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Polypeptides of Hepatitis B Virus Surface Antigen Produced by a Hepatoma Cell Line

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The PLC/PRF/5 cell line derived from a human hepatoma produces hepatitis B surface antigen (HBsAg) in 22-nm particles of the same buoyant density as those found in the serum of infected patients. The HBsAg particles from this cell line were labeled with [^{35}S]methionine and purified, and the polypeptides were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with those of serum-derived particles. The two major polypeptides of serum-derived HBsAg particles (p20 and p23) were found in the same relative amounts in the particles from the cell line. The three smallest of the five minor components observed in HBsAg particles from serum were present in particles from the cell line. These polypeptides (p31, p36, and p43), as well as p20 and p23, were precipitated with anti-HBs-containing serum. The two largest polypeptides of serum particles (p49 and p66) were not detected in particles from these cells. When the PLC/PRF/5 HBsAg particles were radiolabeled with tritiated sugars, p23, and not p20, was found to contain radioactivity, indicating that the pattern of polypeptide glycosylation is similar to that of serum HBsAg. None of the other possible gene products of hepatitis B virus was detected in the PLC/PRF/5-derived HBsAg particles, in the cells, or in the cell supernatants.

Hepatitis B virus (HBV) infection in humans is accompanied by the appearance of viral antigens in liver and blood. Several viral forms in the blood contain hepatitis B surface antigen (HBsAg). One form, the Dane particle, contains an internal antigen designated the hepatitis B core antigen (HBcAg), DNA, and DNA polymerase activity and is considered to be the complete virion (18). A third antigen, hepatitis B e antigen (HBeAg), can be detected in the blood of some infected patients (13). Recent evidence indicates that HBeAg is found in a cryptic form in the interior of the Dane particle (22). The most abundant HBsAg forms are 22-nm spherical particles and filamentous forms consisting of protein, carbohydrate, and lipid, and these are considered to be defective or incomplete viral forms without HBcAg or DNA. Several polypeptides isolated from these particles have been shown to contain both group- and type-specific HBsAg determinants (4, 5, 19). In infected liver, HBsAg is detected in cell cytoplasm and at cell surfaces, and HBcAg is detected in nuclei by immunofluorescence (1, 6).

Numerous studies have demonstrated an association between persistent HBV infection and primary liver cancer in some parts of the world (2, 8, 21). It is not known whether the relationship is a causal one. In 1976, a tissue culture cell line designated PLC/PRF/5 was isolated from a primary liver carcinoma of an African man with persistent HBV infection. The cell line was subsequently shown to produce small amounts of HBsAg, apparently in the form of 22-nm particles (12). The antigenic subtype has been reported to be ad (12). The other antigens associated with HBV, HBcAg and HBeAg, were not detected in the cells. These findings indicate that the cells contain at least some HBV genes.

Since cells in culture have not been successfully infected with HBV, PLC/PRF/5 cells offer the first opportunity to study HBsAg formation in tissue culture.

Here we describe experiments in which PLC/PRF/5 cells were incubated with radiolabeled methionine or sugars, the HBsAg particles were purified from culture medium, and the radioactive polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and compared with HBsAg particles purified from patient serum. The cells and culture medium were also assayed for Dane particles, HBcAg, and HBeAg.

MATERIALS AND METHODS

Cell culture. The PLC/PRF/5 cells were grown in Dulbecco-modified Eagle medium supplemented with 10% fetal bovine serum. The cells have been passaged

weekly and are now at passage 44 in our laboratory. Production of HBsAg has remained at a constant level. The cells are infected with mycoplasma.

Labeling of cells. At 2 to 3 days after reaching confluency in 10-cm culture dishes, PLC/PRF/5 cells were incubated with 50 μCi of L-[^{35}S]methionine (Amersham Corp., 800 to 900 Ci/mmol) in 10 ml of methionine-free medium supplemented with 1% methionine-containing medium and 10% fetal bovine serum. After 3 days, the medium was replaced with fresh medium containing L-[^{35}S]methionine and incubated for another 2 days. The pooled radioactive cell supernatants were centrifuged at 12,000 × *g* and 4°C for 30 min to remove cellular debris.

