Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 11745–11749, December 1992
Immunology

Expression of hepatitis B surface antigen in transgenic plants

(oral vaccine/foreign genes/plants)

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Contributed by Charles J. Arntzen, September 16, 1992

ABSTRACT **Tobacco plants were genetically transformed with the gene encoding hepatitis B surface antigen (HBsAg) linked to a nominally constitutive promoter. Enzyme-linked immunoassays using a monoclonal antibody directed against human serum-derived HBsAg revealed the presence of HBsAg in extracts of transformed leaves at levels that correlated with mRNA abundance. This suggests that there were no major inherent limitations of transcription or translation of this foreign gene in plants. Recombinant HBsAg was purified from transgenic plants by immunoaffinity chromatography and examined by electron microscopy. Spherical particles with an average diameter of 22 nm were observed in negatively stained preparations. Sedimentation of transgenic plant extracts in sucrose and cesium chloride density gradients showed that the recombinant HBsAg and human serum-derived HBsAg had similar physical properties. Because the HBsAg produced in transgenic plants is antigenically and physically similar to the HBsAg particles derived from human serum and recombinant yeast, which are used as vaccines, we conclude that transgenic plants hold promise as low-cost vaccine production systems.**

Hepatitis B virus infection is one of the most widespread viral infections of humans and causes acute and chronic hepatitis and hepatocellular carcinoma (1). The infectious viral particle (Dane particle) is a 43-nm double-shelled sphere that consists of a core containing the 3.2-kilobase (kb) DNA genome bound to the core protein, surrounded by the viral envelope containing phospholipids and the major surface antigen [hepatitis B surface antigen (HBsAg)] (2). In addition to Dane particles, the serum of infected individuals also contains 22-nm subviral particles in great excess over virions. These noninfectious particles contain the elements of the viral envelope, including the major 24-kDa peptide that occurs in glycosylated and unglycosylated forms (2).

Because the host range of hepatitis B virus is limited to humans and chimpanzees, and since the virus cannot be propagated in cell culture, HBsAg for use in vaccines was purified from the serum of infected individuals until a recombinant form (rHBsAg) was produced in yeast (3). The immunogenic yeast-derived rHBsAg occurs in the form of spherical particles with an average diameter of 17 nm. Integration of the peptides into the phospholipid-containing particles greatly enhances their immunogenic properties (4). Subsequent work showed that the peptides present in the yeast-derived particles were much less extensively disulfide-linked than in the human material but that such linking could be induced *in vitro* (5).

Intramuscular injection of serum-derived HBsAg or yeast-derived rHBsAg in healthy individuals results in effective immunization and protection from viral infection (6, 7). In many areas of the developing world, however, the expense of immunization programs for large segments of the population is prohibitive. This has led us to attempt the expression of rHBsAg in plants with the hope of developing a less expensive production system. Further, we hope to find a way to present the rHBsAg in edible plant tissues in a form that would be useful as an oral vaccine. In this paper we describe the transformation of tobacco with the gene encoding HBsAg and its expression in leaf tissue in the form of an antigenic spherical particle with an average diameter of 22 nm. This plant-derived rHBsAg is directly analogous to the rHBsAg from yeast that is now used for commercial vaccines. We view this as a successful first step in a long-term project dedicated to developing technologies for low cost "edible vaccines" for the developing world.

MATERIALS AND METHODS

Construction of Plasmids for Plant Transformation. The HBsAg coding region on the *Pst* I/*Hin*dIII fragment from pMT-SA (kindly provided by Li-he Guo, Chinese Academy of Sciences) was subcloned into pBluescript KS (Stratagene) to form pKS-HBS. The HBsAg gene in pKS-HBS was opened 116 base pairs (bp) 3′ to the termination codon with *Bst*BI and the resulting ends were blunted by filling with Klenow enzyme and dCTP/dGTP. The entire coding region was then excised 16 bp upstream of the *Pst* I site with *Bam*HI. pBI121 (ref. 8; obtained from Clontech) was digested with *Sac* I and the ends were blunted with mung bean nuclease. The GUS coding region was then released with *Bam*HI and the vector was isolated. The HBsAg coding fragment was ligated into the GUS-less pBI121 to yield pHB101 (Fig. 1), where its expression is driven by the cauliflower mosaic virus (CaMV) promoter derived from pBI121.

