JOURNAL OF VIROLOGY, Feb. 1985, p. 543–551
0022-538X/85/020543-09$02.00/0

Vol. 53, No. 2

HBc and HBe Antigenicity and DNA-Binding Activity of Major Core Protein P22 in Hepatitis B Virus Core Particles Isolated from the Cytoplasm of Human Liver Cells

MARIE-ANNE PETIT[1]* AND JACQUES PILLOT[1,2]

Institut National de la Santé et de la Recherche Médicale U131, 92140 Clamart,[1] and Unité d'Immunologie Microbienne, Institut Pasteur, 75724 Paris Cedex 15,[2] France

Received 14 August 1984/Accepted 16 October 1984

Highly purified hepatitis B virus core particles were obtained in large amounts from the cytoplasm of infected human liver cells. This DNA polymerase-negative core preparation had only hepatitis B core antigen-specific antigenicity and showed a surprising stability. Two forms of a single protein of 22,000 molecular weight, P22, were resolved electrophoretically; the slower moving species, P22a, appeared to be a reduced form of the protein, and the faster moving species, P22b, could have represented a conformational isomer containing an intramolecular disulfide bond(s). The immunological properties and DNA-binding activity of the reduced form, P22a, were examined following separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by transfer onto nitrocellulose membranes (Western blotting). We found that the hepatitis B virus *C* gene protein shared the antigenic site responsible for both hepatitis B core and e antigen reactivity. We also demonstrated that the core protein(s) bound specifically the genomic hepatitis B virus DNA in comparison with a plasmid DNA (pBR322). This last observation was further substantiated by a radioimmunological method. P22a was also found to be phosphorylated in vitro by the endogenous protein kinase activity, copurified with the hepatitis B core antigen particles. These findings suggest that P22 is a multifunctional protein which is incorporated into core particles within the cytoplasm of the host cell before DNA encapsidation. A critical role of this protein in hepatitis B virus assembly is suggested.

Hepatitis B virus (HBV) is a 42-nm particle (the Dane particle) consisting of an internal 27-nm nucleocapsid bearing the hepatitis B core (HBc) antigen (HBcAg) surrounded by an antigenically and morphologically distinct lipoprotein surface (HBs) component (HBsAg) (9). In addition, a third HBV-specific antigen, hepatitis B e (HBe) antigen (HBeAg) has been shown to be a cryptic form of core antigen (6). Viral core particles and their major antigen (HBcAg) can be extracted from HBsAg-positive sera containing Dane particles by detergent treatment (3, 9). They are present in the hepatocytes of chronically infected patients (3, 13). A protein of 19,000 molecular weight, P19, has been found to be their major component (14). The complete HBV is a heavy subpopulation of Dane particles containing viral DNA (25) and an endogenous DNA-dependent DNA polymerase (16). Most Dane particles are believed to lack DNA as a DNA-polymerase activity and thus represent defective HBV. So two subfractions of core particles can be isolated either from serum HBV or from liver cells, which have been found to have densities of 1.36 g/ml for the DNA-containing full capsids and 1.30 g/ml for the empty capsids (15).

Recently, a protein kinase (PK) activity was detected in purified preparations of HBV core particles; this enzyme phosphorylates the major core protein in vitro (2). Gerlich et al. (11) have shown that PK is a part of the viral core particles and phosphorylates one or several serine residues of the major core protein per particle at a site sterically protected against enzymatic hydrolysis.

The gene coding for P19, the major protein of the viral core, has been identified on the HBV DNA (21). It produces a polypeptide chain composed of 183 amino acid residues, with a calculated molecular weight of 21,042. The C-terminal region of this polypeptide is remarkable because it manifests a protamine-like structure with repeating arginines separated by serine and proline, suggesting its involvement in binding to the HBV-DNA within the Dane particles. The DNA-binding properties of HBcAg are as yet unknown.

Up to now the antigenicity of HBcAg has not been detected on the two polypeptides P19 and P45 derived from the core of Dane particles by the conventional in vitro assay methods (27, 28). However, an antibody response against HBcAg has been obtained in addition to the antibody against HBeAg after immunization with P19 (28). Furthermore, recently, Takahashi et al. (29) have found HBcAg epitopes on HBeAg polypeptides P15.5 and P19 from cores of Dane particles, by using monoclonal antibodies.

In this study, the Western blotting analysis was used to identify with immunological procedures the core protein(s) electrophoretically separated in gels and to detect the DNA-binding protein(s) of HBV. We found that the more fully reduced and unfolded form of a single core protein, P22, shared HBcAg and HBeAg determinants and bound efficiently the HBV DNA. We provide further evidence that intact empty virus capsids composed of a simple protein (P22) could be isolated from the cytoplasm of hepatocytes in which nuclear HBcAg was detected by immunofluorescence. Moreover, we demonstrate that such core particles had a high HBcAg activity and were stable over a long period of time, in contrast to the instability of the HBcAg obtained from the nucleus.

MATERIALS AND METHODS

Purification procedure of core particles from liver. An HBV-infected human liver obtained from a patient on dialysis and screened for the presence of HBcAg in nuclei by immunofluorescence was the starting material for the purification pro-

* Corresponding author.

cedure. Frozen liver tissue (20 g) was minced and then homogenized in an Ultra turrax homogenizer at low speed with 4 volumes of chilled buffer containing 0.25 M sucrose in TKM (0.05 M Tris-hydrochloride [pH 7.6], 0.025 M KCl, and 0.005 M $MgCl_2$) and two protease inhibitors, 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) and 100,000 U of Iniprol (Laboratoire Choay, Paris). The 20% (wt/vol) homogenate was filtered through four layers of gauze and centrifuged at 750 g for 10 min as previously described for the isolation of nuclei (4). The pellet was suspended in the same volume of the homogenizing medium by using a hand-operated Potter-Thomas homogenizer. The resulting supernatants were pooled and centrifuged again at 3,000 × *g* for 15 min.

Core particles were then purified by the method described by Onodera et al. (20) with minor modifications. The pooled supernatants obtained above were centrifuged at 30,000 rpm (160,000 × *g*) for 12 h in an MSE (Crawley, Sussex, England) swing-out rotor (6 by 14 ml) to condense the core particles. The resulting pellet was suspended in 1 volume of 0.14 M NaCl–0.01 M Tris-hydrochloride, pH 7.6 (TN buffer), and centrifuged at 8,000 × *g* for 30 min. The supernatant was then layered over a 30% (wt/vol) sucrose cushion in an MSE rotor. After 18 h at 27,000 rpm (130,000 × g), the core-rich pellet was suspended in a 0.25 volume of TN buffer. Further purification was carried out by gel filtration from this material containing 2 to 3 mg of protein per ml and a very high titer of HBcAg ($1:2^{11}$ in solid-phase [SP]-radioimmunoassay [RIA]). A 2-ml sample (ca. 5 mg of protein) was passed through a column (100 by 1.6 cm) of Bio-Gel A 0.5-m (200 to 400 mesh), with 4 to 5 ml of TN buffer per h. Absorbancy profiles were determined by measurements at 280 nm on a spectrophotometer (ISCO UA-5, Roucaire, France). The fractions were assayed for HBcAg by SP-RIA. The HBcAg-positive peak fractions were pooled and stored in samples at −80°C. The specific activity of HBcAg obtained was $1:2^{17}$ SP-RIA titers per mg of protein.