Similar cultures were incubated with L-[6-^{3}H]fucose (Amersham Corp., 17 Ci/mmol) and D-[6-^{3}H]glucosamine (Amersham Corp., 20 Ci/mmol) (20 μCi each) in 10 ml of Dulbecco-modified Eagle medium for the same times described for [^{35}S]methionine.

Detection of HBsAg. Solid-phase radioimmunoassay (Ausria II, Abbott Laboratories) was used to detect HBsAg. Samples from CsCl gradient fractions were diluted in TNE buffer (0.01 M Tris-hydrochloride, pH 7.5; 0.15 M NaCl; 0.001 M EDTA) at least eightfold to avoid the effects of concentrated CsCl on the assay.

Purification of HBsAg. Clarified cell supernatants from PLC/PRF/5 cells were centrifuged at 25,000 rpm and 4°C for 24 h in a Spinco SW27 rotor to pellet the HBsAg particles. All but 0.5 ml of the supernatants were removed by suction; the remaining 0.5 ml contained concentrated fetal calf serum proteins and loosely pelleted HBsAg particles. The pellets were suspended in the remaining supernatant and were made up to 7 ml with TNE buffer. Solid CsCl was added to give a density of 1.2 g/ml, and the material was centrifuged at 45,000 rpm and 4°C for 68 h in a Spinco 50 Ti rotor. Fractions were collected from the bottom of the tube and assayed for radioactivity and HBsAg. Peak fractions of HBsAg were pooled, diluted to 7 ml with CsCl at 1.2 g/ml in TNE buffer, and centrifuged a second time. The HBsAg peak fractions were again pooled and dialyzed at 4°C overnight against TNE buffer to remove CsCl. The material (approximately 2 ml) was then layered on top of a 33-ml preformed 5 to 20% sucrose density gradient containing TNE buffer and 1 mg of bovine serum albumin per ml and centrifuged at 25,000 rpm and 4°C for 5 h in a Spinco SW27 rotor. HBsAg peak fractions were dialyzed overnight against TNE buffer to remove the sucrose. The HBsAg was then banded twice more in CsCl density gradients as described above. The fractions containing purified HBsAg particles from the final gradient were then pooled and kept at 4°C until used.

HBsAg from serum was purified in the same manner, but a Lowry protein assay (10) was used to detect total protein in gradient fractions, and HBsAg was detected by complement fixation.

Antisera. A guinea pig anti-HBs/adw-containing serum was a gift from John Gerin of the Oak Ridge National Laboratory. The serum was absorbed with rabbit liver powder and has a complement fixation titer of 1:32.

The immunoglobulin G fraction of serum from HBsAg carriers with high titers of anti-HBc was prepared by ammonium sulfate precipitation and employed in a solid-phase radioimmunoassay for HBcAg (16).

Sera from HBsAg carriers with high titers of anti-HBe were used to detect HBeAg by double immunodiffusion (detection to a serum dilution of 1:128) and by an enzyme-linked immunosorbent assay (14) used in this laboratory (detection to a dilution of 1:64,000).

PAGE. Purified HBsAg particles in CsCl were diluted 1.5-fold with TNE buffer, and 20 μg each of bovine serum albumin and cytochrome *c* were added. A total of 5 to 10 volumes of ethyl alcohol (EtOH) was added, and the precipitated protein was pelleted by centrifugation for 10 min at 10,000 × *g*. The pellets were suspended in sample buffer (0.07 M Tris-hydrochloride, pH 6.8; 11% glycerol; 0.0015% bromophenol blue; 3% SDS; 10% β-mercaptoethanol) and heated at 100°C for 3 min.

