JOURNAL OF VIROLOGY, Nov. 1987, p. 3543–3549
0022-538X/87/113543-07$02.00/0

Vol. 61, No. 11

Expression of Hepatitis B Virus Middle and Large Surface Antigen Genes in *Saccharomyces cerevisiae*

TAKAYUKI IMAMURA,[1] MASATAKE ARAKI,[2] ATSUSHI MIYANOHARA,[1] JUNJI NAKAO,[1] HIROSHI YONEMURA,[1] NOBUYA OHTOMO,[1] AND KENICHI MATSUBARA[2]*

The Chemo-Sero-Therapeutic Research Institute, Shimizu, Kumamoto 860,[1] and Institute for Molecular and Cellular Biology, Osaka University, Yamada-oka, Suita, Osaka 565,[2] Japan

Received 7 May 1987/Accepted 7 August 1987

The hepatitis B virus genome carries the surface antigen (SAg) gene and an open reading frame that encodes two SAg-related polypeptides: SAg with a 55-amino-acid N-terminal extension polypeptide and SAg with a 174-amino-acid N-terminal extension polypeptide. These are termed middle S and large S, respectively. These polypeptides or their glycosylated derivatives have been detected in Dane particles, but their chemical and biological properties have remained largely unknown because of their limited availability. We attempted to produce these proteins in *Saccharomyces cerevisiae* by placing the coding regions under the control of the promoter of the yeast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Yeast cells carrying middle S and large S coding sequences produced 33,000- and 42,000-dalton products, respectively, each of which reacted with anti-S antibody and bound to polymerized human serum albumin, in accordance with the known properties of pre-S proteins from particles in human sera (K. H. Heermann, U. Goldmann, W. Schwartz, T. Seyffarth, H. Baumgarten, and W. H. Gerlich, J. Virol. 52:396–402, 1984; A. Machida, S. Kishimoto, H. Ohnuma, K. Baba, Y. Ito, H. Miyamoto, G. Funatsu, K. Oda, S. Usuda, S. Togami, T. Nakamura, Y. Miyakawa, and M. Mayumi, Gastroenterology 86:910–918, 1984). The middle S polypeptide is glycosylated and can be assembled into particles whose size and density are similar to those of SAg. However, this polypeptide was highly susceptible to proteolytic degradation into 29,000- and 26,000-dalton polypeptides, of which only the former retained the binding activity to polymerized albumin. The large S polypeptides are nonglycosylated, relatively stable, and do not seem to assemble into particles by themselves.

Hepatitis B virus (HBV) is a small DNA virus which infects only livers of humans and chimpanzees. The sera of chronic carriers often display 45-nm (Dane) particles along with small particles, both of which carry 24,000- and 27,000-dalton surface antigen (SAg) polypeptides. These have been shown to be the same polypeptide in nonglycosylated and glycosylated forms. In addition, larger polypeptides have been discovered in Dane particles, as well as in some small particles recovered from eAg-positive patients (7). These proteins bind to polymerized human serum albumin (PHSA) (14).

Studies with cloned HBV DNA (3, 4, 6, 23, 29) have revealed an amino acid-coding region located upstream of and in frame with the SAg-coding region. Depending upon which initiation codon is chosen, the product is SAg with an extension of 174 or 55 amino acids at the N terminus. The DNA sequence starting from the initiation site furthest upstream, viz., nucleotide position 2,720 (6), which encodes SAg with the 174-amino-acid extension, is defined as the large S gene; the sequence that starts from nucleotide position 3,077 and encodes SAg with the 55-amino-acid extension is defined as the middle S gene. The large S and middle S gene products in nonglycosylated form would be 42,000 and 31,000 daltons, respectively. For the sake of clarity, the SAg gene that starts at nucleotide position 28 is called the major S gene.

The extension polypeptides have been claimed to enhance the immunogenicity of SAg in eliciting anti-S antibody (7, 16–18, 20), suggesting that they may play important roles in the process of virus infection and the induction of a defensive body response. However, the limited availability of materials has precluded detailed biological and chemical analyses of these molecules.

In this communication, we report production of the large S and middle S gene products in *Saccharomyces cerevisiae* cells, which have been successfully used for the production of major S in an assembled spherical form (19). The middle S gene products were also recovered in assembled spherical forms which showed binding activity to PHSA.

MATERIALS AND METHODS

Preparation of yeast lysate. The yeast strain AH22 (a *leu2 his4 can1*) (8) was used in all experiments. Leu2$^+$ transformants carrying a desired plasmid were selected, grown in Burkholder minimal medium (2), and collected by centrifugation. To examine SAg-related materials, spheroplasts were induced with Zymolyase 100T (100 μg/ml) (Seikagaku Kogyo, Tokyo, Japan)–14 mM 2-mercaptoethanol–50 mM phosphate buffer (pH 7.2) and then lysed in a lytic mixture (0.1% Triton X-100, 50 mM phosphate [pH 7.2], 1 mM phenylmethylsulfonyl fluoride) containing the protease inhibitor mixture described below. The lysate was cleared by centrifugation at 10,000 rpm in an RPR20 rotor (Hitachi) for 10 min.

Protease inhibitor mixture. A stock solution was made by mixing the following protease inhibitors, each at a concentration of 500 μg/ml: antipain, chymostatin, phosphoramidon, leupeptin, elastatinal, and pepstatin (Protein Research Institute of Osaka University). This mixture was added to the lytic mixture to give a final concentration of each inhibitor of 2.5 μg/ml.

