into spherical or oval immunogenic particles.

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MATERIALS AND METHODS

Strains and Media. Yeast strain AH22 (a leu2 hist4 can1 cir*) (13) and its APase constitutive derivative, AH22 pho80, were from our stock collection. Burkholder minimal medium (10) fortified with histidine (20 mg/liter) was used to prepare high-P i medium (1.5 g of KH2PO4 per liter) or low-P i medium (1.5 g of KCl per liter).

Enzymes and Linkers. Restriction enzymes were purchased from New England Biolabs, Boehringer Mannheim, and Takara Biochemicals (Kyoto, Japan). Exonuclease BAL-31 was purchased from Bethesda Research Laboratories. T4 DNA polymerase, T4 DNA ligase, and polynucleotide kinase were prepared or obtained from Seikagaku Kogyo (Tokyo, Japan). Xho I linker was purchased from Collaborative Research (Waltham, MA) and phosphorylated at its 5' termini before use (14).

Plasmids. The yeast–E. coli shuttle vector pAT77 (unpublished data) consists of a 5.5-kb DNA fragment from yeast that carries markers ars1 (15), 2-μm ori (16), and leu2 (17) and a 3.7-kb DNA fragment from E. coli plasmid pBR322 (18) that carries an ampicillin-resistance marker. Also present in this plasmid is a 2.8-kb DNA fragment of the yeast APase gene that includes the region encoding the NH2-terminal portion of APase (peptide P-60) and its control region (12) (see Fig. 1). Plasmid pHBV4 is a derivative of pACYC177 (19) that carries the entire HBV (subtype adr) genome in double-stranded form inserted in the Xho I site of the vector (unpublished data). The plasmid DNAs were prepared as described by Clewell (20) or by Matsumura et al. (21).

Exonuclease Digestion. pAT77 (1 μg) cleaved by Sal I was incubated with 0.1 unit of exonuclease BAL-31 for 30–60 sec at 30°C in 50 μl of 20 mM Tris-HCl, pH 8.2/12 mM CaCl2/12 mM MgCl2/0.2 M NaCl/1 mM EDTA. During this reaction, 85–130 base pairs (bp) of DNA were deleted. The reaction was stopped by treatment with phenol.

Transformation of Yeast Cells. Transformation was performed as described by Hinnen et al. (13). Leu + transformants were selected on Burkholder minimal medium containing 2% agar.

Yeast Cell Growth, Induction, and Preparation of Extracts. Yeast cells were grown in high-P i Burkholder minimal medium supplemented with histidine (20 μg/ml) at 30°C with aeration. When the cell density reached about 4 × 108 cells per ml, a sample was taken out, centrifuged, and suspended in low-P i minimal medium for induction. Control samples were treated similarly, except that high-P i minimal medium was used. When the density reached 1.5 × 107 cells per ml, 10-ml samples of the induced and noninduced cultures were centrifuged. The

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; APase, yeast acid phosphatase; kb, kilobase(s); bp, base pair(s).
cells were suspended in 3 ml of a solution containing Zymolyase (100 μg/ml), 1.2 M sorbitol, 50 mM phosphate (pH 7.2), and 14 mM 2-mercaptoethanol and were incubated for 30 min at 30°C. The spheroplasts were collected by centrifugation and lysed in 1 ml of 0.1% Triton X-100/50 mM phosphate, pH 7.2/1 mM phenylmethylsulphonyl fluoride. The lysate was clarified by centrifugation at 7,000 rpm for 10 min. Protein concentration was measured by the Lowry method.

Detection of HBV Proteins. Radioimmunoassay for HBV surface antigen (HBsAg), core antigen (HBcAg), and e antigen (HBeAg) (1) were performed on samples of yeast extract containing 50 μg of proteins by using the Abbott radioimmunoassay kit. Electron microscopy of HBsAg particles was performed as described by Hirschman et al. (22).

RESULTS

Construction of the Expression Plasmid. The yeast-E. coli shuttle vector pAT77 contains a fragment of yeast DNA (EcoRI-BamHI-Sal I fragment) that carries the sequence of the NH2-terminal region of the P-60 polypeptide and its upstream control region (Fig. 1B) (12). A Goldberg-Hogness sequence T-A-T-A-T-A-A, which is believed to be the eukaryotic promoter sequence (23), is located at positions -95 to -101 when the A in the initiator codon AUG for the P-60 polypeptide is assigned position +1. The sequence T-T-C-A-T-C-T-C-T, believed to be the transcription initiation site (23), is located at positions -51 to -59. The unique Sal I site located at position +83 in the P-60 coding region defines the junction between this DNA and pBR322. To allow production of nonfused HBsAg, we completely eliminated the P-60 polypeptide coding sequence and replaced it with the HBsAg coding sequence that carries the initiator codon. To do this, we cleaved pAT77 with Sal I, treated it with exonuclease BAL-31 so that 85–130 bp of DNA were removed from the Sal I cleavage-site ends, and then ligated with Xho I linkers. The exact deletion endpoints were determined by DNA sequence analyses. One of the resulting plasmids, pAM82, which had lost 116-bp of the P-60 gene from the Sal I site of pAT77, was found to have the intact APase promoter sequence. This plasmid was chosen for further studies as an expression vector. The 3' terminus of the APase promoter region was found to be at position -33, where a Xho I linker had been joined (Fig. 1).