The polypeptides were separated by electrophoresis in vertical slab gels as described by Laemmli (9). Gels of 13 or 8 to 20% polyacrylamide were run usually at 19 mA/13.5 cm for 5 h. Gels were fixed in 20% methanol-7% acetic acid, stained in 0.2% Coomassie brilliant blue in 50% methanol-7% acetic acid, and destained in the fixative. To enhance detection of radioactivity, we performed fluorography as described by Bonner and Laskey (3) on all gels before drying with a slab gel dryer from Hoefer Scientific Instruments. The dried gels were exposed to XR-5 X-ray film (Eastman Kodak Co.) at −70°C and developed. Protein standards of known molecular weights were β-galactosidase, bovine serum albumin, ovalbumin, aldolase, chymotrypsinogen A, and cytochrome *c*.

Immunoprecpitation. Antigens were immunoprecipitated with staphylococcal protein A prepared by the method of Kessler (7). One volume of HBsAg particles was diluted with either 3 volumes of TNE buffer plus 0.1% β-mercaptoethanol and 1 mg of bovine serum albumin per ml (TNEMEBSA) or 2 volumes of 1X RIPA buffer (0.01 M Tris, pH 7.2; 0.15 M NaCl; 1% deoxycholate; 1% Triton X-100; 0.1% SDS) plus 1 volume of 2× RIPA buffer. Serum containing anti-HBs (25 μl/ml) was added, and the mixture was incubated for 1 h at 37°C. A suspension of protein A (10%, vol/vol) was then added (50 μl/5 μl of antiserum), and the mixture was incubated for 30 min at room temperature. After centrifugation (10,000 rpm in a Brinkman 3200 centrifuge for 10 min), the pellet was washed once with 0.5 ml of TNEMEBSA or 1× RIPA buffer, repelleted, and resuspended in 50 μl of sample buffer containing 6% SDS. Samples were heated at 100°C for 3 to 5 min, the protein A was removed by centrifugation, and samples were analyzed by SDS-PAGE.

RESULTS

Purification of HBsAg particles from PLC/PRF/5 cells. As culture fluids of PLC/PRF/5 cells contained greater amounts of HBsAg than did disrupted cells, these fluids were used as the source of HBsAg particles for purification. After removal of cellular debris by low-speed centrifugation, the HBsAg particles were pelleted in the ultracentrifuge. The particles were then purified as described above by four equilibrium centrifugations in CsCl and one

rate sedimentation in a sucrose gradient. The final CsCl gradient served mainly to concentrate the particles without the losses incurred when other concentrating methods, such as pelleting or ultrafiltration, were used.

The first and last CsCl centrifugation steps of [^{35}S]methionine-labeled HBsAg particle purification are shown in Fig. 1. Figure 1A shows trichloroacetic acid-precipitable ^{35}S and HBsAg measured by solid-phase radioimmunoassay in the fractions of the first gradient. The HBsAg particles accounted for such a small fraction of the acid-precipitable [^{35}S]methionine that these particles could not be distinguished as a separate ^{35}S component in this early purification step. ^{35}S-labeled HBsAg particles became detectable only at the third or, rarely, second purification step. In the final gradient (Fig. 1B), the major ^{35}S peak and that of HBsAg coincided. The peak fractions of the final gradient were pooled and stored at 4°C. Several purification steps for HBsAg were needed because of the large amount of non-HBsAg ^{35}S-labeled material released by these cells. ^{35}S-labeled HBsAg represented about 1% of the total trichloroacetic acid-precipitable radioactivity in the clarified cell supernatants. SDS-PAGE analysis of the non-HBsAg ^{35}S-labeled material revealed a continuous distribution of polypeptides with molecular weights of 5,000 (5K) to 100K, and no predominant components were visible.

HBsAg particles from serum were found at the same buoyant density (1.23 g/ml) in CsCl density gradients.