The CaMV 35S promoter with duplicated enhancer linked to the tobacco etch virus (TEV) 5′ nontranslated leader sequence, which acts as a translational enhancer (9), was excised from pRTL2-GUS (10) as follows. pRTL2-GUS was digested with *Nco* I and the ends were blunted with mung bean nuclease. The promoter-leader fragment was then released by digestion with *Hin*dIII. pHB101 was digested with *Hin*dIII and *Sma* I to release the 35S promoter fragment, and the vector was purified. The promoter-leader fragment was then ligated into the *Hin*dIII/*Sma* I-digested pHB101 to yield pHB102 (Fig. 1). The HBsAg coding region lies upstream of the nopaline synthase terminator in both constructs. The plasmids contain the left and right border regions, which denote the limits of the DNA that is integrated into the plant genomic DNA via *Agrobacterium tumefaciens*-mediated transformation, as well as the neomycin phosphotransferase gene, which allows selection with kanamycin.

Plant Transformation. *Agrobacterium* strain LBA4404 cells were transformed by the direct method (11) with the plasmids prepared from *Escherichia coli* clones, and the

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Abbreviations: CaMV, cauliflower mosaic virus; HBsAg, hepatitis B surface antigen; rHBsAg, recombinant HBsAg; TEV, tobacco etch virus.

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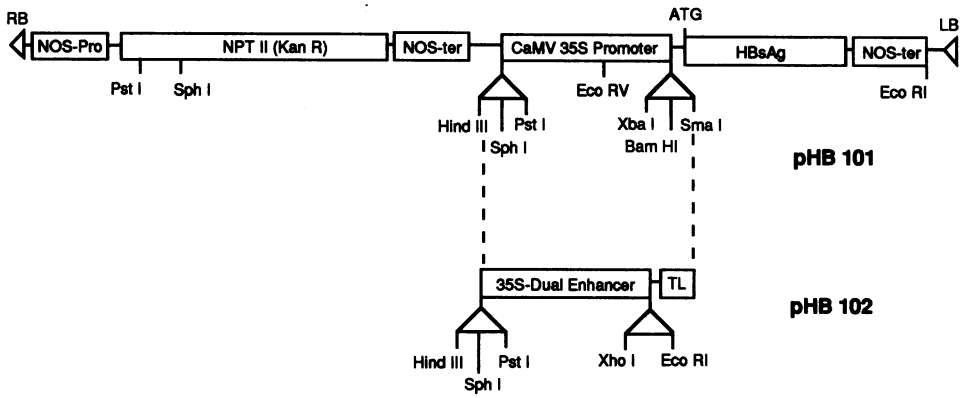

FIG. 1. Structure of plasmids pHB101 and pHB102. The constructs carry the left and right borders (LB, RB) of the transferred DNA that demarcates the sequences that are incorporated into the plant genomic DNA via *Agrobacterium*-mediated transformation. The HBsAg coding region lies downstream of the CaMV 35S promoter in pHB101 and is followed by the nopaline synthase (NOS) terminator. In pHB102, the 35S promoter is replaced by a modified CaMV 35S promoter containing a duplicated transcriptional enhancer region, linked to the TEV 5′ nontranslated leader (TL). Restriction endonuclease cleavage sites are indicated. Blunt-ended ligation has removed the *Nco* I site at the 3′ end of the leader sequence.

structure of the plasmids was verified by restriction digestion. Tobacco (*Nicotiana tabacum* cv. Samsun) was transformed by cocultivating leaf discs (12) with *Agrobacterium* strains transformed with pHB101 or pHB102. Shoots were generated from transformed callus selected on medium containing 0.2 mg of kanamycin per ml and 0.2 mg of cefotaxime per ml. Shoots were rooted in medium containing 0.1 mg of kanamycin per ml, transplanted to soil, and watered with one-half strength Hoagland medium.