Polymerized albumins. Crystallized and lyophilized serum albumins (human, bovine, and rabbit) and ovalbumin were

* Corresponding author.

purchased from Sigma Chemical Co. Chromatographically purified serum albumins (human, horse, goat, monkey, and dog) were purchased from Cooper Biomedical, Inc. Polymerized albumins were prepared by treatment with glutaraldehyde (10).

Detection of anti-S antibody-binding activity and PHSA-binding activity. Binding to antibody against SAg was assayed with an AUSRIA-II radioimmunoassay (RIA) kit (Abbott Laboratories). This kit detects SAg in particle form but not as free polypeptide. PHSA-binding activity was assayed by passive hemagglutination of sheep erythrocytes coated with PHSA as described by Imai et al. (10). Briefly, sheep erythrocytes were coated with PHSA in 1% glutaraldehyde and adjusted to a 1% suspension in phosphate-buffered saline (PBS) (10 mM phosphate, 150 mM NaCl [pH 7.2]) containing 1% rabbit serum. Portions (25 μl) of this suspension were incubated at 37°C for 1 h in microplates with serial twofold dilutions of sample. PHSA-binding activity is expressed as the highest dilution showing hemagglutination. Three human sera (eAg^+ SAg^+, e antibody positive [eAb^+] SAg^+, and normal human serum) were used as controls.

Endo H treatment. Samples were digested with 0.1 U of endo-β-*N*-acetylglucosaminidase H (endo H) (EC 3.2.1.96; Seikagaku Kogyo) in 100 μl of 150 mM sodium citrate (pH 5.0) at 37°C for 2 h.

Western blotting (immunoblotting). The method developed by Towbin et al. (27) was used with some modifications. The polypeptides were run in a 15% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS) (13) and transferred to a nitrocellulose sheet (Schleicher & Schuell, Inc.) with a Transblot apparatus (Bio-Rad Laboratories) at a constant current (250 mA) for 3 h in a buffer containing glycine (192 mM), Tris hydrochloride (pH 8.3; 25 mM), and methanol (20%). The nitrocellulose sheet was soaked with blocking buffer containing 1% bovine serum albumin and 0.1% Tween 20 in PBS at 37°C for 1 h with shaking and then analyzed for the polypeptides that bound to anti-S antibody or PHSA. For detection of anti-S antibody binding, the sheet was incubated with rabbit anti-S antibody in blocking buffer for 12 h at room temperature with shaking. After being washed with Tween 20 (0.1%) in PBS (PBS-Tween), the sheet was incubated for 2 h with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (Bio-Rad) in blocking buffer at 37°C with shaking. The sheet was again washed with PBS-Tween, and the HRP-labeled polypeptides were detected with diaminobenzidine. For detection of PHSA binding, the sheet was incubated with PHSA (20 μg/ml) in blocking buffer at room temperature for 12 h and then washed with PBS-Tween. The sheet was then incubated with guinea pig anti-PHSA antibody in blocking buffer at 37°C for 2 h. After being washed with PBS-Tween, the sheet was incubated with HRP-conjugated goat anti-guinea pig IgG in blocking buffer at 37°C for 2 h. After the sheet was washed, the HRP-labeled polypeptides were detected with diaminobenzidine.

Preparation of PHSA-binding materials in lysates. Polyvinyl microtiter plates were coated with PHSA by adding 100 μl of PHSA in PBS (100 μg/ml) to each well and keeping them at 4°C overnight. The wells were washed with PBS-Tween, incubated with 0.1% bovine serum albumin in PBS at 37°C for 1 h, and washed again with PBS-Tween. Portions (100 μl) of yeast cell lysates prepared in the presence of protease inhibitors were then added to the wells and incubated at 37°C for 1 h, the wells were washed with PBS-Tween, and the bound materials were eluted with Laemmli sample buffer (0.1 M Tris hydrochloride, [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1% bromophenol blue).

Sedimentation analyses. Yeast lysate (1 ml) was layered on top of 11 ml of a 5 to 20% (wt/wt) linear sucrose gradient in PBS and centrifuged at 25,000 rpm for 5 h in an RPS40T rotor (Hitachi). Fractions were collected and assayed by RIA for anti-S antibody-binding activity and by passive hemagglutination for polymerized albumin-binding activity. The fractions of interest were pooled and concentrated in a microconcentrator (Centricon-10; Amicon Corp.). The concentrated samples were subjected to a second cycle of sedimentation analyses by isopycnic centrifugation, layering on top of 5 ml of CsCl step gradients consisting of 11, 17, 23, 29, and 35% CsCl (wt/wt) in PBS, and centrifugation at 36,000 rpm for 17 h in an RPS40T rotor (Hitachi).

Electron microscopy. The peak fractions for SAg in a CsCl gradient were concentrated, stained with uranyl acetate, and examined under a JEOL 100CX electron microscope.

RESULTS

Construction of expression systems for large S and middle S. The *S. cerevisiae-Escherichia coli* shuttle vector pYG100 consists of, in order of appearance on the map, the promoter and terminator of the yeast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, the *E. coli* pBR322 replicon, the ampicillin resistance gene, the yeast *leu2* gene, and the yeast 2μm DNA replicon (Fig. 1). To express the major S gene product, a 1,272-base-pair (bp) *Xho*I-*Bam*HI fragment of HBV DNA (6) was inserted with *Sal*I linkers into the single *Sal*I site of pYG100, which is located about 120 bp downstream of the TATA box of the GAPDH promoter and the resulting plasmid (pYG100S) was used to transform *S. cerevisiae*. For the middle S and large S genes, a 1,417-bp *Mst*II-*Bam*HI fragment and a 1,799-bp *Bst*EII-*Bam*HI fragment of the HBV genome, respectively, were inserted into the *Sal*I site of pYG100 to yield pYG100M and pYG100L (Fig. 1) and each plasmid was used to transform *S. cerevisiae*.