Comparison of the polypeptides of [^{35}S]-methionine-labeled HBsAg with HBsAg from human serum. The polypeptides of purified [^{35}S]methionine-labeled HBsAg particles (subtype ad) from PLC/PRF/5 cells and of purified HBsAg (subtype adw) from patient serum that was HBeAg positive were analyzed by electrophoresis in an 8 to 20% SDS-polyacrylamide gradient gel as described above.

The polypeptides of the serum HBsAg preparation, shown with cytochrome *c* in Fig. 2, track b, appear to be similar to previous findings of others (18). Two polypeptides corresponding to molecular weights of 20 and 23K (p20 and p23) constitute the majority of the Coomassie brilliant blue-staining material. p20 was present in greater amounts than p23. Several minor Coomassie brilliant blue-staining components in positions corresponding to molecular weights of 31, 36, 43, 49, and 66K (p31, p36, p43, p49, and p66) were also regularly observed. p66 comigrates with human serum albumin. An eighth polypeptide migrating at 97K was frequently observed, although not in the gel shown.

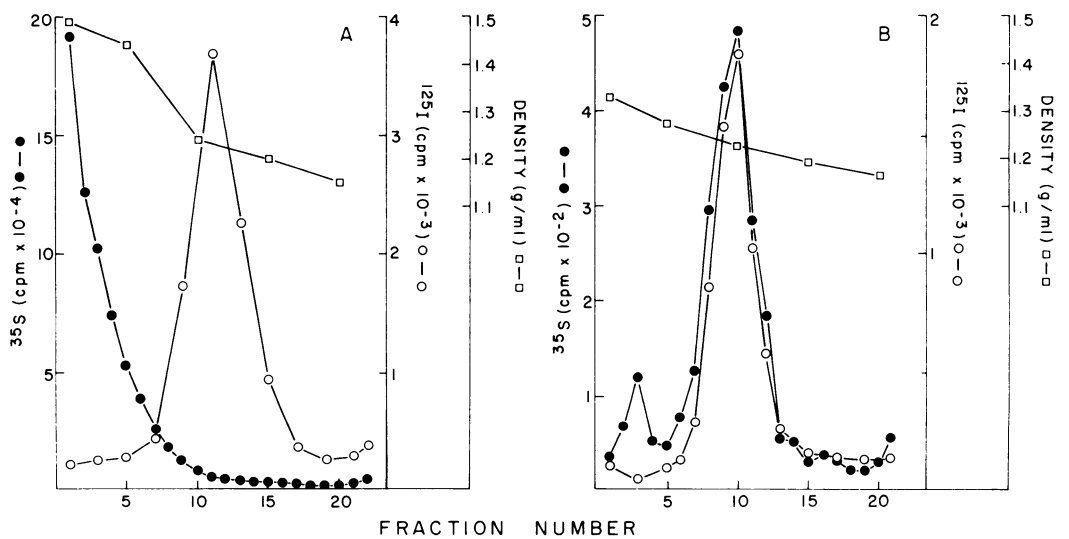

FIG. 1. *Isopycnic centrifugation of ^{35}S-labeled HBsAg particles in CsCl gradients. (A) The first equilibrium CsCl density gradient centrifugation for ^{35}S-labeled HBsAg purification was carried out as described in the text, and fractions were collected from the bottom of the tube. The trichloroacetic acid-precipitable radioactivity was determined on 1 μl of each fraction, and the amount of HBsAg activity was measured in every other fraction by radioimmunoassay after 100-fold dilution of 2.5 μl. (B) The final equilibrium CsCl density gradient centrifugation step in ^{35}S-labeled HBsAg particle purification was carried out as described in the text, and fraction collection, radioactivity, and HBsAg assays were carried out as described in (A), using 5 μl for the ^{35}S determination and 5 μl diluted 50-fold for the antigen assay.*