Whole HBV DNA was recovered from pHBV4 by digestion with Xho I. Fig. 2A shows the cleavage sites of some restriction enzymes and the locations of the coding sequences of HBsAg and HBcAg. The Xho I terminus is located 27 bp upstream of the initiation codon for HBsAg. HBsAg consists of 226 amino acids.

The whole HBV DNA with Xho I termini was inserted into the Xho I site of the pAM82 expression vector, and the product was named pAH203. Similarly, a 1.3-kb Xho I–BamHI fragment carrying the HBsAg gene was inserted into the same site of pAM82, and the product was named pAH301. In this case, the

![Diagram](image-url)
**BamHI** terminus of the 1.3-kb fragment was converted to an Xho I terminus by filling in with T4 DNA polymerase, followed by ligation with Xho I linkers. In the resulting recombinant plasmids, the genetic elements were joined in the following order: APase promoter–Xho I linker–HBsAg gene–Xho I linker–pBR322 (Fig. 2B).

The recombinant plasmids propagated in *E. coli* χ1776 were transformed into a yeast recipient strain, AH22, or into its APase constitutive derivative, AH22 pho80, by standard transformation procedure, selecting for Leu" colonies.

**Induction and Production of HBsAg in Yeast Cells.** The Leu" cells were grown in liquid medium and then induced in phosphate-free medium. At appropriate times, cells were collected, converted to spheroplasts, and lysed by Triton X-100. After a high-speed centrifugation, the extracts were examined by radioimmunoassay for HBsAg (Table 1). A substantial amount of HBsAg was detected in an extract from induced AH22 cells, whereas extracts from noninduced AH22 cells showed only the background level of HBsAg activity—namely, the level in the extract of cells that carried only vector plasmid. A constitutive mutant of AH22 synthesized a moderate level of HBsAg without induction. This strain seems to be an incompletely derepressed derivative, as HBsAg gene expression was partially induced by P, starvation. The results were essentially the same irrespective of whether the recombinant plasmid carried whole HBV DNA or the 1.3-kb fragment carrying only the HBsAg gene. Note, however, that in both recombinant plasmids, the junction between APase promoter and the HBsAg gene is identical. An important conclusion to be drawn is that in both cases the HBsAg gene expression is controlled by yeast APase promoter, which is under regulation by P, concentration in the medium.

To assess the practical significance of the recombinant plasmids, we first examined their stability. After growth for six gen-

**Table 1. Content of HBsAg polypeptide in yeast extracts**

<table>
<thead>
<tr>
<th>Host</th>
<th>Plasmid</th>
<th>Induction</th>
<th>HBsAg* (µg/ml)</th>
<th>Molecules per cell†</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH22</td>
<td>pAH203</td>
<td>+</td>
<td>2.5</td>
<td>400,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>&lt;0.001</td>
<td>&lt;200</td>
</tr>
<tr>
<td>AH203</td>
<td>pAH301</td>
<td>+</td>
<td>2.8</td>
<td>450,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>&lt;0.001</td>
<td>&lt;200</td>
</tr>
<tr>
<td>AH22</td>
<td>pAM82</td>
<td>+</td>
<td>&lt;0.001</td>
<td>&lt;200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>&lt;0.001</td>
<td>&lt;200</td>
</tr>
<tr>
<td>AH22</td>
<td>pAH203</td>
<td>+</td>
<td>2.1</td>
<td>340,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>1.0</td>
<td>160,000</td>
</tr>
<tr>
<td>AH203</td>
<td>pAM82</td>
<td>+</td>
<td>3.4</td>
<td>540,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>1.8</td>
<td>280,000</td>
</tr>
<tr>
<td>AH22</td>
<td>pAM82</td>
<td>+</td>
<td>&lt;0.001</td>
<td>&lt;200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>&lt;0.001</td>
<td>&lt;200</td>
</tr>
</tbody>
</table>

Yeast cells carrying one of the plasmids as indicated were grown and induced as described above. At the density of 1.5 × 10⁷ cells per ml, cells (induced and noninduced) were collected, lysed, and subjected to radioimmunoassay for HBsAg (Table 1).