Analysis of RNA from Transformed Tobacco. Total RNA from leaves of plants transformed with pHB101 was isolated as described (13). The RNA was denatured with formaldehyde, fractionated on 1% agarose gels (5 μg per lane), blotted to nylon, and probed with ^{32}P-labeled random-primed DNA using a 700-bp *Bam*HI/*Acc* I fragment from pKS-HBS that includes most of the coding region for HBsAg. Blots were hybridized at 68°C in 0.25 M sodium phosphate, pH 7.0/1 mM EDTA/7% SDS, washed with 40 mM sodium phosphate, pH 7.0/5% SDS at 68°C, and exposed to X-Omat AR film for 4 hr.

Analysis of Protein from Transformed Tobacco. Protein was extracted from leaf tissues by homogenization with a Ten-Broek ground glass homogenizer (clearance, 0.15 mm) in 5 volumes of buffer containing 20 mM sodium phosphate (pH 7.0), 0.15 M NaCl, 20 mM sodium ascorbate, 0.1% Triton X-100, and 0.5 mM phenylmethylsulfonyl fluoride at 4°C. The homogenate was centrifuged at 1000 × *g* for 5 min, and the supernatant was centrifuged at 27,000 × *g* for 15 min. The 27,000 × *g* supernatant was centrifuged at 100,000 × *g* for 1 hr, and the pellet was resuspended in extraction buffer. Protein in the different fractions was measured by the Coomassie dye-binding assay (Bio-Rad). HBsAg was assayed with the Auszyme monoclonal kit (Abbott), using the positive control (HBsAg derived from human serum) as a standard. The positive control was diluted to give HBsAg levels of 0.09–1.8 ng per assay, and the absorbance at 492 nm after color development gave a linear relationship in this range.

Immunoaffinity Purification of HBsAg from Transgenic Tobacco. Monoclonal antibody against HBsAg (clone ZMHB1) was obtained from Zymed Laboratories. The immunogen source for this antibody is human serum. The antibody was bound to Affi-Gel Hz hydrazide gel (Bio-Rad) according to the instructions supplied with the kit. Soluble material that was resuspended from the 100,000 × *g* pellet was made to 0.5 M NaCl and mixed with the immobilized antibody-gel by end-over-end mixing for 16 hr at 4°C. The gel was washed with 10 volumes of 10 mM sodium phosphate, pH 7.0/0.5 M NaCl, and 10 volumes of 10 mM sodium phosphate, pH 7.0/0.15 M NaCl, and bound HBsAg was eluted with 0.2 M glycine (pH 2.5). The eluate was immediately neutralized with Tris base, and particles were pelleted at 109,000 × *g* for 1.5 hr at 5°C. The pelleted material was negatively stained with phosphotungstic acid and visualized with transmission electron microscopy using a Phillips CM10 microscope.

Sucrose and CsCl Gradient Analysis of HBsAg from Transgenic Tobacco. Extracts of leaf tissues were made as described above and 0.5 ml of the 27,000 × *g* supernatants was layered on linear 11-ml 5–30% sucrose gradients made in 10 mM sodium phosphate, pH 7.0/0.15 M NaCl or discontinuous 12-ml CsCl gradients (1.1–1.4 g/ml) made in 10 mM sodium phosphate at pH 7.0 (3 ml each of 1.1, 1.2, 1.3, and 1.4 g of CsCl per ml). Positive control HBsAg from the Auszyme kit was also layered on separate gradients. The sucrose gradients were centrifuged in a Beckman SW41Ti