In Fig. 2, track a, the positions of [^{35}S]methionine-labeled polypeptides from the PLC/PRF/5 HBsAg preparation electrophoresed in the same gel can be observed after autoradiography to be similar to the positions of the polypeptides of serum-derived HBsAg, with certain exceptions. p20 and p23 are present in the same general proportions. p31, p36, and p43 are also present, although the high background makes it difficult to distinguish them. The two preparations differed by the presence of a 27K (p27) ^{35}S-polypeptide less heavily labeled than p20 and p23, but more in evidence than the other minor components and not detected by Coomassie brilliant blue staining of the serum HBsAg polypeptides. p27 was present in the same relative amounts in two other preparations of ^{35}S-labeled HBsAg particles.

Carbohydrate in HBsAg from PLC/PRF/5 cells. One of the major polypeptides of purified serum HBsAg (our p23) has been observed by others (18) to stain with periodic acid-Schiff, suggesting that it was glycosylated. Minor components migrating at 27, 32 or 35, and 53.3K have also appeared to contain carbohydrate in previous studies (18).

To determine whether any of the polypeptides in the HBsAg preparation from PLC/PRF/5 cells were glycosylated, we incubated the cells with both L-[^{3}H]fucose and D-[^{3}H]glucosamine and purified the HBsAg particles in the culture supernatants as described above. The ^{3}H and HBsAg profiles in the final CsCl gradient coincided as shown in Fig. 3. The ^{3}H-labeled HBsAg particles were EtOH precipitated and analyzed by SDS-PAGE alongside EtOH-precipitated [^{35}S]methionine-labeled HBsAg. A major problem with this analysis was the very low level of radioactivity incorporated into HBsAg particles in these experiments. Although 2.5 mCi of [^{35}S]methionine incubated with 10^8 cells as described yielded a purified particle preparation containing approximately 150,000 cpm, the same amount of tritium in the sugars used at the same specific activity yielded no more than 37,000 cpm in purified particles in any experiment and required fluorography for detection by autoradiography. The pattern of ^{3}H-labeled polypeptides can be seen in track b of Fig. 4 and compared with the ^{35}S-labeled polypeptides shown in track a. A major amount of ^{3}H appears in p23,

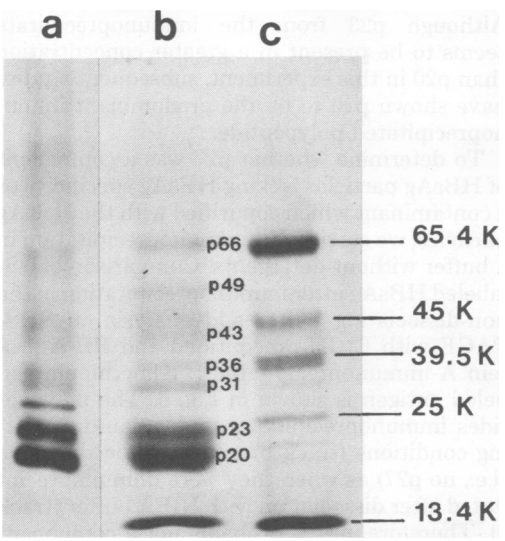

FIG. 2. *Comparison of the polypeptides of HBsAg particles from PLC/PRF/5 cells and from human serum. HBsAg particles from serum and [^{35}S]methionine-labeled particles from PLC/PRF/5 cells were purified and precipitated with EtOH as described in the text, and the polypeptides of each were separated on an 8 to 20% SDS-polyacrylamide gradient gel. Electrophoresis was carried out for 16 h at 40 V. (Track a) Polypeptides of ^{35}S-labeled HBsAg particles (8,000 cpm) after fluorography for 3 weeks; (track b) polypeptides of serum HBsAg particles stained with Coomassie brilliant blue; (track c) marker proteins run in the same gel and stained with Coomassie brilliant blue were bovine serum albumin (65.4K), ovalbumin (45K), aldolase (39.5K), chymotrypsinogen A (25K), and cytochrome c (13.4K).*

