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A Recombinant Hepatitis B Core Antigen Polypeptide with the Protamine-Like Domain Deleted Self-Assembles into Capsid Particles but Fails To Bind Nucleic Acids

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We have cloned in *Escherichia coli* both the complete core gene of hepatitis B virus and a truncated version of it, leading to the synthesis of high levels of a core-antigen-equivalent polypeptide (r-p22) and of an e-antigen-equivalent polypeptide (r-p16), respectively. We then compared the structural and antigenic properties of the two polypeptides, as well as their ability to bind viral nucleic acids. r-p16 was found to self-assemble into capsid-like particles that appeared similar, when observed under the electron microscope, to those formed by r-p22. In r-p16 particles, disulfide bonds linked the truncated polypeptides in dimers, assembled in the particle by noncovalent interactions. In r-p22 capsids, further disulfide bonds, conceivably involving the carboxy-terminal cysteines of r-p22 polypeptides, joined the dimers together, converting the structure into a covalently closed lattice. The protamine-like domain was at least partly exposed on the surface of r-p22 particles, since it was accessible to selective proteolysis. Finally, r-p22, but not r-p16, was shown to bind native and denatured DNA as well as RNA. Taken together, these results suggest that the protamine-like domain in core polypeptides is a nucleic acid-binding domain and is dispensable for the correct folding and assembly of amino-terminal and central regions.

Hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg) are two topologically separated antigens produced in the course of acute or chronic infection by hepatitis B virus (HBV). HBcAg is synonymous with a 28-nm nucleocapsid core, whose main structural constituent is the core polypeptide (13). Free HBcAg is confined to infected hepatocytes. In contrast, HBeAg is a nonparticulated form detected in the bloodstream (23, 45). It has been recently shown that a single viral open reading frame precore-core (16, 20, 31, 34, 47, 48), is capable of coding for both antigens (22, 44) by utilizing either of two in-frame start codons. Initiation of translation at the 3′ start codon leads to the synthesis of a core polypeptide which self-assembles intracellularly into a capsid particle. Initiation of translation at the 5′ start codon results in the addition of 29 amino acids (precore region) to the amino terminus of the core polypeptide. The precore region most likely acts as a signal peptide, directing the nascent protein into the secretory pathway (4, 26, 33, 37, 42, 46, 49). Mature HBeAg is generated from this precore-core precursor after the precore region has been cotranslationally processed (4) and 36 amino acids, constituting a protamine-like domain, have been removed from the carboxy terminus by an as yet unidentified protease (4, 42, 44).

The biological role of secreted HBeAg is still enigmatic (4, 9, 40). Moreover, a great many unanswered questions remain concerning the antigenic duality of HBcAg and HBeAg and their interaction with nucleic acids. HBeAg shares with the core polypeptide the region from the core amino terminus to the boundary of the protamine-like domain. The antigenic individuality of HBcAg and HBeAg seems therefore to involve the masking of common epitopes (14, 38); some HBe-specific epitopes could be buried in the interior of core particles, whereas core-specific epitopes could be hidden by immunoglobulins, carrier proteins, or both in circulating HBeAg (44). Other issues of concern are the role of the protamine-like domain in HBcAg antigenicity, in capsid particle assembly, and in the ability of the core polypeptide to bind nucleic acid (35). A recent report suggests that circulating HBeAg is also a DNA-binding protein (25).

In an attempt to address some of these questions, in this report we compare the structural and functional properties of two recombinant polypeptides synthesized in *Escherichia coli*, an HBcAg-like polypeptide (r-p22) and an HBeAg-like polypeptide (r-p16). Several laboratories have already exploited recombinant DNA technology to express in bacteria the core polypeptides capable of self-assembly into capsid-like particles (6, 12, 29, 46). Here we show also that r-p16 can form capsid-like particles and that it is devoid of nucleic acid binding activity. In contrast, r-p22 does bind native and denatured DNA as well as RNA. A tentative model for disulfide bond arrangement and protamine-like domain location in core particles is also proposed.

MATERIALS AND METHODS

Construction of pKKcore5. Plasmid pAM6 (28) containing a complete HBV genome (subtype adw) was digested with *Mst*I, which cuts at map position 1804 in the viral sequence (11 base pairs [bp] upstream from precore ATG), treated with Bal 31 slow (International Biotechnologies, Inc.) (0.5 U/μg of DNA; 37°C, 45 min) to shorten the generated fragments by approximately 90 bp at each end, and recut with *Bam*HI (cleaving at HBV map positions 30 and 1402). The resulting ca.-1,330-bp fragments, spanning the HBV sequence from the shortened end upstream from the core ATG to the *Bam*HI site at map position 30, were inserted

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into plasmid pKK223-3 (11; obtained from Pharmacia), which had been digested with *Sma*I and *Bam*HI (partial). *E. coli* JM101 was transformed with the ligation products, and 10 recombinant clones were partially sequenced to define the 5′ end of core gene insert. Clone pKKcore5, in which the 5′ end of the HBV insert starts 3 nucleotides upstream from the core ATG (5′GGCATG...3′), was chosen for further manipulations.