Human polyclonal anti-HBc. Human anti-HBc immunoglobulin G (IgG) was obtained from HBeAg-positive sera of asymptomatic HBsAg carriers highly positive for anti-HBc (RIA titer, $1:10^5$) and negative for anti-HBe in RIA (CORAB and Abbott-HBe, Abbott Laboratories, North Chicago, Ill.). The IgG fraction was separated by gel filtration on Sephadex G200 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and by chromatography on DEAE Trisacryl M (Industrie Biologique Française, Clichy, France). The anti-HBc reagent (10 mg of protein per ml for an RIA titer of $1:10^5$) was negative for HBsAg, HBeAg, anti-HBs, and anti-HBe by commercial RIA kits from Abbott Laboratories (AUSRIA II, AUSAB, and Abbott-HBe). For the determination of anti-HBe activity in the Abbott-HBe test, we analyzed the neutralizing reagent, which was found to be reactive for HBeAg and HBsAg and also to be highly positive for anti-HBc. Consequently, we concluded that the HBe reagent was an infectious human serum negative for HBcAg as confirmed by the selective detection of HBcAg activity in this reagent by our RIA method as described below.

Sandwich SP-RIA of HBcAg. Core particles isolated from the liver preparation were tested for HBcAg activity by a sandwich SP-RIA developed in our laboratory for the specific detection of HBcAg in serum HBV particles (M.-A. Petit, F. Capel, A. Budkowska, and J. Pillot, submitted for publication). Briefly, polycarbonate Removawell Strips (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with human anti-HBc IgG at a concentration of 50 μg of protein per ml. The plate was then saturated with 2% bovine serum albumin. The test sample was incubated for 18 h at room temperature, and the bound antigen was detected by the same anti-HBc IgG labeled with ^{125}I to a specific activity of 0.5 μCi/μg by the modified procedure of Fraker and Speck (10). The plate was further incubated, and the radioactivity bound to each well was counted. Results were expressed as the ratio of the counts per minute of the test sample (P) to the average counts per minute of five negative controls (N); a test sample was considered positive if the P/N ratio was ≥2.1.

Selective determination of HBeAg. HBeAg was measured by a modified method with the Abbott-HBe. Because the antiserum used in this commercial test contains anti-HBe as well as anti-HBc, titers of both HBeAg and HBcAg were assayed by the kit. Therefore, to determine specifically HBeAg in the core preparation, it was necessary to add an excess of human anti-HBc IgG (prepared as described above) to the test sample to neutralize the HBcAg activity. The blocking was successful since the test sample became negative for HBcAg as measured by our selective method described above. Positive control for Abbott-HBe, which is reactive for HBeAg and HBsAg and negative for HBcAg (highly positive for anti-HBc), was found to be unaffected by such a neutralizing procedure.

Polyacrylamide gel electrophoresis and transfer to nitrocellulose sheets. Core particles were solubilized by incubation for 10 min at 60°C in 0.0125 M Tris-hydrochloride–0.1% sodium dodecyl sulfate (SDS), pH 6.8, containing 2% SDS with or without 5% 2-mercaptoethanol (2-ME), 10% glycerol, and 0.02% bromophenol blue (sample buffer). SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on 12.5% separating slab gels in the discontinuous system of Laemmli (17), with a ratio of acrylamide to bisacrylamide of 37:1. As molecular weight markers, a set of proteins (Pharmacia) with the following molecular weights was used: 14,400, 20,100, 30,000, 43,000, 67,000, and 94,000.

After electrophoresis, the gel was cut into two pieces. One part was stained with Coomassie brilliant blue (R-250) in a methanol-acid acetic mixture (31), whereas the other part was submitted to a transfer procedure adapted by Burnette (8) from Towbin et al. (30). The electrophoretic transfer of proteins to nitrocellulose membranes (NCM; BA85 membrane filters, 0.45 μm, Schleicher & Schuell, Dassel, Federal Republic of Germany) was done as previously described (23).

Immunoreactivity of core polypeptides. For determination of the immunoreactivity of proteins bound to NCM, the NCM were incubated either with ^{125}I-labeled human anti-HBc IgG, used for the SP-RIA and negative for anti-HBe, at 37°C for 4 h or with anti-HBe-anti-HBc, from the Abbott-HBe test kit, at 45°C for 3 h. After extensive washings with TN buffer, the NCM were dried and subjected to autoradiography (23).

DNA binding. The DNA-binding activity was determined by the method described by Bowen et al. (5). After protein transfer, the NCM were immersed in standard binding buffer (0.01 M Tris-hydrochloride [pH 7.0], 0.001 M Na-EDTA, 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinyl pyrrolidone containing 0.05 M NaCl) overnight at 4°C to reduce background binding slightly. The NCM were then incubated either with a ^{32}P-labeled pBR322 plasmid or with the cloned ^{32}P-labeled HBV DNA for 1 h at room temperature to permit DNA binding. The DNA probes (supplied by C. Bréchot) were labeled by nick translation to a specific activity of 2×10^8 to 4×10^8 cpm/μg. After incubation, the NCM were extensively washed in standard

binding buffer. (If desired, the NCM can be washed in binding buffer containing a higher NaCl concentration [0.2 or 0.5 M].) After that, the NCM were dried and exposed to X-ray film. The protein bands detected by autoradiography were excised, and the radioactivity was counted in a Beta SL 30 counter (Kontron Analytical, Montigny-le-Bretonneux, France).

The DNA-binding activity was also detected by using a radioimmunological method. After solubilization in 2% SDS and 5% 2-ME, as described for SDS-PAGE, followed by extensive dialysis for 3 to 5 days against TN buffer, core particles were incubated with polycarbonate Removawell Strips (Dynatech) coated with either a pBR322 plasmid or the genomic HBV DNA at the same concentration of 1 ng/ml for 1 h at room temperature. The bound core protein was revealed by ^{125}I-labeled anti-HBe and anti-HBc (anti-HBe reagent from Abbott), and the radioactivity bound to each well was counted as described for the determination of HBcAg. A test control was performed by coating wells of Removawell Strips with TN buffer containing 2% bovine serum albumin. For detection of DNA binding, anti-HBe reagent rather than specific ^{125}I-labeled anti-HBc IgG was used to achieve maximal test sensitivity.