Cells carrying one of these plasmids were grown, lysed, and assayed for reactivity with anti-S antibody and for binding to PHSA. All the lysates except the control (pYG100) had anti-S antibody-binding activity (Table 1). In addition, PHSA-binding activity was observed with middle S and large S samples but not with the major S and control samples. The anti-S antibody-binding and PHSA-binding activities in the lysates were affected by the addition of inhibitors of proteolysis. When the lysates were prepared in the absence of protease inhibitors, the PHSA-binding activity in middle S and large S samples was reduced, whereas reactivity with anti-S antibody was elevated. These changes may have been due to the degradation of the polypeptides in question, as well as elimination of the materials inhibitory for assembly. The problems associated with protease digestion are discussed below.

Glycosylation and degradation of the gene products. The lysates were subjected to Western blotting to determine polypeptides that react with anti-S antibody. The results (Fig. 2) demonstrate that yeast cells carrying coding regions for major S, middle S, and large S, respectively, produced polypeptides of 24,000, 33,000, and 42,000 daltons as major components. The 24,000 and 42,000 values are in complete agreement with those predicted from the DNA sequences, whereas the 33,000 value is larger than that expected for middle S (31,000). This suggests that major S and large S polypeptides are not or are only poorly glycosylated,

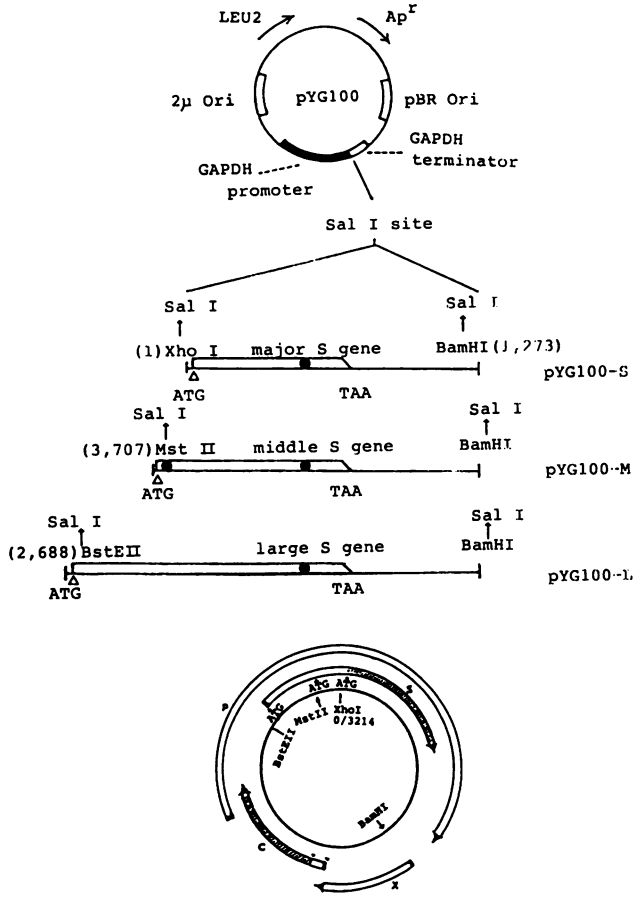

FIG. 1. Construction of three plasmids, pYG100S, pYG100M, and pYG100L, that allow expression of major S, middle S, and large S genes, respectively, under control of the yeast GAPDH promoter and terminator. For explanation of symbols and the sources of the DNA fragments from HBV, see the text. The GAPDH promoter carried by pYG100 is approximately 1 kilobase in size and contains a TATA box and cap site (1). Nucleotide positions of restriction enzyme cleavage sites (6) and possible N-glycosylation sites (22) (●) are indicated. At the bottom, the HBV genome is shown with relevant restriction sites and open reading frames.

whereas middle S is glycosylated to a significant extent. The 33,000-molecular-weight component (33K component) of the middle S gene product was converted to 31K upon treatment with endo H (Fig. 3, lane D), showing that this protein is in the N-glycosylated form. Based on their susceptibility to endo H, the sugars are likely to be mannose-rich glycans (26). As expected, the major S and large S gene products did not change upon endo H treatment (Fig. 3, lanes B and F). These observations suggest that the N-glycosylation site located at amino acid 81 (Asn) from the common C terminus of these three polypeptides must have been nonglycosylated or glycosylated in a practically nondetectable form. In the blood of patients, on the other hand, approximately 40% of the polypeptides are glycosylated at this position by a complex-type glycan (7). This must reflect a difference in the glycosylation systems in mammalian and yeast cells, but the mechanism is not clear.

Some smaller components (29K and 26K), possibly degradation products, appeared in the protease inhibitor-free samples of middle S and large S preparations (Fig. 2 and 3).

TABLE 1. Reactivity with anti-S antibody or binding activity to PHSA expressed in yeast cells

Plasmid[a]	Expected product	Reactivity with anti-S antibody (cpm)[b]		PHSA-binding activity[c]	
		+[d]	−[d]	+	−
pYG100S	Major S	1,100	6,400	$<2^1$	$<2^1$
pYG100M	Middle S	1,400	6,800	2^8	2^4
pYG100L	Large S	570	2,400	2^6	2^3
pYG100		210	220	$<2^1$	$<2^1$

[a] Yeast cells carrying the indicated plasmid were grown to 2×10^7 cells per ml in 10 ml of minimal medium at 30°C, collected, lysed with 1 ml of lytic mixture, and cleared by centrifugation as described in the text.
[b] Assayed by AUSRIA-II RIA kit (Abbott) after 100-fold dilution with PBS.
[c] Assayed by passive hemagglutination (see the text).
[d] + and −, Presence and absence, respectively, of the protease inhibitor mixture during preparation of cell lysates.