Construction of pKKe4. The DNA of pKKcore5 was digested with *Eco*RI and *Bam*HI, and the 1,338-bp fragment corresponding to the HBV insert was recut with *Hpa*II, which cleaves at HBV map position 2332 (within the core gene and just upstream from the region coding for the protamine-like domain). The 5′ sub-fragment thus generated was inserted into plasmid Blue Scribe (Stratagene) doubly cut with *Eco*RI and *Acc*I. The recombinant plasmid (pBSe) was cut with *Eco*RI and *Pst*I (3′ to *Acc*I in the Blue Scribe polylinker), and the excised insert was cloned into plasmid pEX-2, which had been cut with the same enzymes. The resulting plasmid was partially digested with *Xba*I (in order to cleave just downstream from the stop codon box present in pEX-2), and 5′ protruding ends were blunted with mung bean nuclease (Promega Biotec) (0.5 U/μg of DNA) (20 min, 37°C). DNA was finally digested with *Eco*RI, and the 485-bp fragment was inserted into plasmid pKK223-3, which had been cut with *Eco*RI and *Sma*I. The resulting plasmid (pKKe4) was used to transform *E. coli* JM101.

Purification of r-p22 and r-p16 particles. Overnight cultures of *E. coli* JM101 harboring pKKcore5 or pKKe4 were diluted 1:100 into 500 ml of fresh 2× TY-ampicillin broth and grown at 37°C for 2 h. Isopropyl-β-D-thiogalactopyranoside was added to 0.5 mM, and the cultures were incubated for two more hours. Phenylmethylsulfonyl fluoride was then added to 0.5 mM, and the cultures were chilled on ice for 30 min. Soluble proteins were extracted by a modification of a previously published procedure (8). Cells were collected by centrifugation (3,000 × *g*, 15 min, 4°C), washed, and suspended in 10 ml of cold lysis buffer (20 mM Tris hydrochloride, pH 7.5, 10 mM disodium EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Lysozyme was added to 0.02%, and the cell suspension was incubated on ice for 30 min, followed by freezing in a dry ice-methanol bath and quick thawing at 37°C. The lysate was then sonicated, NaCl was added to 0.5 M, and the extract was centrifuged at 14,000 × *g* for 15 min at 4°C. To the supernatant, ammonium sulfate was added to 35% solubility. The resulting precipitate was collected by centrifugation and dissolved in 2.5 ml of column buffer (20 mM Tris hydrochloride, pH 7.5, 10 mM disodium EDTA, 1 mM phenylmethylsulfonyl fluoride, 5% sucrose). The solution was briefly sonicated and cleared by centrifugation. The supernatant was applied to a 150-ml Sephacryl S-300 (Pharmacia) column equilibrated in column buffer. Proteins eluting in the void volume were precipitated with ammonium sulfate, redissolved in column buffer, and applied to a 30-ml 20 to 50% sucrose gradient in column buffer. After being centrifuged for 20 h at 26,000 rpm (SW27 rotor; Beckman Instruments), fractions including the leading peak were pooled and precipitated as described above. The final ammonium sulfate pellet contained over 95% r-p22 or r-p16.

Enzyme immunoassay of HBc and HBe reactivity. HBe-specific immunoreactivity in r-p22 or r-p16 preparations was tested by a commercial enzyme immunoassay, ETI-EBK (SORIN Biomedica), which utilizes monoclonal antibodies 904 (anti-HBe/a) and 905 (anti-HBe/b) (19) in a sandwich design. Natural HBcAg shows no reactivity in this assay. HBc-specific immunoreactivity was detected by a modification of enzyme immunoassay ETI-CORE-IGMK (Sorin Biomedica), originally designed for detection of anti-HBcAg immunoglobulin M (IgM) in serum. In the original scheme, insolubilized rabbit anti-IgM IgG binds sample anti-core IgM that can then be revealed by HBcAg plus peroxidase-conjugated anti-HBc. The anti-HBc conjugate is the purified IgG fraction from an anti-core-positive, anti-HBe- and HBeAg-negative human serum, which does not recognize natural HBeAg. We reversed the use of the kit by presaturating immobilized anti-IgM with control anti-core IgM and then substituting our samples for bridging HBcAg. Under these conditions, the assay is specific for HBcAg reactivity. It is, however, expected to recognize HBc-α epitopes (as defined by Takahashi et al. [44]) on natural or recombinant HBeAg polypeptides.