PK reaction. The PK reaction was carried out as described by Gerlich et al. (11) in 100 μl with 20 to 60 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham France S.A., Les Ulis) and increasing HBcAg concentrations from 7 to 60 μg of protein. After 1 h at 37°C, 10 μl of sample buffer containing 20% SDS and 50% 2-ME was added. The mixture was incubated at 60°C for 10 min and then analyzed by SDS-PAGE with a 12.5% polyacrylamide gel, as described above. After staining and drying of the gel (Slab Gel Dryer Unit, LKB Instruments, Inc., Bromma, Sweden), the phosphorylated protein was localized by autoradiography (23).

Other assays. DNA polymerase activity was analyzed as reported by Alberti et al. (1). Protein concentration was determined by the method of Peterson (22). Electron microscopy was performed, after phosphotungstic acid staining, with a Siemens microscope.

RESULTS

Physicochemical and morphological characteristics of cytoplasmic core particles (HBcAg) isolated from liver. HBc particles were isolated from the soluble fraction of a human liver homogenate after removal of nuclei by low-speed centrifugation (750 × *g*). They were further purified by centrifugation in TKM buffer and sucrose cushion followed by gel filtration (20). Contrary to core particles isolated from nuclear extracts, which showed a gradual loss ($t_{1/2}$, 22 days) of the HBc antigenicity when stored at 4°C (7), our purified core preparation had a high HBcAg activity specifically detected up to a dilution of $1:2^{11}$ in SP-RIA (Fig. 1). Our preparation was very stable when stored either at 0°C or frozen at −20 and −80°C. In fact, cytoplasmic core particles in liver revealed a notable morphological stability for over 1 year without any loss of HBc antigenicity or release of HBeAg.

Throughout the purification procedure, we did not use CsCl density gradient centrifugation, to avoid releasing HBeAg from core particles by affecting their morphology (19). When our HBcAg preparation was tested specifically for HBeAg activity, an RIA titer of $<1:2^5$ was found, corresponding to an HBeAg/HBcAg activity ratio of <0.02. HBV-infected cells expressing HBsAg in the cytoplasm were also assayed, and such activity was detected by RIA only in the core preparation without dilution, corresponding to an HBsAg/HBcAg activity ratio of <0.005.

The buoyant density of this material was determined by layering a 200-μl sample of fractions pooled from gel filtration onto a discontinuous CsCl gradient (1.1 to 1.5 g/ml) and by centrifugation in a TGA-65 ultracentrifuge with a TST 41 rotor (Kontron) for 18 h at 35,000 rpm (200,000 × *g*). A single peak of HBcAg activity was recovered from the gradient at a density of 1.30 g/ml (Fig. 2), indicating that the material consisted almost entirely of light core particles. These cores were negative for specific DNA polymerase activity (data not shown).

Electron-microscopic examination of the HBcAg preparation (Fig. 3) revealed the presence of spherical particles 27 nm in diameter.

These results indicate that the physicochemical and morphological properties of cytoplasmic HBcAg in liver are similar to those described earlier for cores obtained from the nuclei of hepatocytes (7, 19). The physical stability reported here, however, has never been observed before.

Immunological properties: HBcAg and HBeAg reactivity on the core proteins. The polypeptide composition of the cytoplasmic HBcAg was determined by SDS-PAGE after treatment at 60°C for 10 min in 2% SDS with or without 5% 2-ME. The sample prepared in SDS alone showed a pair of closely migrating bands in the region of polyacrylamide gel

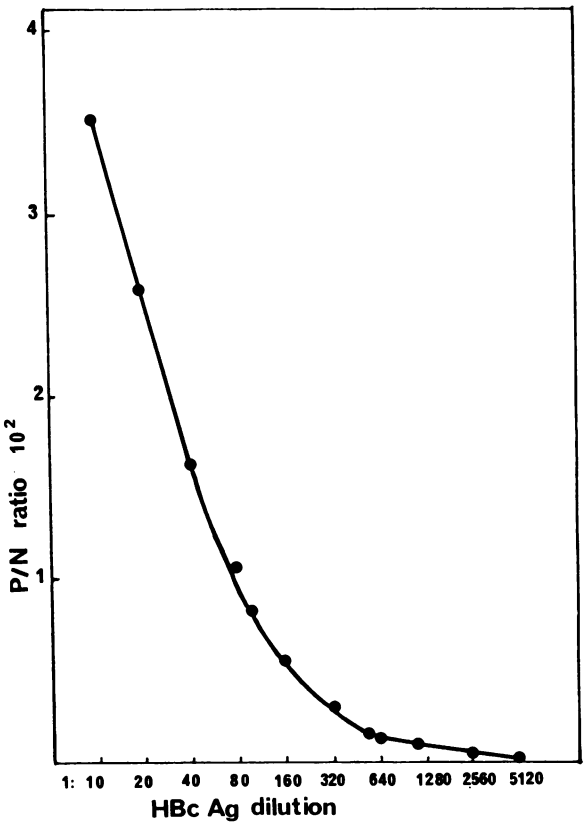

FIG. 1. Titration of HBcAg in purified core preparations derived from the supernatant of liver homogenate with an anti-HBc IgG-coated solid phase, HBcAg dilutions, and ^{125}I-labeled anti-HBc IgG (SP-RIA). The results are expressed as the ratio of the counts per minute of the test sample (P) to the average counts per minute of five negative controls (N); a test sample was considered positive if the P/N was ≥2.1.

corresponding to ca. 21,000 daltons, after Coomassie blue staining. The molecular weights of the two bands were determined to be 20,000 and 22,000 by comparison with the migration of proteins of known molecular weight (Fig. 4, lanes A and B). Because we believe these two bands to be two different forms of the major core protein P22, we named the slower migrating band P22a and the faster migrating band P22b. When the core particles were prepared in sample buffer containing 5% 2-ME, only the P22a form was observed (Fig. 4, lane C). These results argue in favor of a P22a form representing the more fully reduced form of P22, which could be converted by oxidation to the disulfide-bonded form (P22b) which migrated faster owing to a folded structure. Presumably, the two polypeptide species were present in the core preparation, and the faster moving species, P22b, was converted to the slower moving species, P22a, by high concentrations of reducing agent in the gel sample buffer. Moreover, we noticed that the major core proteins could be obtained by treatment with SDS alone, suggesting a possible noncovalent association between the P22 molecules.