The size of these components did not change upon endo H treatment.

There are three arginine residues in the extension polypeptide of middle S, at positions 16, 18, and 48 from the N terminus (6, 24). These sites are candidates for cleavage by a trypsinlike protease, which would yield 29.8K, 29.6K, and 26.1K products. The 29K and 26K components in Fig. 2, lanes D and F, may correspond to these components. Because these components were unaffected by endo H treatment, they are not glycosylated. These observations suggest that the site of glycosylation in the 33K middle S polypeptide is in the N-terminal extension polypeptide region and that the 29K and 26K products must have lost this site. A candidate sequence is Met-Gln-Trp-Asn-Ser-Thr located at the N terminus. Note that there is no N glycosylation in the C-terminal end of the molecule, although the candidate Asn-X-Thr sequence begins at amino acid 81 from the C terminus. In contrast to middle S, large S is not glycosylated either in the extension polypeptide or in the

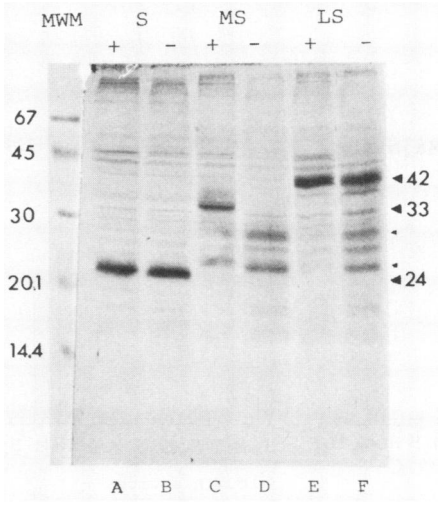

FIG. 2. Western blotting profiles of the products in lysates from cells carrying coding regions for major S, middle S, and large S. The gene products were detected with anti-S antibody. Lanes: S, lysate of pYG100S; MS, lysate of pYG100M; LS, lysate of pYG100L; + and −, presence and absence, respectively, of protease inhibitors during lysate preparation; MWM, polypeptide size markers (Bio-Rad). The numbers indicate molecular size in kilodaltons. Unlabeled arrowheads denote degradation polypeptides.

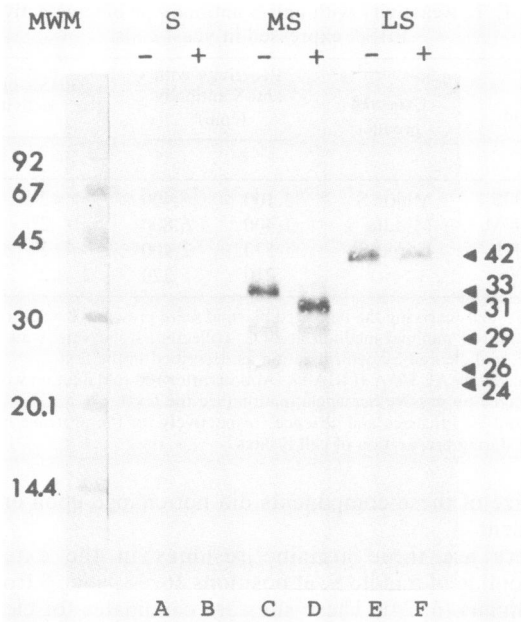

FIG. 3. Endo H treatment of products from major S (S), middle S (MS), and large S (LS) genes. Lysates were treated (+) or not treated (−) with endo H before SDS-gel electrophoresis. The polypeptides were detected with anti-S antibody. MWM, Polypeptide size markers. The numbers indicate molecular size in kilodaltons.

region common to major S, although it carries the glycosylation site in common with middle S. This difference is probably due to the difference in the overall structure of the molecule.

The amounts of major S, middle S, and large S products produced by the three cell preparations were roughly the same, as judged from the intensities of bands in Western blots of the same amount of lysates (Fig. 2). However, the anti-S antibody-binding activities measured by RIA, which assays only assembled SAg and its related molecules, were low in lysates made in the presence of protease inhibitors (Table 1). These results suggest that the removal of other proteins by protease in the process of lysate preparation promotes more efficient assembly of the antigen molecules. For large S, elimination of the extension polypeptide may enhance assembly of the protein molecules.

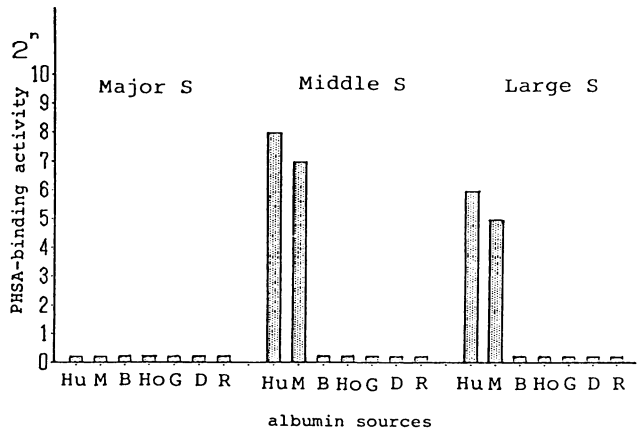

FIG. 4. Specificity of polymerized serum albumin-binding activity. The polymerized albumin-binding activity was examined in the same amount of lysates from cells carrying pYG100S, pYG100M, and pYG100L by using passive hemagglutination as described in the text. All lysates were prepared in the presence of protease inhibitors. Albumin sources were as follows: Hu, human; M, monkey (*Macaca fascicularis*); B, bovine; Ho, horse; G, guinea pig; D, canine; R, rabbit.