Analytical sucrose gradients. Purified r-p22 or r-p16 (200 μg) was applied to 12-ml 20 to 50% sucrose gradients in column buffer with or without 1 M urea. In the former case, samples were treated with 8 M urea for 30 min at 60°C and then extensively dialyzed against 1 M urea in column buffer before they were applied to the gradient. Gradients were centrifuged for 13 h at 35,000 rpm (SW40 rotor; Beckman Instruments) at 4°C and recovered in 31 0.4-ml fractions.

PAGE. Bacterial suspensions or purified recombinant proteins were solubilized by being mixed with an equal volume of 2× sample buffer (25 mM Tris hydrochloride, pH 6.8, 2% sodium dodecyl sulfate [SDS], 6% [vol/vol] β-mercaptoethanol [β-ME], 0.02% bromophenol blue). Samples were heated for 5 min at 95°C before they were applied to the gel. To perform electrophoretic separation under nonreducing conditions, β-ME was omitted from sample buffer and samples were heated at 60°C for 30 min. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as previously described (21) on a 15% resolving gel.

Immunoblotting. Total protein from induced *E. coli* JM101 harboring plasmid pKKcore5, pKKe4, or control pKK223-3 was separated by SDS-PAGE, transferred to nitrocellulose (NC) sheets, and assayed for HBc-HBe immune reactivity as already described (18). Monoclonal antibody 905, which recognizes both core and HBe denatured polypeptides (44), was utilized as a core-HBe-specific reagent.

Nucleic acid binding assays. Nucleic acid binding activity was assayed by the protein blotting method (3, 36), with minor modifications. Proteins were incubated in sample buffer with β-ME for 10 min at 60°C and were then separated by SDS-PAGE (13% running gel). After incubation of the gel for 4 h at room temperature in refolding buffer (10 mM Tris hydrochloride, pH 7.5, 1 mM disodium EDTA, 50 mM NaCl, 1 mM dithiothreitol, 4 M urea), proteins were electrophoretically transferred to an NC membrane in 25 mM Tris hydrochloride (pH 8.3)–192 mM glycine buffer. Transfer efficiency was assessed by staining with amido black (Fig. 5A). NC membranes were presaturated in binding buffer (10 mM Tris hydrochloride, pH 7.2, 0.1 mM disodium EDTA, 1 mM dithiothreitol, 50 mM NaCl, 2× Denhardt solution) overnight at 4°C and then incubated for 1 h at room temperature in binding buffer containing 5 ng of nick-translated plasmid pAM6 DNA (either double stranded or denatured, approximately 10^8 cpm/μg) per ml or an RNA probe produced by in vitro transcription of plasmid pBSadw with T7 RNA polymerase. pBSadw includes the complete HBV genome from pAM6 inserted at its *Bam*HI site (map position 30) into plasmid Blue Scribe (Stratagene), in the sense orientation with respect to the T7 promoter. The presence of full-length transcripts was verified by formamide-PAGE and

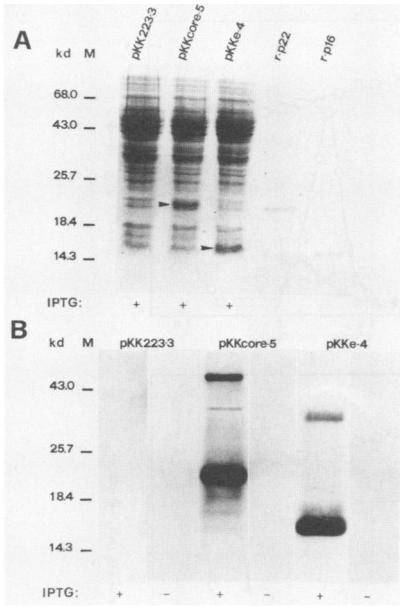

FIG. 1. Expression of HBV core gene in *E. coli*. Total protein from *E. coli* JM101 harboring the indicated plasmid was fractionated by SDS-PAGE (15 or 13% running gel) and stained with Coomassie blue (A) or blotted onto NC membranes and incubated with monoclonal anti-HBe antibody 905 (B). Immune recognition was revealed with an alkaline phosphatase conjugate of goat anti-human IgG. Presence (+) or absence (−) of inducer (0.5 mM isopropyl-β-D-thiogalactopyranoside) is indicated. Purified r-p22 and r-p16 are also shown in gel A. M, Molecular mass markers; kd, kilodalton.

autoradiography. Approximately 5×10^5 cpm of RNA probe per ml was used in the binding assay. After incubation, NC sheets were extensively washed in binding buffer, dried under vacuum, and exposed to X-ray film. Alternatively, proteins were directly immobilized onto NC membranes in a multiwell vacuum filtration device (Schleicher & Schuell, Inc.) and filters were processed and hybridized as described above. To test binding activity in r-p22 and r-p16 monomers and dimers, the respective particle preparations were dissociated in 8 M urea containing 1% (vol/vol) β-ME (60°C, 30 min) and then subjected to a refolding treatment by quasiequilibrium dialysis as previously described (7).