The polypeptides separated electrophoretically were then transferred to nitrocellulose paper and incubated with specific ^{125}I anti-HBc IgG or ^{125}I IgG containing both anti-HBe and anti-HBc activities (anti-HBe reagent from Abbott) (Fig. 5). Thus, the slower moving species, P22a, largely present on gels after reduction, appeared as the shared antigenic site responsible for HBcAg and HBeAg reactivity. One clearly visible band corresponding to 22,000 daltons was detected on the autoradiographs after incubation with anti-HBc IgG (prepared by us) which did not contain any anti-HBe activity

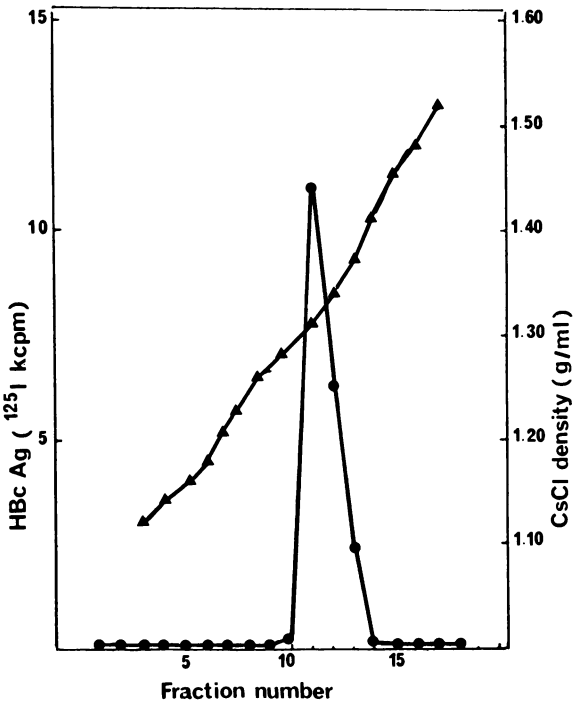

FIG. 2. Isopycnic banding of purified cytoplasmic core particles in CsCl. A 200-μl sample of core particle preparation (RIA titer of HBcAg, $1:2^{11}$) which had been purified by Bio-Gel A 0.5-m column chromatography was layered onto a discontinuous CsCl gradient (1.1 to 1.5 g/ml) and centrifuged in a TST 41 Kontron rotor for 18 h at 35,000 rpm. Fractions collected by bottom puncture were assayed for HBcAg activity (●) at a dilution of 1:4 by SP-RIA and for CsCl density (▲) by refractometry.

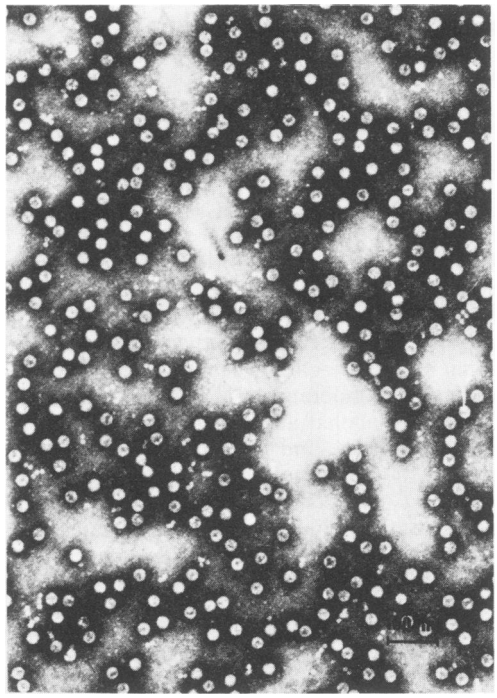

FIG. 3. Electron micrograph of core particles isolated from the cytoplasm of a human liver cells after purification by gel filtration (at a final concentration of 140 μg of protein per ml).

detectable in RIA (Fig. 5, lane A). This protein, P22a, also revealed immunoreactivity to anti-HBe after incubation with anti-HBe reagent, and a very weak band at 41,000 daltons (P41; not stained by Coomassie blue in gels and only suspected after anti-HBc reaction) was detected (Fig. 5, lane

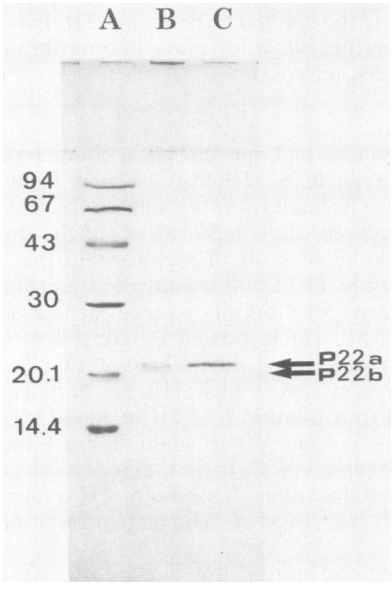

FIG. 4. SDS-polyacrylamide gel analysis of marker proteins (A) and of purified cytoplasmic core particles (RIA titer of HBcAg, $1:2^{11}$) which were dissolved in gel sample buffer containing 2% SDS in the absence (B) or presence (C) of 5% 2-ME and electrophoresed on a 12.5% polyacrylamide gel. The polypeptides were visualized by Coomassie blue staining. Molecular weights of marker proteins ($\times 10^3$) are on the left.

B). This last positive reaction was caused by anti-HBe and did not result from the presence of anti-HBc in the anti-HBe reagent. To examine this reaction, we performed triplicate blots which were all first incubated with anti-HBc IgG that was free of detectable anti-HBe activity, and we subsequently again incubated two of these blots either with anti-HBc IgG (as in the first assay) or with the Abbott anti-HBe reagent. An intensified labeling of bands compared with those in the control blot, incubated once, was observed only after a second incubation in the presence of anti-HBe antibodies, in contrast to observations after successive incubations with anti-HBc IgG alone (data not shown). This result suggests an effective blocking of the HBcAg determinant(s) and a further detection of the HBeAg determinant(s).

These results indicate that the more fully reduced form of the core protein, P22a, released from empty HBV capsids, could react with both anti-HBc and anti-HBe IgG under our experimental conditions. The immunological properties of the possible conformational isomer containing an intramolecular disulfide bond(s) (P22b) could not be detected due to an insufficient protein concentration in the gels.

PK activity. Cytoplasmic HBcAg particles from HBV-infected liver cells were examined for endogenous PK activity. After PK reaction in the presence of [γ-^{32}P]ATP, radiolabeled polypeptides were identified by SDS-PAGE under reducing conditions and subsequent autoradiography. The only phosphorylated product had an electrophoretic mobility corresponding to an apparent molecular weight of 22,000 (P22a) (Fig. 6). The amount of ^{32}P bound to P22a followed quantitatively the core protein concentration layered on the polyacrylamide gel (7 and 14 μg/ml [Fig. 6, lanes a and c, respectively]). For the same HBcAg concentration (7 or 14 μg of protein per ml), doubling the concentration of [γ-^{32}P]ATP (3,000 Ci/mmol) in the PK reaction mixture (13 instead of 7 pmol) produced a doubling of the band intensity (compare Fig. 6, lanes a and c with lanes b and d, respectively) only up to 20 pmol. This supports the results obtained by Gerlich et al. (11) and indicates a strong endogenous PK activity associated with DNA polymerase-negative light core particles isolated from hepatocyte cytoplasm.