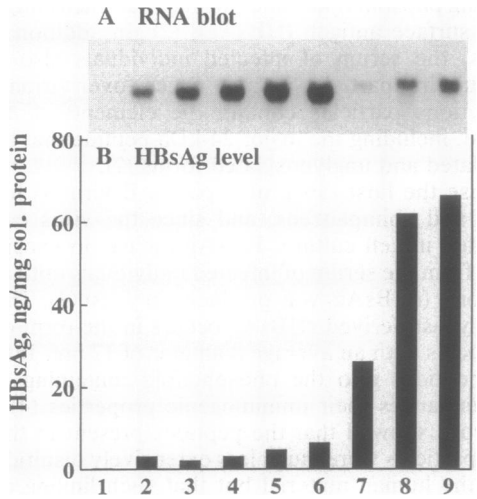

FIG. 2. HBsAg mRNA and protein levels in transgenic tobacco plants. (*A*) Total RNA from wild-type untransformed or independent transgenic tobacco lines carrying either the pHB101 or the pHB102 construct was hybridized with a probe specific for the HBsAg coding region. (*B*) Protein extracts from the same leaves were tested for HBsAg with the Auszyme monoclonal kit (Abbott), and HBsAg levels were quantified using a standard curve of human serum-derived HBsAg. Numbers: 1, wild-type control plant; 2–6, independent transformants harboring the construct in pHB101; 7–9, independent transformants harboring the construct in pHB102.