†The amount of HBsAg in the extract was calculated from an standard curve obtained with native HBsAg in human sera by assuming that HBsAg is detectable in both yeast and human sera react similarly.

The number of HBsAg molecules per cell was calculated by assuming that the HBsAg subunit in yeast extract has a molecular weight of 25,000.
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EYAHAG-positive spherical and oval particles appearing in induced yeast cell lysates. Extracts (100 µl) from the induced yeast cells were incubated with an equal volume of anti-HBsAg rabbit IgG (passive hemagglutinin titer, 1:2^2) for 12 hr at room temperature. The mixture was then centrifuged at 12,000 × g for 30 min. The pellet was stained with uranyl acetate. (Bar = 20 nm.)

orations, more than 99.9% of the cells retained the Leu' character, showing that the recombinant plasmids are as stable as the parental 2-µm plasmid (26). The expression level of HBsAg was calculated to be about 5 × 10^6 HBsAg subunits per yeast cell, assuming that the HBsAg in yeast extracts reacts with anti-
HBsAg antibody with an efficiency equal to that of HBsAg in serum. This level is roughly the same as that reported for production of interferon D under control of the yeast alcohol dehydrogenase I promotor (27).

Some Properties of the HBsAg Produced in Yeast Cells. The HBsAg in the yeast extracts was precipitated by anti-HBsAg antibody and examined by electron microscopy. Aggregates of 20- to 22-nm spherical or oval particles were observed (Fig. 3), which did not appear in control samples. Thus, HBsAg produced by yeast seemed to be assembled into particles similar in size and shape to those small particles found in HBV-infected patients' sera. Note, however, that these particles do not carry DNA and are smaller than Dane particles (28).

The yeast extract containing HBsAg was injected subcutaneously into guinea pigs (3400 ng of HBsAg per animal). After two further boosters with a 1-wk interval, the sera were examined for anti-HBsAg antibody titer by using the AUSAB radioimmunoassay (Abbott). The injected animals produced high levels of anti-HBsAg antibody, which proves that the antigen produced in yeast cells was sufficiently immunogenic (Table 2).

We were not able to detect other HBV-related antigens, such as HBeAg and HBcAg, by radioimmunoassay of extracts from yeast cells carrying the complete HBV genome, plasmid pAH203.

DISCUSSION

In this paper we demonstrated (i) that a cloned HBsAg gene from subtype adr HBV can be expressed efficiently in yeast cells under control of the yeast Aαase promotor, (ii) that the gene products are sufficiently immunogenic, and (iii) that the products are assembled into spherical or oval particles similar to those found in patients' sera. Because we did not leave any coding sequences between the Aαase promotor and the initiation codon of the HBsAg gene in the expression plasmid, the product should be a nonfused complete HBsAg polypeptide. This has yet to be proven by amino acid sequence studies.

Several attempts have been made to prepare HBsAg in E. coli cells. However, none of them have resulted in the production of nonfused HBsAg polypeptides. The cause for difficulty is not known, but in E. coli cells, the newly produced HBsAg polypeptides seem to be quickly degraded (29); in addition, they seem to cause effects that are deleterious to the host cells. The use of a eukaryotic host, yeast, seems to overcome these problems. However, what feature or structure of yeast cells allowed accumulation of a polypeptide that is restricted in E. coli cells remains to be elucidated.

The efficient expression of the HBsAg gene in nonfused complete polypeptide form in yeast not only will provide us with

Table 2. Immunogenic activity of HBsAg in yeast extracts

<table>
<thead>
<tr>
<th>Injected antigens</th>
<th>Anti-HBsAg antibody titer, cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg-positive yeast extract*</td>
<td>6,180</td>
</tr>
<tr>
<td>HBsAg-negative yeast extract†</td>
<td>102</td>
</tr>
<tr>
<td>Purified HBsAg particles‡</td>
<td>10,955</td>
</tr>
</tbody>
</table>

Numbers represent titer in guinea pig sera as measured by radioimmunoassay. Results of duplicate experiments are shown. AUSUB control, PCE: 11,505; NCE: 110.
* Extract of cells carrying plasmid pAH203 (equivalent to 400 ng of HBsAg).
† Extract of cells carrying plasmid pAM82.
‡ Purified HBsAg particles from human serum (400 ng of HBsAg).
an opportunity to make vaccines but also will allow us to study
other important mammalian or plant gene products. It also will
facilitate greatly studies of the DNA signals controlling the
expression of eukaryotic genes.

During the preparation of this manuscript, a similar work was
published (30).

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nology Agency of Japan.

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