Polymerized albumin-binding activity. Imai et al. (10) have shown that virus particles recovered from eAg-positive sera bind to PHSA, and this activity resides in the so-called pre-S region, the 55-amino-acid N-terminal extension polypeptide region in middle S. Yeast lysates carrying middle S and large S, but not major S, showed binding activity to PHSA (Table 1). This binding activity was specific for polymerized albumins from humans and monkeys (*Macaca fascicularis*), among the animals tested (Fig. 4).

To examine which component(s) in the lysate bound to PHSA, yeast cell lysates were added to PHSA-precoated polyvinyl microplates, the plates were washed, and the bound materials were eluted (see Materials and Methods). The recovered materials were electrophoresed in duplicate in the presence of SDS and then transferred to two filters, one of which was stained with anti-S antibody (see Materials and Methods). The results (Fig. 5a) show that products from the middle S and large S genes, but not from the major S gene, bound to PHSA. In the middle S sample, two components (29K and 26K) were detected in addition to the 33K component. As noted above, they must have been the products of proteolysis. Further action by protease resulted

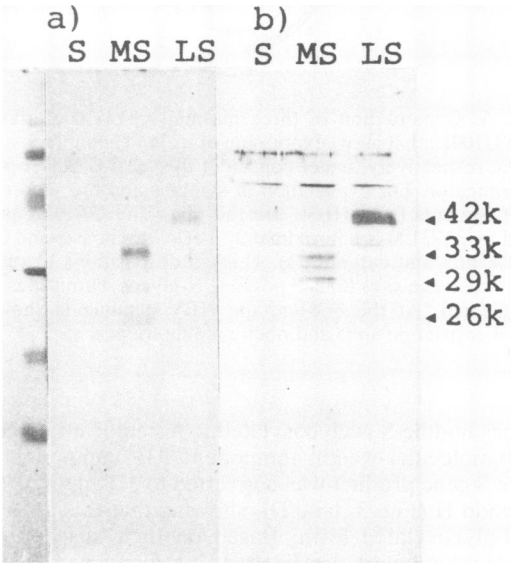

FIG. 5. Western blotting profiles of bound materials eluted from PHSA. Portions (100 μl) of lysates carrying products from major S (S), middle S (MS), or large S (LS) genes prepared in the presence of protease inhibitors were incubated in PHSA-coated polyvinyl microplates at 37°C for 1 h, the plates were washed with PBS-Tween, and the bound materials were eluted with Laemmli sample buffer (see the text). The eluted materials were electrophoresed in duplicate in the presence of SDS and then blotted onto two filters (Schleicher & Schuell). (a) One of the filters was incubated with rabbit anti-S antibody and then stained with HRP-conjugated goat anti-rabbit IgG. (b) The other filter was again incubated with PHSA, then with guinea pig anti-PHSA antibody, and finally with HRP-conjugated goat anti-guinea pig IgG. It was then exposed to diaminobenzidine. The leftmost lane contained polypeptide size markers.

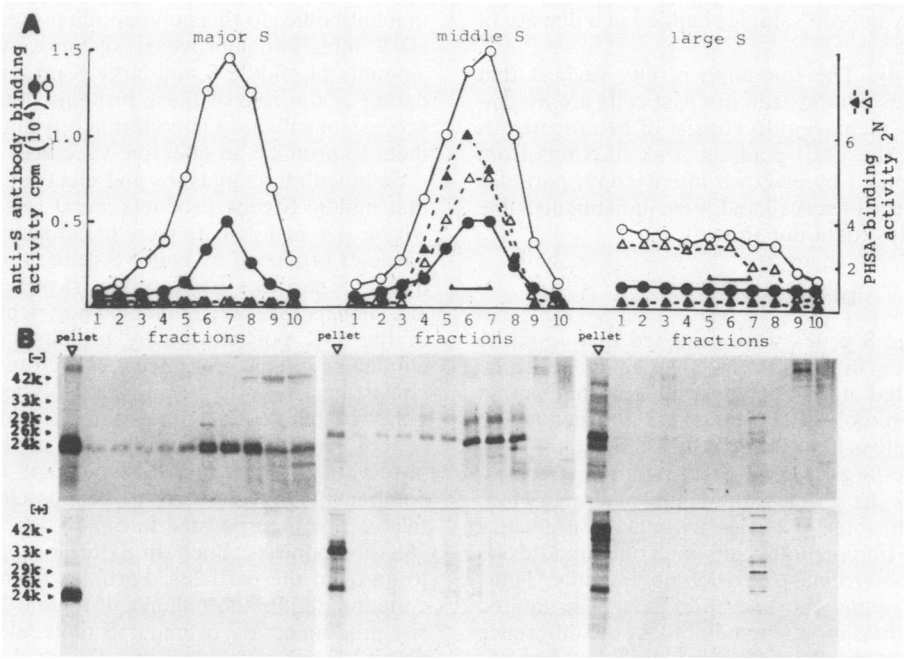

FIG. 6. Analyses of particles consisting of major S, middle S, and large S gene products. (A) Sedimentation profiles of anti-S antibody-binding and PHSA-binding activities. Cell lysates prepared in the presence or absence of protease inhibitors were layered without purification on 5 to 20% (wt/wt) sucrose linear gradients and centrifuged at 25,000 rpm in an RPS40T rotor (Hitachi) for 5 h (see the text). Sedimentation is from right to left. After the run, each fraction was assayed by RIA for anti-S antibody-binding activity (circles) or by passive hemagglutination for PHSA-binding activity (triangles). Filled symbols denote samples prepared in the presence of protease inhibitors, and open symbols denote their absence. (B) SDS-polyacrylamide gel electrophoresis profiles of polypeptides in each sucrose fraction. Each fraction was concentrated to 100 μl in a microconcentrator, and a 10-μl sample was electrophoresed, blotted onto a filter, and then stained by using anti-S antibody. Pellets were washed with PBS and resuspended in 100 μl of PBS, and a 10-μl sample was subjected to Western blotting. + and −, Presence and absence, respectively, of protease inhibitors during lysate preparation.

in particles consisting only of a 26K component that no longer bound to PHSA (see below).