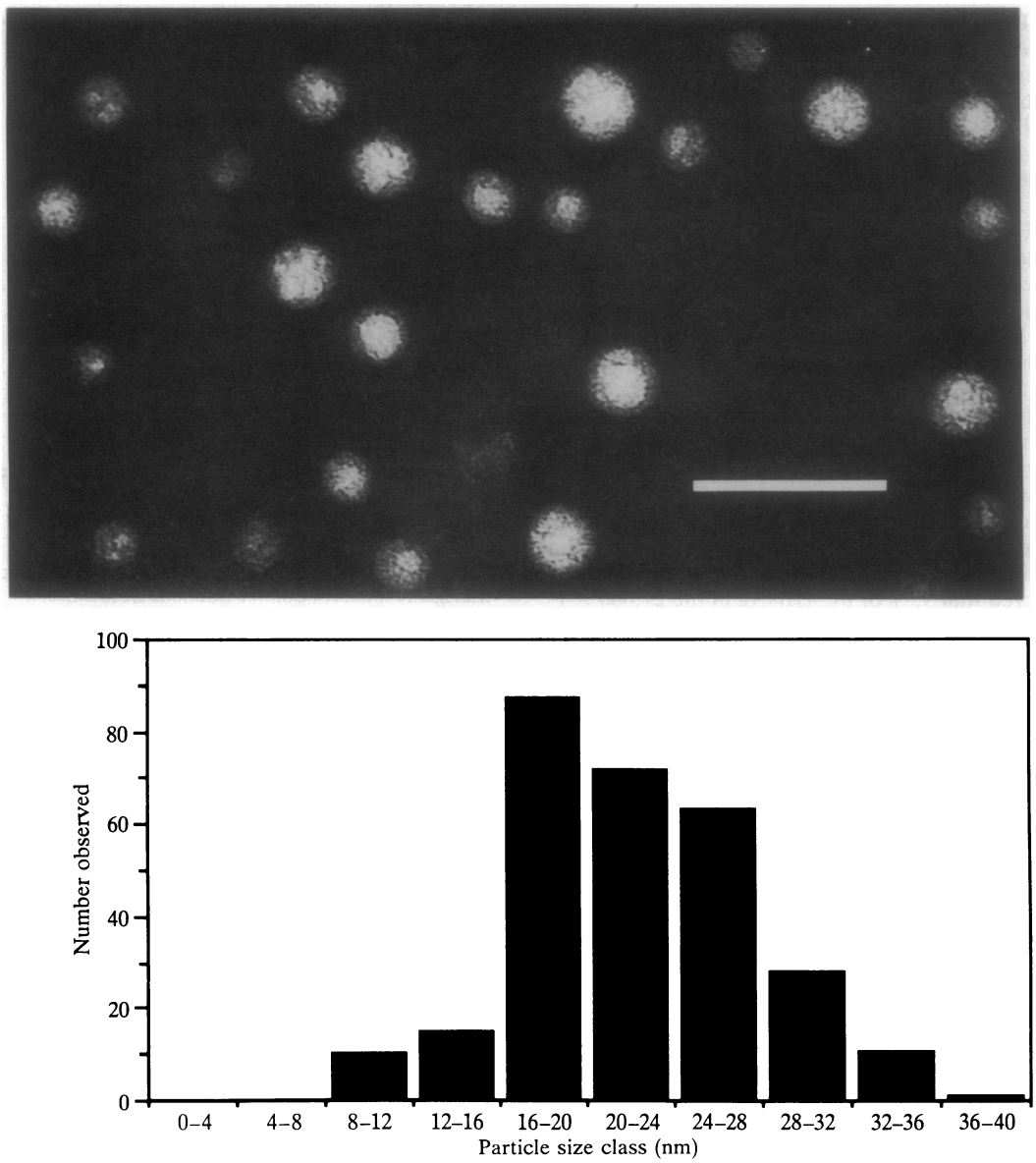

FIG. 3. (*Upper*) Electron micrograph of immunoaffinity purified rHBsAg. HBsAg from a transgenic tobacco plant harboring construct pHB102 was purified by immunoaffinity chromatography, negatively stained with phosphotungstic acid, and visualized by transmission electron microscopy. (Bar = 100 nm.) (*Lower*) Histogram generated by measuring the diameters of the particles observed in the representative field.

rotor at 33,000 rpm for 5 hr at 5°C and fractionated into 1-ml fractions while monitoring the A_{280}. The CsCl gradients were centrifuged in a Beckman SW40Ti rotor at 30,000 rpm for 25 hr at 5°C and fractionated into 1.0-ml fractions. HBsAg in the gradient fractions was assayed using the Auszyme kit as described above. The density of gradient fractions was measured by weighing aliquots with an analytical balance.

RESULTS AND DISCUSSION

The plasmid HB101 (Fig. 1) was constructed by inserting the coding region for HBsAg from pMT-SA between the *Bam*HI and *Sac* I sites in the plant transformation vector pBI121 after removal of the GUS coding region. In this construct the expression of HBsAg is driven by the CaMV 35S promoter. A modification of pHB101 was made by inserting the CaMV 35S promoter with dual transcriptional enhancer linked to the TEV 5′ nontranslated leader (10) in the place of the original 35S promoter to form pHB102 (Fig. 1). The TEV leader acts as a translational enhancer to increase the amount of protein made using a given amount of template mRNA (9).

The plasmids were used to transform tobacco by the leaf disc method using *Agrobacterium*, and regenerated kanamycin-resistant transformants were analyzed by hybridizing RNA samples with a labeled probe encompassing the coding region of the HBsAg gene. Fig. 2*A* shows the results of an experiment where selected transformants harboring either the pHB101 or the pHB102 construct and a wild-type control were probed. The signals were variable between transformants, as expected due to effects of position of insertion into the genomic DNA and differing copy number. The transcripts from the pHB101 transformants (Fig. 2*A*, lanes 2–6) were ≈1.2 kb in length by comparison with RNA standards (H.S.M., unpublished data), which is consistent with the expected size. The pHB102 transcripts were slightly larger (Fig. 2*A*, lanes 7–9), owing to the 5′ addition of the TEV leader sequence. The nontransformed control leaf RNA (Fig. 2*A*, lane 1) showed no detectable signal at this stringency of hybridization. Thus, mRNA that hybridizes specifically with HBsAg probe was present in the leaves of selected transformants, and there is no inherent transcriptional limitation to the expression of HBsAg in tobacco leaves.