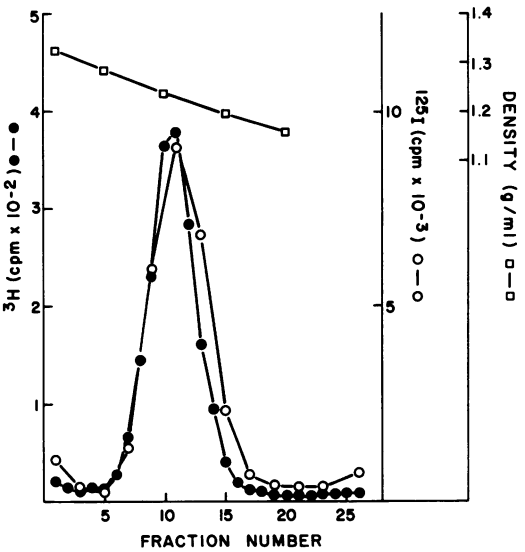

FIG. 3. *Final isopycnic banding of HBsAg particles labeled with L-[6-^{3}H]fucose and D-[6-^{3}H]glucosamine. The particles were radiolabeled and partially purified as described in the text. Fractions collected from the final CsCl density gradient were analyzed for radioactivity and HBsAg as described in the legend to Fig. 1B, except that the ^{3}H content of 10 μl of each fraction was measured.*

and none is detected in p20. Although not visible in Fig. 4, radioactivity is also present in p31 and p36, indicating that the pattern of glycosylation in the PLC/PRF/5 HBsAg polypeptides is similar to that of HBsAg from patient serum.

Immunospecificity of labeled polypeptides of PLC/PRF/5 HBsAg. To determine which ^{35}S-labeled polypeptides have HBsAg determinants, we dissociated ^{35}S-labeled polypeptides of purified particles with RIPA buffer and immunoprecipitated them with a guinea pig serum containing anti-HBs/adw and protein A. Forty to fifty percent of the radioactivity in various particle preparations was precipitated and recovered by this method, compared with 50 to 70% recovery by EtOH precipitation. The SDS-polyacrylamide gel pattern of polypeptides precipitated by HBsAg-specific antiserum is shown in Fig. 5, track b, and compared with the same amount of EtOH-precipitated HBsAg from the same preparation in track a. The high background along the track of EtOH-precipitated polypeptides is not present with the immunoprecipitated material, and only p20, p23, p31, p36, and p43 are detected in similar proportions to the polypeptides of serum HBsAg. The third major polypeptide found in EtOH-precipitated HBsAg, p27, was not detected, and there are no visible bands corresponding to the serum-derived HBsAg components at 49, 66, and 97K.

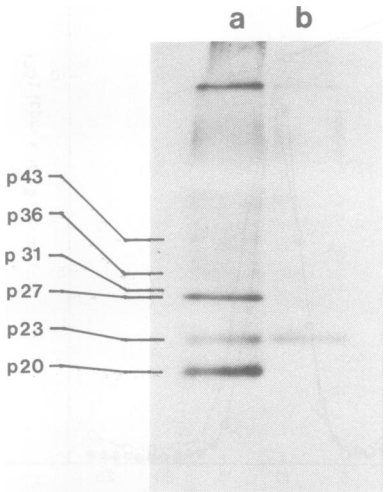

FIG. 4. *Pattern of glycosylation of the polypeptides of HBsAg particles produced by PLC/PRF/5 cells. HBsAg particles labeled with L-[^3H]fucose and D-[^3H]glucosamine (track b) and [^{35}S]methionine-labeled particles (track a) were purified, EtOH precipitated, and analyzed by SDS-PAGE (13% gel, 19 mA for 5 h) as described in the text. Both samples contained 12,000 cpm, and the fluorograms were exposed for 3 weeks.*