To determine directly the polypeptides that bound to PHSA on the nitrocellulose sheet, another filter was stained with PHSA (see Materials and Methods). The middle S product (33K) and its 29K component, but not the 26K component, had PHSA-binding activity (Fig. 5b). The fact that the 26K polypeptide was recovered from PHSA-bound particles but did not itself bind to PHSA suggests that the particles consist of 33K, 29K, and 26K polypeptides, of which only the first two carry the PHSA-binding region. Thus, the binding site must be located in the N-terminal region of the extension polypeptide of middle S.

In the large S sample, the 42K component showed PHSA-binding activity.

Sedimentation analyses. The major S gene products synthesized in yeast cells can be recovered as particles similar in size to the small particles observed in human sera. The polypeptides are assembled by yeast membranes (9, 15, 19, 30), as is the case with particles from mammalian cells. To determine whether the middle S and large S gene products synthesized in yeast cells are similarly assembled, lysates prepared in the presence and absence of the protease inhibitors were sedimented through sucrose gradients (Fig. 6A). The major S and middle S products displayed the anti-S antibody-binding peak, as measured by RIA, in fraction 7. The PHSA-binding activity peak sedimented slightly faster than the anti-S antibody-binding peak. The large S gene product showed broad sedimentation profiles with respect to both anti-S antibody-binding and PHSA-binding activities. These activities were low compared with those in other samples.

The Western blotting profiles of each sucrose fraction are shown in Fig. 6B. The major S particles consisted of 24K polypeptides, and the middle S particles consisted of heterogeneous components. This observation is consistent with our previous notion that heterogeneous polypeptides, at least the 33K, 29K, and 26K components, are assembled into particles, although many intact 33K polypeptides were detected in the pellet. It is not possible to determine whether each particle carries multiple components or whether some particles consist solely of intact 33K middle S polypeptide. In the presence of protease inhibitors, large amounts of polypeptides were detected in the pellets. They may have represented the products that had not assembled into the regular-size particles.

Large S polypeptides themselves do not seem to assemble into similar particles, and almost all of the gene products were detected in the pellet. Although several bands appeared in fraction 7, their distribution in the sucrose gradient did not parallel that of anti-S antibody-binding activity. In fact, most cellular components sedimented in this fraction. There is a possibility that proteolytic elimination of the extension polypeptide from large S helped to produce the heterogeneously assembled products. However, since their level was too low for further studies, we did not attempt to elucidate their structures.

The peak fractions (5 to 7) of major S and middle S products were pooled, concentrated, and subjected to isopycnic centrifugation in CsCl gradients. The anti-S anti-

body-binding activity in both samples banded at a density of 1.2.

Electron microscopy. The foregoing results suggest that middle S polypeptides synthesized in yeast cells are assembled into particles, which seem to consist of heterogeneous polypeptides. When the CsCl gradient peak fractions from middle S were examined by electron microscopy, particles 20 nm in diameter were observed and were indistinguishable from major S particles (data not shown).

DISCUSSION

Middle S and large S genes were designed that could be expressed themselves in yeast cells. The middle S gene product is glycosylated at a site close to its N terminus that is lost upon partial proteolysis. Based on the deduced amino acid sequence, this site is likely to be Asn, amino acid 4 from the N terminus. The large S gene product is not glycosylated, although it has the glycosylation site. Heerman et al. (7) have shown that some particles in human sera carry middle S and large S polypeptides, of which only middle S is glycosylated in the N-terminal extension polypeptide. Thus, the yeast products mimic the liver products. The limited reactivity of the glycosylation site may reflect the difference in conformation of the pre-S region in middle S and large S. Alternatively, a difference in the intracellular transport pathway that brings the nascent polypeptide into contact with glycosylating enzymes cannot be ruled out, although it is less likely. Another common glycosylation site, at amino acid 81 from the C terminus of the major S, middle S, and large S gene products, is not glycosylated in yeast cells, although about 40% of these sites are glycosylated in the polypeptides recovered from human sera. No attempts have been made to pursue the cause of this difference.

Western blotting showed that some of the gene products are readily attacked by protease. Thus, the middle S product was degraded to 29K and 26K components, and the cleavage profiles agree with the locations of Arg in the N-terminal extension polypeptide, viz., amino acids 16, 18, and 48 from the N terminus. Valenzuela et al. (28) constructed a plasmid designed to produce middle S polypeptide in yeast cells. Their products were not glycosylated and were only 28,000 daltons in size. Since they did not use protease inhibitors, it is likely that their products were partially degraded materials. Large S was also degraded to 29K and 26K derivatives by the protease.