Using the HBsAg assay kit, we tested leaf extracts for the presence of material that reacts specifically with monoclonal antibody to serum-derived HBsAg. Fairly low levels were observed for the pHB101 transformants, ranging from 2 to 6 ng/mg of soluble protein (Fig. 2*B*, nos. 2–6). The pHB102 transformants showed substantially greater levels of HBsAg, ranging up to 66 ng/mg of soluble protein (Fig. 2*B*, nos. 7–9). The reaction was specific because wild-type tobacco showed no detectable HBsAg (Fig. 2*B*, no. 1). The levels of HBsAg observed in the individual transformants were roughly proportional to the levels of specific mRNA encoding HBsAg for a given construct. The pHB102 transformants, containing the 5′ TEV leader, showed much higher accumulations of HBsAg for a given amount of mRNA than did the pHB101 transformants (Fig. 2). The translational enhancement observed in mRNAs carrying the TEV leader appears to involve a cap-independent competition for translation initiation factors (9).

HBsAg from human serum occurs as ≈22-nm spherical particles, consisting of protein embedded in a phospholipid bilayer. Since rHBsAg from plasmid-transformed yeast also occurs as particles of a similar size class, we sought evidence that the recombinant material in tobacco is present as particles. We observed that 95% of the HBsAg in the 27,000 × *g* supernatants of transgenic tobacco leaf extracts pelleted at 200,000 × *g* for 30 min (H.S.M., unpublished data), suggesting a particle form. We purified HBsAg by immunoaffinity chromatography using a monoclonal antibody raised against human serum-derived HBsAg. Inspection of this material by negative staining and transmission electron microscopy revealed the presence of particles ranging in diameter between 10 and 40 nm (Fig. 3). Most of the particles were between 16 and 28 nm (Fig. 3 *Lower*); the average diameter was 22 nm. These are very similar to the particles observed in human serum (2), although we observed no rods. The rHBsAg particles from yeast occur in a range of sizes with a mean of 17 nm (3). We conclude that the rHBsAg made in transgenic tobacco retains the capacity for self-association and thus has the physical properties of human serum-derived HBsAg and rHBsAg from yeast, both of which are highly immunogenic in the particle form.

We obtained further evidence of particle behavior from sedimentation and buoyant density studies of transgenic tobacco leaf extracts. Fig. 4 shows a sucrose gradient profile of HBsAg activity from transgenic tobacco harboring the construct in pHB102. The plant-derived HBsAg sedimented with a peak near the 60S ribosomal subunit, and the serum-derived material sedimented in a somewhat sharper peak just slightly slower. These data are consistent with the finding that human HBsAg sediments at 55 S (14). The observation that the plant material sedimented slightly faster and with a broader peak than the human HBsAg is also consistent with the larger mean size of the plant particles and wider range of sizes (Fig. 3). The buoyant density of the rHBsAg from transgenic tobacco in CsCl was found to be ≈1.16 g/ml (Fig. 5), whereas the human particle showed a density of about 1.20 g/ml. Thus, the rHBsAg from transgenic tobacco exhibits sedimentation and density properties that are very similar to the subviral particles obtained from human serum. Importantly, HBsAg in the particle form is found to be much more immunogenic than that in the form of the peptide alone (4).

The subcellular localization of the HBsAg in our transgenic plants has not been characterized. Particles are observed in the lumen of the endoplasmic reticulum in infected liver cells (15) and appear to be secreted by the constitutive secretory pathway. The peptides contain two signal sequences, one N-terminal and one internal, that together determine a transmembrane orientation of the molecules (2). Whether the rHBsAg particles in leaf cells are secreted to the extracellular space or retained within the cytoplasm is a question that needs to be addressed. Although the HBsAg coding region is part of a larger open reading frame in the viral genome (2), the lack of the pre-S peptide does not alter the formation of particles in transfected mammalian cells (16) or yeast (3). Our construct also contains no pre-S sequences and is thus similar to that used in those studies. We were unable to analyze the size of the HBsAg peptides produced in transgenic tobacco because the monoclonal antibodies that we used failed to recognize the SDS-denatured peptides on SDS/PAGE blots. The antibodies did recognize undenatured HBsAg in dot blots of leaf extracts or blots of whole leaves or seedlings, however (H.S.M., unpublished data).