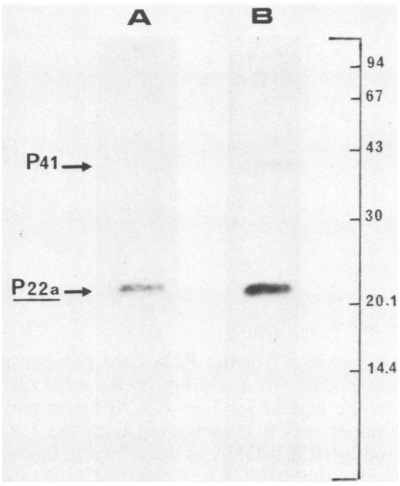

FIG. 5. Immunoreactivity of core proteins electrophoretically separated in gels under reducing conditions (as described in the legend to Fig. 4C) after transfer to nitrocellulose paper (Western blotting). (A) Incubation with specific ^{125}I-labeled anti-HBc IgG (prepared as described in the text). (B) Incubation with ^{125}I-labeled anti-HBe and anti-HBc IgG (Abbott reagent). Autoradiographs were exposed for 7 h. Molecular weights of marker proteins ($\times 10^3$) are on the right. Core polypeptides are indicated by arrows on the left.

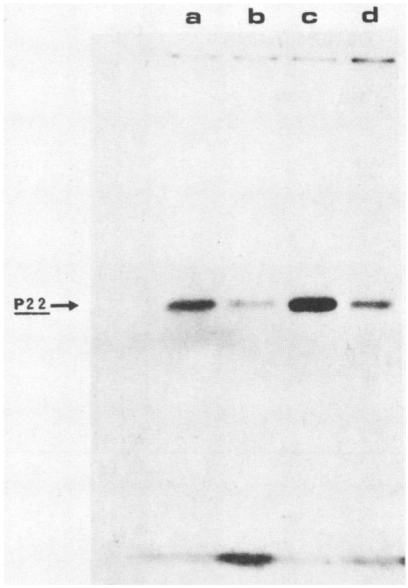

FIG. 6. SDS-PAGE of PK ^{32}P-labeled core polypeptides. Cytoplasmic core particles were purified, and PK reactions were carried out as described in the next. Radioactive polypeptides were analyzed by SDS-PAGE under reducing conditions and detected by autoradiography of the dried gel. Core proteins (7 μg [lanes a and b] or 14 μg [lanes c and d] per ml) were layered on the gel. There was 7 pmol (lanes b and d) or 13 pmol (lanes a and c) of [γ-^{32}P]ATP, present in the PK reaction mixture.

Demonstration of a specific binding of the core proteins to the HBV DNA. We first tested the ability of the core proteins to bind viral and nonviral DNA by using the protein blotting method, which combines the high resolving power of gel electrophoresis with a nitrocellulose filter binding assay, requiring small amounts of the labeled probe. For this assay, after protein transfer, the NCM were incubated with DNA probes, either ^{32}P-labeled pBR322 plasmid (Fig. 7A) or cloned ^{32}P-labeled HBV DNA (Fig. 7B), in the same concentration of 1 ng per ml to permit DNA binding. After washing in standard binding buffer containing 0.05 M NaCl, the 22,000-dalton major core polypeptide, P22a, efficiently bound the HBV DNA. It appeared that P22a was also able to bind plasmid DNA but that such a nonspecific binding represented only ca. 40% of the specific binding activity of P22a for HBV DNA (Fig. 7C). Consequently, we propose that P22a is a DNA-binding protein of HBV. We determined the stability of this HBV DNA protein complex in buffers of increasing salt concentrations (0.05 to 0.5 M NaCl). NaCl (0.2 M) removed the majority (ca. 95%) of HBV DNA from core protein P22a (Fig. 8A). After extensive washings in buffer containing 0.2 or 0.5 M NaCl, bands were, however, still visualized in autoradiography after a longer exposure time (24 h instead of 3.5 h in 0.05 M binding buffer [Fig. 8B]) in contrast to nonspecific interactions with plasmid DNA. The filters were extensively washed to increase the degree of specificity, and we noticed that no binding was observed for the marker proteins (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin) analyzed in parallel by the same technique (Fig. 8C).

By this method, which required highly purified proteins as our core preparation, it was demonstrated that P22a bound

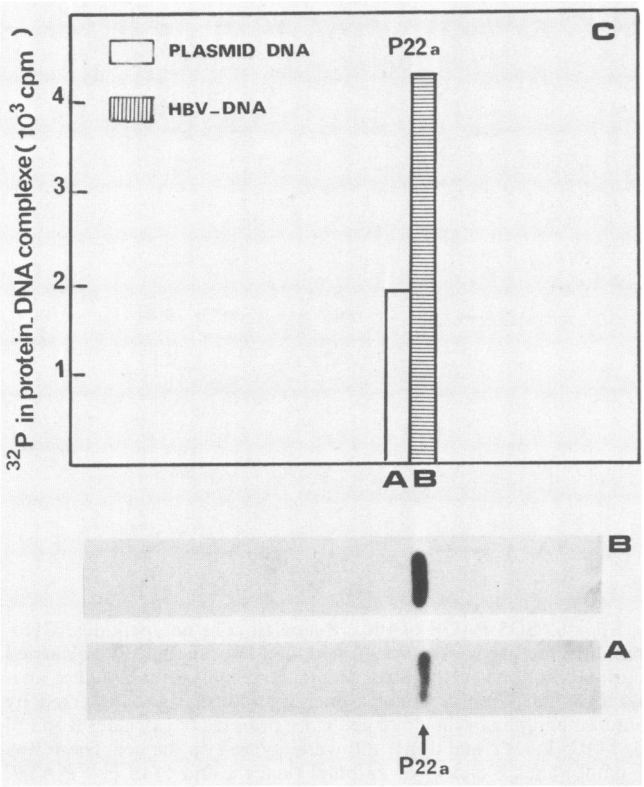

FIG. 7. Specific binding of the P22a core polypeptide to HBV DNA as determined by filter binding assay. Core particles (RIA titer of HBcAg, 1:2^{11}) were treated with 2% SDS–5% 2-ME (as described in the legend for Fig. 4C) before SDS-PAGE. After protein transfer, the nitrocellulose paper was incubated either (A) with a ^{32}P-labeled pBR322 plasmid or (B) with the ^{32}P-labeled HBV DNA. (C) The radioactive band which migrated like a protein of 22 kilodaltons (as determined by appropriate marker proteins; cf. Fig. 4A) on autoradiograms (A) and (B) was excised from the nitrocellulose paper, dissolved in Dynagel (Baker), and counted in a scintillation counter. After incubation with ^{32}P-labeled DNA probes, the filters were washed in binding buffer containing 0.05 M NaCl. Autoradiographs were exposed for 3.5 h.

the HBV DNA with a greater affinity that it bound a plasmid DNA. Therefore, to further explore this observation, we developed a DNA-binding immunoassay. With this second method, protein-DNA complexes were identified by reaction with ^{125}I-labeled anti-HBe-anti-HBc reagent (Abbott). The results obtained by both procedures, filter binding and immunoprecipitation, were fully compatible. HBcAg, after treatment with 2% SDS and 5% 2-ME at 60°C for 10 min (which resulted in only a single 22,000 polypeptide immunoreactive to anti-HBc and anti-HBe, P22a), bound the HBV DNA with a high specificity in contrast to its binding of pBR322 plasmid DNA (Fig. 9). In this experiment, we used TN buffer containing 0.14 M NaCl, and we observed that the nonspecific activity of P22a for plasmid DNA was insignificant at this salt concentration compared with its specific activity for the HBV DNA (Fig. 9).