The PHSA-binding activity was detected only for middle S and large S, not major S. It was specific to human and monkey albumins. This strict specificity is identical to that observed with particles recovered from human sera. Western blotting analyses of the middle S polypeptides showed that the 33K and 29K components, but not the 26K component, bound to PHSA. Therefore, the PHSA-binding activity must lie close to the N terminus of the 29K polypeptide, in a region that is missing in the 26K polypeptide, namely, between amino acids 19 and 48 from the N terminus of the middle S product. The PHSA-binding activity of the large S polypeptide may also be carried by the same region. Independently, Itoh et al. (11) and Cheng and Moss (5) showed that the product of the middle S gene in yeast or mammalian cells has PHSA-binding activity. In contrast to our observations, Itoh et al. (11) detected two glycosylated polypeptides (37K and 34K) in yeast cells. This discrepancy remains unexplained. The PHSA-binding activity is thought to be related to the process of infection of liver cells by HBV (25), and Neurath et al. (20, 21) and Itoh et al. (12) have claimed that antibodies to this polypeptide protect chimpanzees from HBV infection. Our yeast systems, which produce large amounts of middle S and large S proteins, will allow elucidation of the role of these proteins, and if their role in the process of infection is confirmed, it may be possible to use them to produce an effective vaccine.

Sedimentation analyses and electron microscopy showed that middle S gene products were assembled into particles whose size and density were indistinguishable from those of particles produced by major S gene products. However, the middle S particles seem to consist of heterogeneous materials, such as 33K, 29K, and 26K polypeptides, since cleavage of the 33K polypeptide by protease was not completely eliminated. It is not clear, therefore, whether particles consisting solely of intact 33K polypeptide can be made, viz., whether the 55-amino-acid extension polypeptide at the N terminus hinders the assembly of the polypeptide. Our observations show that a significant number of the 33K components that carry the 55-amino-acid extension polypeptide can be incorporated into particles, along with 29K and 26K components. Such an extension polypeptide may protrude from the particles. Particles from human sera, while containing a limited number of middle S polypeptides, consist predominantly of major S molecules. Here again, data obtained with human serum samples do not tell us whether the middle S polypeptides by themselves can form particles. The yeast-produced middle S particles differ from those of human sera in their very high content of the 33K component.

In contrast to middle S, the large S molecules, which have a 174-amino-acid extension polypeptide, did not readily assemble into the well-ordered structure that is typical of major S. It is likely, therefore, that the large S molecules form particles only by assembling with major S polypeptides. Determining whether the presence of large S molecules affects the shape or diameter of the particles awaits further elucidations in a system in which the ratio of the large S and major S components can be changed. A similar argument may apply to the middle S gene products.

Our analyses also showed that efficient assembly of major S gene products into 20-nm particles was achieved by partial proteolysis, although such treatment did not affect the major S polypeptide itself. Apparently, elimination of some cellular component(s) enhances the assembly process. The middle S and large S gene products are more susceptible to protease, and their partial proteolytic products, such as the 29K and 26K components, would behave similarly. The difference in reactivity to anti-S antibody between lysates prepared in the presence and absence of the protease inhibitors may reflect this effect.

We have discussed only the assembly of proteins into 20-nm particles observed in cell lysates. The question of the time and process of particle formation cannot yet be answered. The time is likely to be that of cell lysis, when membranes that have assembled proteins are disrupted and reform into small vesiclelike structures. The detailed mechanism of this assembly process awaits further elucidation.

ACKNOWLEDGMENTS

We are grateful to A. Toh-e, Department of Technology, Hiroshima University, Hiroshima, Japan, for providing the yeast GAPDH gene and to S. Nishimura, The Chemo-Sero-Therapeutic Research Institute, for electron microscopy.

LITERATURE CITED

1. **Bitter, G. A., and K. M. Egan.** 1984. Expression of heterologous genes in *Saccharomyces cerevisiae* from vectors utilizing the