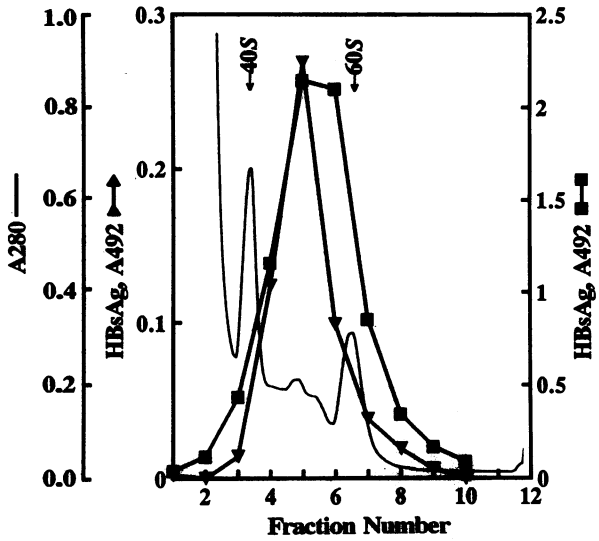

FIG. 4. Sucrose density gradient sedimentation of HBsAg from transgenic tobacco. Soluble fractions from a transformant harboring the pHB102 construct or human serum-derived HBsAg were sedimented in 5–30% sucrose gradients, fractionated, and assayed for HBsAg. The solid curve represents the absorbance profile at 280 nm of the plant extract. The top of the gradient is at the left; positions of the 40S and 60S ribosomal subunits are indicated. ■, HBsAg in transgenic plant extract; ▼, HBsAg in serum-derived material; solid line, absorbance of tobacco leaf extract at 280 nm.

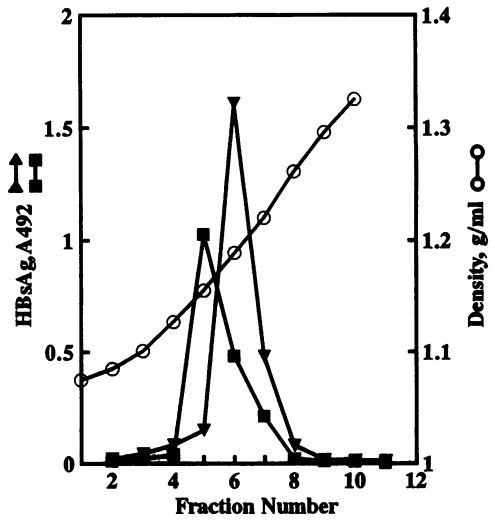

FIG. 5. Buoyant density in CsCl of HBsAg from transgenic tobacco. Supernatant fractions of transgenic tobacco harboring construct pHB102 and human serum-derived HBsAg were banded in a 1.1–1.4 g/ml CsCl gradient. Fractions were assayed for HBsAg activity. ■, HBsAg in plant extract; ▼, HBsAg in serum-derived material; ○, density of gradient fractions.

In conclusion, we have shown that HBsAg can be expressed in plant tissues via stable transformation with foreign DNA. Furthermore, the rHBsAg from transgenic tobacco is recognized specifically by monoclonal antibodies directed against human serum-derived HBsAg and is processed properly after translation so that the antigenic particle form is observed. These studies indicate the feasibility of expression of foreign antigens in plants for possible use as oral vaccines. Presently, the maximal levels of HBsAg we have found in transgenic plants represent ≈0.01% of the soluble leaf protein. This is an inadequate level for the efficient use of plants as production systems for rHBsAg for vaccine use. Further studies must be done to increase the accumulation of HBsAg, such as using other transcriptional regulatory elements to increase mRNA levels. The processing of the HBsAg peptides in plant tissues must also be examined, specifically with regard to glycosylation and intermolecular disulfide bonding. The HBsAg system may be useful for determining the feasibility of targeting foreign antigens to specific subcellular compartments.

We thank Professor Li-He Guo of the Chinese Academy of Sciences for providing the pMT-SA plasmid, Shaun Stallings for construction of the figures, Qing-Yi Zeng for electron microscopy, and Jian-Jian Shi for technical assistance.

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