All of these results indicate that the reduced form, P22a, is a DNA-binding protein of HBV. Furthermore, we showed that both forms of the core protein were resolved in SDS-PAGE without reduction. P22a, P22b, and P41 (probably a dimeric form of P22) were detected on the autoradiographs after the filter DNA-binding assay (Fig. 10). Certainly P22b was not clearly resolved from P22a because they migrated very close to each other. A broad band from 20,000 to 22,000 daltons was, however, stained under these conditions, whereas the staining was closely localized at 22,000 daltons as a limited band after resolution in SDS-PAGE with reduction (Fig. 10 compared with Fig. 7). Consequently, we believe that all of these forms bound specifically the HBV DNA. Such a finding suggests that these polypeptides had identical C-terminal amino acid sequences where the DNA-binding site was probably localized (11). It also indicates that P22 was a polypeptide monomer which exhibited different conformational states within core particles, and all of the polypeptides represented the DNA-binding proteins of HBV.

DISCUSSION

DNA polymerase-negative light core particles were isolated from the cytoplasm of HBV-infected hepatocytes after

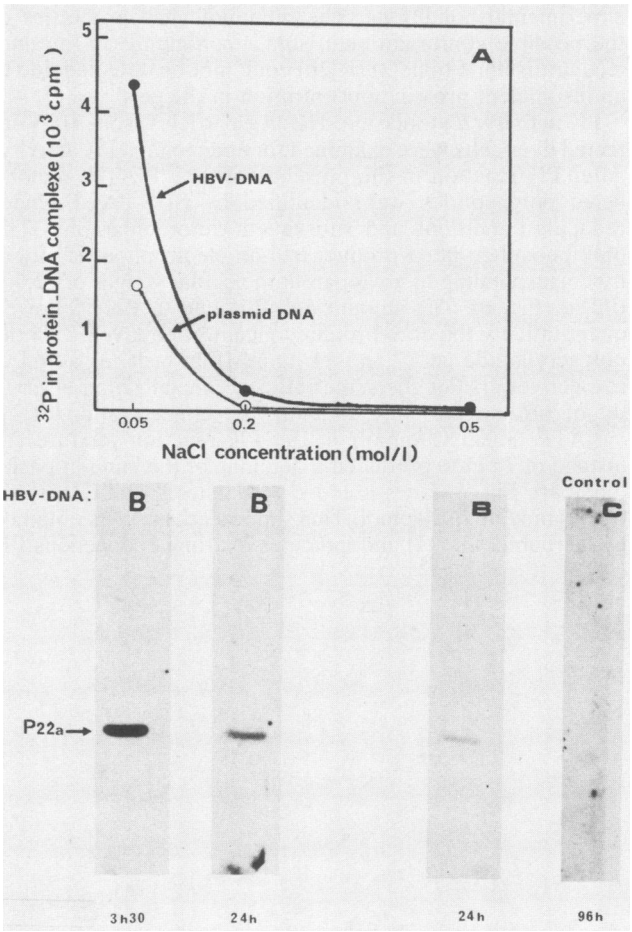

FIG. 8. Salt sensitivity of the P22a core polypeptide binding to specific and nonspecific DNA. Core proteins were applied to the gel as described in the legend for Fig. 4C. After protein transfer, the nitrocellulose paper was first incubated with the ^{32}P-labeled HBV DNA or plasmid (pBR322) DNA as described in the legend to Fig. 7 and then washed with 0.05, 0.2, or 0.5 M NaCl. (A) Radioactivity in the protein-HBV DNA complex (●) or in the protein-plasmid DNA complex (○) measured as described in the legend to Fig. 7. (B) Autoradiograms made from filters washed in binding buffer containing increasing salt concentrations for the protein-HBV DNA complex. (C) Control (marker) proteins applied to the gel as described in the legend for Fig. 4A. After protein transfer and incubation with the ^{32}P-labeled HBV DNA, an autoradiogram was made from the filter washed in binding buffer containing 0.05 M NaCl. Exposure times for autoradiograms are below the filters.

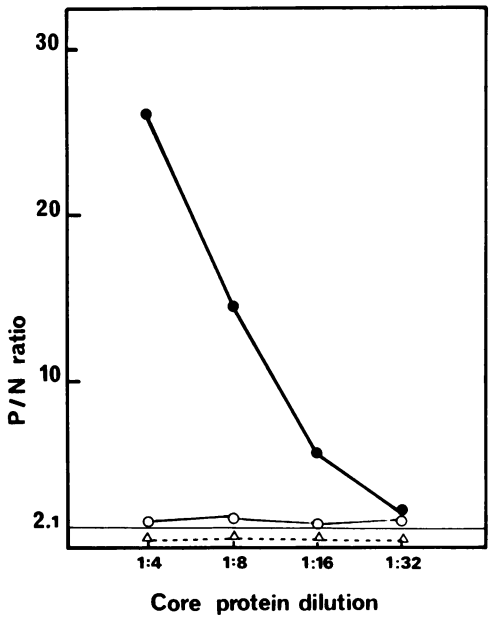

FIG. 9. Immunological assay for DNA binding of the P22a core polypeptide. Purified core particles (200 μg of protein per ml) treated with 2% SDS–5% 2-ME for 10 min at 60°C and extensively dialyzed were incubated in wells coated either with TN buffer containing 2% bovine serum albumin as a control (△) or with plasmid pBR322 (○), or with the genomic HBV DNA (●). Bound protein was subsequently identified by the immunoprecipitation technique, carried out as described in the text, with ^{125}I-labeled anti-HBe reagent (Abbott). We present the ratio (P/N) of the counts per minute obtained for the test sample (P) to the average counts per minute of three negative controls (N) as a function of core protein dilution. Samples were considered positive when the P/N ratio was ≥2.1.

removal of the nuclei by low-speed centrifugation and were further purified by gel filtration rather than by conventional CsCl banding. This confirms the presence of large amounts of cytoplasmic HBcAg as empty particles in liver (12, 26). Unlike core particles isolated from nuclear extracts of liver cells (7), HBcAg activity associated with such cytoplasmic particles shows a surprising stability when stored at −80°C or at 0°C. Therefore, it was noteworthy that in the same liver homogenate, there was a complete loss of HBc antigenicity in nuclei preparations after the procedure for isolation of the nuclear protein matrix, whereas the HBe antigenicity was still present (data not shown). These findings suggest that excess HBcAg, stable both morphologically and immunologically, may be present in hepatocyte cytoplasm as empty capsids, although small amounts of nuclear HBcAg are readily degraded, thus releasing HBeAg activity. Such hepatocytes might represent cells undergoing HBV DNA replication, with or without mature virion formation (12). We conclude that core particles with high HBcAg activity are localized into the cytoplasm of the host cell as empty capsids showing a strong physical stability. Low HBcAg activity is only found in nuclear extracts where degradative processes seem to exist.