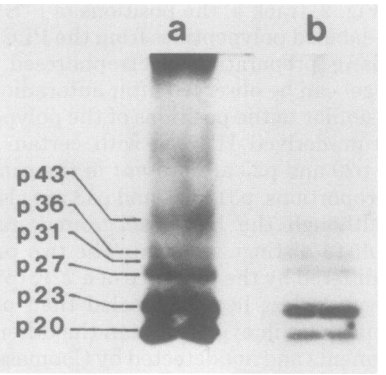

FIG. 5. *Immunoprecipitation of ^{35}S-labeled HBsAg polypeptides with guinea pig anti-HBs/adw. Purified HBsAg particles labeled with [^{35}S]methionine were divided into two parts. The first part (track a) was concentrated by precipitation with EtOH, and the second (track b) was precipitated in RIPA buffer with the anti-HBs and protein A as described in the text. The polypeptides were analyzed by SDS-PAGE as described in the legend to Fig. 4.*

Although p23 from the immunoprecipitate seems to be present in a greater concentration than p20 in this experiment, subsequent studies have shown p20 to be the predominant immunoprecipitated polypeptide.

To determine whether p27 was a component of HBsAg particles lacking HBsAg specificity or a contaminant which copurified with the HBsAg particles, we carried out immunoprecipitation in a buffer without detergents. Comparison of ^{35}S-labeled HBsAg after immunoprecipitation in the non-dissociating buffer and analysis on SDS-PAGE with EtOH-precipitated and RIPA-protein A-immunoprecipitated [^{35}S]methionine-labeled antigen is shown in Fig. 6. The polypeptides immunoprecipitated under non-dissociating conditions (track b) appear to be the same (i.e., no p27) as when they were immunoprecipitated after dissociation with RIPA buffer (track c). Therefore, p27 is probably not a component of HBsAg particles, but a contaminant of the purified HBsAg preparations.

To determine whether p27 or any of the other polypeptides contained HBcAg or HBeAg specificities, we incubated equal amounts of purified [^{35}S]methionine-labeled HBsAg particles with purified human immunoglobulin G containing anti-HBc and with human serum containing anti-HBe. The latter serum also contained anti-HBs. After precipitation with protein A, the polypeptides were compared by SDS-PAGE with polypeptides from equal amounts of the same particle preparation precipitated, respectively, with EtOH, anti-HBs (Fig. 6, tracks a, b, and c), and normal human serum (track f). It

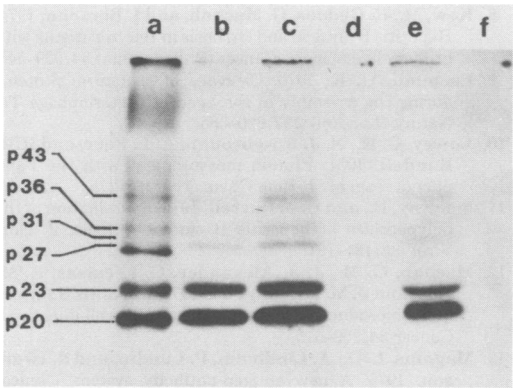

FIG. 6. *Immunoprecipitation of ^{35}S-labeled HBsAg with anti-HBs, anti-HBc, anti-HBe, and normal human serum. Purified and ^{35}S-labeled HBsAg particles were divided into six equal fractions which were precipitated with the following: (track a) EtOH, (track b) anti-HBs/adw serum and protein A in a non-dissociating buffer, (track c) anti-HBs/adw with protein A in a dissociating buffer (RIPA), (track d) anti-HBc and protein A in RIPA buffer, (track e) anti-HBe plus anti-HBs with protein A in RIPA buffer, and (track f) normal human serum and protein A in RIPA buffer. Each was then centrifuged, and the pellet was analyzed by SDS-PAGE as described in the text and in the legend to Fig. 4.*

can be seen that no discernible polypeptide was precipitated with anti-HBc (track d) or normal human serum (track f), and only the polypeptides precipitated by anti-HBs were precipitated by the serum with anti-HBe and anti-HBs (track e). Thus, there was no evidence for HBeAg- or HBcAg-reactive polypeptides in the purified HBsAg particle preparations in these experiments.