glyceraldehyde-3-phosphate dehydrogenase gene promoter. Gene **32**:263–274.
2. **Bostian, K. A., J. M. Lemire, L. E. Cannon, and H. O. Halvorson.** 1980. In vitro synthesis of repressible yeast acid phosphatase: identification of multiple mRNAs and products. Proc. Natl. Acad. Sci. USA **77**:4504–4508.
3. **Burrell, C. I., P. Mackay, P. J. Greenaway, P. H. Hofschneider, and K. Murray.** 1979. Expression in *Escherichia coli* of hepatitis B virus DNA sequences cloned in plasmid pBR322. Nature (London) **279**:43–47.
4. **Charnay, P., C. Pourcel, A. Louise, A. Fritsch, and P. Tiollais.** 1979. Cloning in *Escherichia coli* and physical structure of hepatitis B virion DNA. Proc. Natl. Acad. Sci. USA **76**:2222–2226.
5. **Cheng, K.-C., and B. Moss.** 1987. Selective synthesis and secretion of particles composed of the hepatitis B virus middle surface protein directed by a recombinant vaccinia virus: induction of antibodies to pre-S and S epitopes. J. Virol. **61**:1286–1290.
6. **Fujiyama, A., A. Miyanohara, C. Nozaki, T. Yoneyama, N. Ohtomo, and K. Matsubara.** 1983. Cloning and structural analyses of hepatitis B virus DNAs, subtype *adr*. Nucleic Acids Res. **11**:4601–4610.
7. **Heermann, K. H., U. Goldmann, W. Schwartz, T. Seyffarth, H. Baumgarten, and W. H. Gerlich.** 1984. Large surface proteins of hepatitis B virus containing the pre-*s* sequence. J. Virol. **52**: 396–402.
8. **Hinnen, R. A., J. B. Hicks, and G. R. Fink.** 1978. Transformation of yeast. Proc. Natl. Acad. Sci. USA **75**:1929–1933.
9. **Hitzeman, R. A., C. Y. Chen, F. E. Hagie, E. J. Patzer, C. C. Liu, D. A. Estell, J. V. Miller, A. Yaffe, D. G. Kleid, A. D. Levinson, and H. Oppermann.** 1983. Expression of hepatitis B virus surface antigen in yeast. Nucleic Acids Res. **11**:2745–2763.
10. **Imai, M., Y. Yanase, T. Nojiri, Y. Miyakawa, and M. Mayumi.** 1979. A receptor for polymerized human and chimpanzee albumins on hepatitis B virus particles co-occurring with HBeAg. Gastroenterology **76**:242–247.
11. **Itoh, Y., T. Hayakawa, and Y. Fujisawa.** 1986. Expression of hepatitis B virus surface antigen P31 gene in yeast. Biochem. Biophys. Res. Commun. **138**:268–274.
12. **Itoh, Y., E. Takai, H. Ohnuma, K. Kitajima, F. Tsuda, A. Machida, S. Mishiro, T. Nakamura, Y. Miyakawa, and M. Mayumi.** 1986. A synthetic peptide vaccine involving the product of the pre-S(2) region of hepatitis B virus DNA: protective efficacy in chimpanzees. Proc. Natl. Acad. Sci. USA **83**:9174–9178.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Machida, A., S. Kishimoto, H. Ohnuma, K. Baba, Y. Ito, H. Miyamoto, G. Funatsu, K. Oda, S. Usuda, S. Togami, T. Nakamura, Y. Miyakawa, and M. Mayumi.** 1984. A polypeptide containing 55 amino acid residues coded by the pre-S region of hepatitis B virus deoxyribonucleic acid bears the receptor for polymerized human as well as chimpanzee albumins. Gastroenterology **86**:910–918.
15. **McAleer, W. J., E. B. Buynak, R. Z. Maigetter, D. E. Wampler, W. J. Miller, and M. R. Hillman.** 1984. Human hepatitis B vaccine from recombinant yeast. Nature (London) **307**:178–180.
16. **Michel, M. L., P. Pontisso, E. Sobczak, Y. Malpiece, R. E. Streeck, and P. Tiollais.** 1984. Synthesis in animal cells of hepatitis B surface antigen particles carrying a receptor for polymerized human serum albumin. Proc. Natl. Acad. Sci. USA **81**:7708–7712.
17. **Milich, D. R., M. K. McNamara, A. McLachlan, G. B. Thornton, and F. V. Chisari.** 1985. Distant H-2-linked regulation of T-cell response to the pre-S and S region of the same hepatitis B surface antigen polypeptide allows circumvention of nonresponsiveness to the S region. Proc. Natl. Acad. Sci. USA **82**: 8168–8172.
18. **Milich, D. R., G. B. Thornton, A. R. Neurath, S. B. Kent, M. L. Michel, P. Tiollais, and F. V. Chisari.** 1985. Enhanced immunogenicity of the pre-S region of hepatitis B surface antigen. Science **228**:1195–1199.
19. **Miyanohara, A., A. Toh-e, C. Nozaki, F. Hamada, N. Ohtomo, and K. Matsubara.** 1983. Expression of hepatitis B surface antigen gene in yeast. Proc. Natl. Acad. Sci. USA **80**:1–5.
20. **Neurath, A. R., S. B. H. Kent, and N. Strick.** 1984. Location and chemical synthesis of a pre-S gene coded immunodominant epitope of hepatitis B virus. Science **224**:392–395.
21. **Neurath, A. R., S. B. H. Kent, N. Strick, P. Taylor, and C. E. Stevens.** 1985. Hepatitis B virus contains pre-S gene-encoded domains. Nature (London) **315**:154–156.
22. **Peterson, D. L., N. Nath, and F. Gavilanes.** 1982. Structure of hepatitis B surface antigen. J. Biol. Chem. **257**:10414–10420.
23. **Sninsky, J. J., A. Siddiqui, W. S. Robinson, and S. N. Cohen.** 1979. Cloning and endonuclease mapping of the hepatitis B viral genome. Nature (London) **279**:346–348.
24. **Stibbe, W., and W. H. Gerlich.** 1983. Structural relationships between minor and major proteins of hepatitis B surface antigen. J. Virol. **46**:626–628.
25. **Takahashi, K., S. Kishimoto, H. Ohnuma, A. Machida, E. Takai, F. Tsuda, H. Miyamoto, T. Tanaka, K. Matsushita, K. Oda, Y. Miyakawa, and M. Mayumi.** 1986. Polypeptides coded for by the region pre-S and gene S of hepatitis B virus DNA with the receptor for polymerized human serum albumin: expression on hepatitis B particles produced in the HBeAg or anti-HBe phase of hepatitis B virus infection. J. Immunol. **136**:3467–3472.
26. **Tarentino, A. L., and F. Maley.** 1974. Purification and characterization of an endo-β-*N*-acetylglucosaminidase from *Streptomyces griseus*. J. Biol. Chem. **249**:811–817.
27. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
28. **Valenzuela, P., D. Coit, and C. H. Kuo.** 1985. Synthesis and assembly in yeast of hepatitis B surface antigen particles containing the polyalbumin receptor. Bio/Technology **3**:317–320.
29. **Valenzuela, P., P. Gray, M. Quiroga, J. Zaldiver, H. M. Goodman, and W. J. Rutter.** 1979. Nucleotide sequence of the gene coding for major protein of hepatitis B virus surface antigen. Nature (London) **280**:815–819.
30. **Valenzuela, P., A. Medina, W. J. Rutter, G. Ammerer, and B. D. Hall.** 1982. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature (London) **298**:347–350.