Our preparation of HBcAg isolated from hepatocyte cytoplasm had both morphological and isopycnic entities identical to those of light core particles extracted from nuclei (7). Its density in CsCl was 1.30 to 1.32 g/ml, and it consisted mainly of empty spherical particles 27 nm in diameter.

The PK activity associated with the cytoplasmic HBcAg reported in this study is similar to the PK activity found in cores from nuclei of infected liver cells reported previously (2, 11). A single polypeptide with a molecular weight of 22,000, P22, was obtained by SDS-PAGE under reducing conditions (18, 19). So far we have identified two protein bands which migrate near P22 when subjected to electrophoresis without reduction. It is conceivable that the relationship between the two forms of P22 could be precursor to product. We envision a conversion by oxidation of the reduced P22 molecule to a disulfide-bonded, folded form within the infected cell. The disulfide bonding (also between two P22 molecules leading to a dimeric form, P41) may be facilitated by the tertiary structure of the protein, which shows three cysteines (at positions 48, 61, and 107) in the first 140 residues of its amino-terminal portion (21). We suggest that this conformational change corresponds to a stage of maturation related to the function of the protein. However, further work is needed to determine the exact relationship between the two P22 polypeptide species.

Furthermore, by Western blotting, the P22 molecule was shown to bear a strong HBeAg activity, which is not recovered after it has been assembled as a core protein that displays only HBcAg determinants. Such a production of viral core antigen (HBcAg) was supported by HBeAg synthesis in HBcAg-negative mouse cells with active core antigen genes (24). We have also identified additional HBc

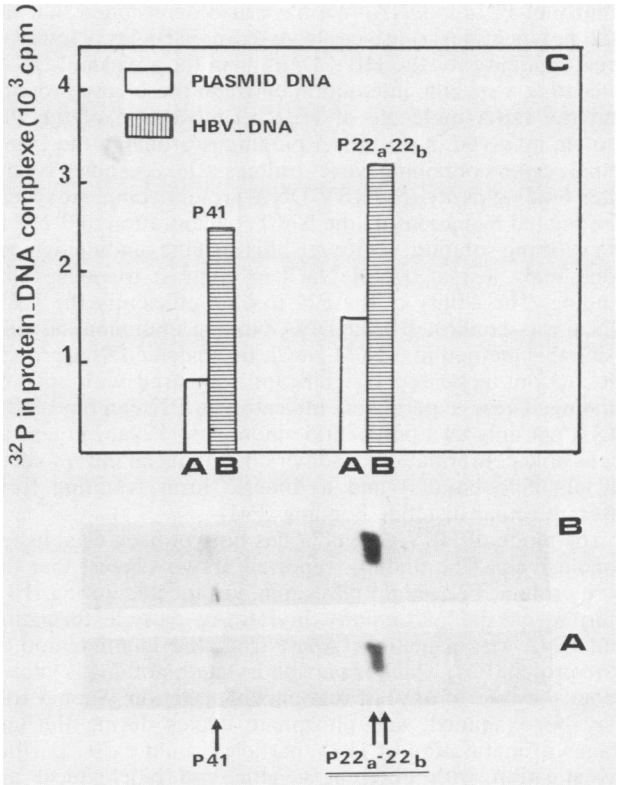

FIG. 10. Specific binding of different forms of the core polypeptide P22 to HBV DNA as determined by filter binding assay. Core particles were treated with 2% SDS alone (cf. Fig. 4B) before SDS-PAGE followed by protein transfer. The nitrocellulose paper was subsequently incubated either (A) with the ^{32}P-labeled plasmid DNA(pBR322) or (B) with the ^{32}P-labeled HBV DNA as described in the legend to Fig. 7. (C) Radioactivity in the protein-DNA complexes, measured as described in the legend to Fig. 7. Autoradiographs were exposed for 3.5 h.

antigenicity on the core particle-derived polypeptide of 22,000 daltons, P22, by a protein transfer method with selective anti-HBc IgG, which does not contain any detectable anti-HBe reactivity. This result seems to be a variance with data from Ohori et al. (18) which indicate that a single polypeptide with a molecular weight of 21,500 released from core particles is immunoreactive only to anti-HBeAg-1 and anti-HBeAg-2 but not to anti-HBcAg. We suppose that this apparent contradiction results from the experimental variations carried out by those authors upon the basic method of protein transfer (8, 30). In fact, the immunoreactivity of P22 molecules from core particles to anti-HBcAg could be preferentially lost during the Western blot because of a greater sensitivity to denaturation and subsequent renaturation of the core protein rather than the immunoreactivity to anti-HBeAg. Our finding can, however, explain the immunogenicity of HBcAg in an HBeAg polypeptide (P19) (27) and confirms the results recently obtained by Takahashi et al. (29), with monoclonal antibodies to P19. Moreover, we have observed that the HBcAg activity associated with the P22 molecules released from core particles can be detected not only by immunoautoradiography after electroblotting but also by using a double antibody specificity RIA with a solid-phase anti-HBc and labeled anti-HBe-anti-HBc (Petit et al., submitted for publication). This would suggest that there is an HBc-specific determinant(s) on HBeAg molecules.

In this paper, we demonstrate for the first time the binding affinity of P22 for DNA. We have also determined that the P22 polypeptides released from core particles showed a greater affinity for the HBV DNA than for a plasmid DNA, indicating a specific interaction between these core proteins and the DNA molecule of HBV. The portion of the P22 protein involved in this DNA binding is probably the C-terminal region containing basic residues (21). As shown by the filter binding assay (5), HBV DNA-protein complexes were dissociated by increasing the NaCl concentration to 0.2 M in the washing solution. However, this specific binding was not completely lost at 0.2 M NaCl in contrast to nonspecific binding. The ability of the P22 to bind efficiently the HBV DNA was confirmed in a DNA-binding immunoassay. By using this method in 0.14 M NaCl, nonspecific DNA-protein interactions appeared insignificant compared with specific binding. These experiments indicate that P22 can bind HBV DNA not only as a polypeptide monomer, P22a, but also as a possible conformational isomer containing an intramolecular disulfide bond(s) and a dimeric form resulting from intermolecular disulfide bonding (P41).