We did not find any other possible gene products of HBV associated with the PLC/PRF/5 cells. No HBcAg was detectable by radioimmunoassay of supernatant fluids concentrated 150-fold by ultracentrifugation or of cells disrupted by freeze-thawing at a concentration of 2×10^7 cells per ml. No HBeAg was found in the same materials with an enzyme-linked immunosorbent assay. No DNA polymerase activity was measurable in the concentrated supernatant fluids.

DISCUSSION

We have radiolabeled and purified the HBsAg particles produced by the PLC/PRF/5 cell line derived from a human hepatoma and compared the polypeptides with those found in particles from human serum. The major [^{35}S]methionine-labeled polypeptides (p20 and p23) and three of the minor polypeptides (p31, p36, and p43) were immunoprecipitated with anti-HBs and were indistinguishable in position and relative amount from polypeptides in serum-derived particles detected by Coomassie brilliant blue staining after SDS-PAGE. However, the higher-molecular-weight minor polypeptides of serum HBsAg, which migrate at 49, 66, and 97K in our system, were not detected in the PLC/PRF/5-produced HBsAg. One of the HBsAg polypeptides, p66, has been found by others to comigrate with human albumin, a protein found in preparations of HBsAg particles purified from serum (20). p66 has been the component varying the most in amount in different preparations (20). Its absence in the hepatoma HBsAg raises the possibility that p66 has actually represented only human albumin in HBsAg preparations from serum in previous studies and that the HBsAg reactivity of p66 found in some experiments was only due to a trailing of lower-molecular-weight antigen along the gel track. p27, a component found in purified HBsAg preparations from PLC/PRF/5 cells and not in HBsAg from serum, appeared to be a contaminant, since it failed to precipitate when intact HBsAg particles were immunoprecipitated with anti-HBs. Thus, it did not react with anti-HBs and was not a component of HBsAg particles.

The presence of five separate ^{35}S-labeled polypeptides in an immunoprecipitate of purified particles, using anti-HBs-containing serum and a dissociating buffer, indicates that multiple HBsAg-reactive polypeptides are found in HBsAg particles from hepatoma cells, as well as in serum particles. This multiplicity is probably not due to degradation by serum proteases (11), unless such proteases are also present in these PLC/PRF/5 cells.

Peterson et al. (15) have proposed that the two main polypeptides in the serum antigen particles (our p20 and p23) differ only by the carbohydrate moiety found in p23. This post-translational modification of a polypeptide with HBsAg reactivity can account for the presence of at least these two components of different apparent molecular weight and similar antigenicity. Consistent with this concept are our findings that the hepatoma cells incorporate ^{3}H-labeled sugars into p23 and not p20 and that both are precipitated by anti-HBs. As the relative proportions of p20 and p23 appear to be the same in HBsAg particles from both PLC/PRF/5 cells and serum, these two components in this ratio may be a necessary feature of the particle structure. How the remaining three minor components (p31, p36, and p43) may be related to p20, to p23, and to each other remains to be determined. Since others have found similar antigenic specificity in several polypeptides isolated from HBsAg particles (4, 19), different polypeptides must share some amino acid se-

quences, and one or more of the larger polypeptides may be uncleaved precursors of the smaller ones.

Although a large proportion of HBV (Dane particle) DNA base sequences appears to be present in PLC/PRF/5 cells (unpublished observations), only HBsAg, and not other probable HBV gene products, e.g., HBcAg, HBeAg, or Dane particles, has been found in the cells.

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