The mode of HBV assembly has not yet been established conclusively. The findings reported above suggest that the core protein, P22, might play an important role during HBV morphogenesis, particularly in HBcAg particle formation and DNA encapsidation. Apart from the identification of core protein P22, which is phosphorylated, nothing is known about the role, if any, of this phosphorylation. Such a role may be examined, and phosphate cycles during the late stages of maturation of HBV particles could exist. Further investigation with different genetic and biochemical approaches is required to characterize the DNA-binding activity of the HBV nucleocapsid antigen and to determine the role of post-translational modifications of this protein in the virus replication cycle.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Marie-José Briantais. We thank C. Bréchot and his colleagues (Pasteur Institute, Paris) for providing ^{32}P-labeled DNA probes and D. Plana (Instituto Malbran, Buenos Aires) for the autopsy liver. We also thank Dr. Chevance and M. Lesourd of the Pasteur Institute for electron micrographs, Giang-Tuyet Pham for typing the manuscript, and Z. Brahmi for reviewing the manuscript.

This work was supported by research grants from the Institut National de la Santé et de la Recherche Médicale.

LITERATURE CITED

1. **Alberti, A., P. Pontisso, and G. Realdi.** 1981. Changes in hapatitis B virus DNA-polymerase activity in patients with chronic infection. J. Med. Virol. **8**:223–229.
2. **Albin, C., and W. S. Robinson.** 1980. Protein kinase activity in hepatitis B virus. J. Virol. **34**:297–302.
3. **Barker, L. F., J. D. Almeida, J. H. Hoofnagle, R. J. Gerety, D. R. Jackson, and P. P. McGrath.** 1974. Hepatitis B core antigen: immunology and electron microscopy. J. Virol. **14**:1552–1558.
4. **Blobel, G., and V. R. Potter.** 1966. Nuclei from rat liver: isolation method that combines purity with high yield. Science **154**:1662–1665.
5. **Bowen, B., J. Steinberg, U. K. Laemmli, and H. Weintraub.** 1980. The detection of DNA-binding proteins by protein blotting. Nucleic Acids Res. **8**:1–12.
6. **Budkowska, A., B. Kalinowska, and A. Nowoslawski.** 1979. Identification of two HBeAg subspecificities revealed by chemical treatment and enzymatic digestion of liver derived HBcAg. J. Immunol. **123**:1415–1416.
7. **Budkowska, A., J. W.-K. Shih, and J. L. Gerin.** 1977. Immunochemistry and polypeptide composition of hepatitis B core antigen (HBcAg). J. Immunol. **118**:1300–1305.
8. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
9. **Dane, D. S., C. H. Cameron, and M. Briggs.** 1970. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. Lancet **i**:695–698.
10. **Fraker, P. G., and J. C. Speck.** 1978. Protein and cell membrane iodinations with sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a, 6a-diphenyl-glycouryl. Biochem. Biophys. Res. Commun. **80**:849–857.
11. **Gerlich, W. H., U. Goldmann, R. Müller, W. Stibbe, and W. Wolff.** 1982. Specificity and localization of the hepatitis B virus-associated protein kinase. J. Virol. **42**:761–766.
12. **Gowans, E. J., C. J. Burrell, A. R. Jilbert, and B. P. Marmion.** 1983. Patterns of single- and double-stranded hepatitis B virus DNA and viral antigen accumulation in infected liver cells. J. Gen. Virol. **64**:1229–1239.
13. **Hoofnagle, J. H., R. J. Gerety, and L. F. Barker.** 1973. Antibody to hepatitis B virus core in man. Lancet **ii**:869–873.
14. **Hruska, J. F., and W. S. Robinson.** 1977. The proteins of hepatitis B Dane particle cores. J. Med. Virol. **1**:119–131.
15. **Kaplan, P. M., E. C. Ford, R. H. Purcell, and J. L. Gerin.** 1976. Demonstration of subpopulations of Dane particles. J. Virol. **17**:885–893.
16. **Kaplan, P. M., R. L. Greenman, J. L. Gerin, R. H. Purcell, and W. S. Robinson.** 1973. DNA polymerase associated with human hepatitis B antigen. J. Virol. **12**:995–1005.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Ohori, H., N. Shimizu, E. Yamada, S. Onodera, and N. Ishida.** 1984. Immunological and morphological properties of HBeAg subtypes (HBeAg/1 and HBeAg/2) in hepatitis B virus core particles. J. Gen. Virol. **65**:405–414.
19. **Ohori, H., M. Yamaki, S. Onodera, E. Yamada, and N. Ishida.** 1980. Antigen conversion from HBcAg to HBeAg by degradation of hepatitis B core particles. Intervirology **13**:74–82.
20. **Onodera, S., H. Ohori, M. Yamaki, and N. Ishida.** 1982. Electron microscopy of human hepatitis B virus cores by

negative staining-carbon film technique. J. Med. Virol. **10**:147–155.

21. **Pasek, M., T. Goto, W. Gilbert, B. Zink, H. Schaller, P. Mac Kay, G. Leadbetter, and K. Murray.** 1979. Hepatitis B virus genes and their expression in *E. coli*. Nature (London) **282**:575–579.
22. **Peterson, G. L.** 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. **83**:346–356.
23. **Pillot, J., and M.-A. Petit.** 1983. Immunochemical structure of the hepatitis B surface antigen vaccine. I. Treatment of immobilized HBsAg by dissociation agents with or without enzymatic digestion and identification of polypeptides by protein blotting. Mol. Immunol. **21**:53–60.
24. **Price, P., S. Ostrove, C. Flordellis, M. A. Sells, S. Thung, M. Gerber, J. Christman, and G. Acs.** 1983. Characterization of RNA transcripts and virally coded proteins synthesized in mouse fibroblasts transfected with hepatitis B DNA: HBeAg synthesis in HBcAg-negative cells with active core antigen genes. Biosci. Rep. **3**:1017–1026.
25. **Robinson, W. S., D. A. Clayton, and R. L. Greenman.** 1974. DNA of human hepatitis B virus candidate. J. Virol. **14**:384–391.
26. **Sakamoto, Y., G. Yamada, M. Mizymo, T. Nishihara, S. Kinoyama, T. Kobayashi, T. Takahashi, and H. Nagashima.** 1983. Full and empty particles of hepatitis B virus in hepatocytes from patients with HBsAg-positive chronic active hepatitis. Lab. Invest. **48**:678–682.
27. **Takahashi, K., Y. Akahane, T. Gotanda, T. Mishiro, M. Imai, Y. Miyakawa, and M. Mayumi.** 1979. Demonstration of hepatitis B e antigen in the core of Dane particles. J. Immunol. **122**:275–279.
28. **Takahashi, K., M. Imai, M. Nomura, A. Oinuma, A. Machida, G. Funatsu, Y. Miyakawa, and M. Mayumi.** 1981. Demonstration of the immunogenicity of Hepatitis B core antigen in a Hepatitis B e antigen polypeptide (P19). J. Gen. Virol. **57**:325–330.
29. **Takahashi, K., A. Machida, G. Funatsu, M. Nomura, S. Usuda, S. Aoyagi, K. Tachibana, H. Miyamoto, M. Imai, T. Nakamura, Y. Miyakawa, and M. Mayumi.** 1983. Immunochemical structure of Hepatitis B e antigen in the serum. J. Immunol. **130**:2903–2907.
30. **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. U.S.A. **76**:4350–4354.
31. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulphate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.