RESULTS

Expression of r-p22 and r-p16 in *E. coli*. Plasmid pKKcore5 was constructed by inserting a fragment containing the entire core coding region into the polylinker of expression vector pKK223-3. The construct was expected to express a 185-amino-acid polypeptide identical in its primary sequence to the natural product. We also generated a deletion variant (pKKe4) of the above-described construct by replacing the carboxy-terminal region of the core gene with a stop codon box. The deleted construct was expected to express a 150-amino-acid polypeptide sharing with core polypeptide the sequence from Met-1 to Pro-144 and bearing a nonspecific six-amino-acid carboxy-terminal tail (-Thr-Cys-Ser-Asn-Ala-Cys-COOH) introduced by the cloning strategy.

When the corresponding transformed *E. coli* strains were induced, both plasmids directed the synthesis of large amounts of polypeptides with the expected size: a 22-kilodalton polypeptide (r-p22) and a 16-kilodalton polypeptide (r-p16), respectively (Fig. 1A). Both products were correctly recognized in immunoblots (Fig. 1B) by monoclonal antibody 905 (anti-HBe-b [19]), which reacts with both core and HBe-dissociated polypeptides (44).

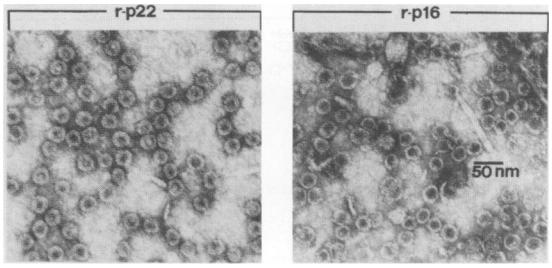

FIG. 2. Electron micrographs of purified r-p22 and r-p16 capsid-like particles. Samples were negatively stained with uranyl acetate. Electron micrographs were taken with a Philips EM201. Magnification, ×70,000. Bar, 50 nm.

Physical and immunological characterization of r-p22 and r-p16. Recombinant polypeptides were extracted from bacterial cells as high-molecular-weight forms that remained in suspension in cleared lysates and were quickly purified by a gel filtration step followed by centrifugation through a 20-to-50% sucrose gradient. The pooled leading peak from the gradient yielded a more than 95% pure preparation of either protein (Fig. 1A).

Electron micrographs of purified r-p22 showed spherical particles averaging 28 nm in diameter (Fig. 2), strictly resembling previously published pictures of natural or recombinant cores (2, 10, 17, 29, 32). Interestingly, also r-p16, when examined under the electron microscope, appeared organized into particles with an external morphology similar to that of r-p22 (Fig. 2), although somewhat larger (about 30 nm in diameter). In both cases, particle contours presented an indentation reminiscent of capsomeric units.

When purified r-p22 and r-p16 were tested for HBcAg immunoreactivity by enzyme immunoassay, both showed a strong core-specific reactivity (Table 1). When assayed for HBe reactivity, r-p22 remained below the boundary of positivity, whereas significant HBe reactivity was present in r-p16. In order to identify the molecular species that were recognized by HBe-specific antibodies, purified r-p22 and r-p16 preparations were fractionated by sucrose gradient sedimentation. HBc- or HBe-specific reactivity was then determined for each fraction. Under these nondissociating conditions, r-p16 sedimented as a high-molecular-weight peak (approximately 80S), followed by lower-molecular-

TABLE 1. HBcAg and HBeAg reactivity in purified r-p22 and r-p16

Sample	Plasmid	Test[a]	P/N[b]
Purified r-p22	pKKcore5	ETICORE	24.6 ± 0.7
Purified r-p16	pKKe4	ETICORE	20.2 ± 0.5
Cleared lysate	pKK223-3	ETICORE	0
Purified r-p22	pKKcore5	ETI-EBK	1.7 ± 0.9
Purified r-p16	pKKe4	ETI-EBK	7.0 ± 0.6
Cleared lysate	pKK223-3	ETI-EBK	0

[a] All tests were performed with the indicated kits from SORIN Biomedica. See Materials and Methods for a description of the assays.

[b] Positive/negative variation interval. Negative (control) values were obtained with cleared lysates from induced *E. coli* JM101(pKK223-3). All values represent the average of three replicas.

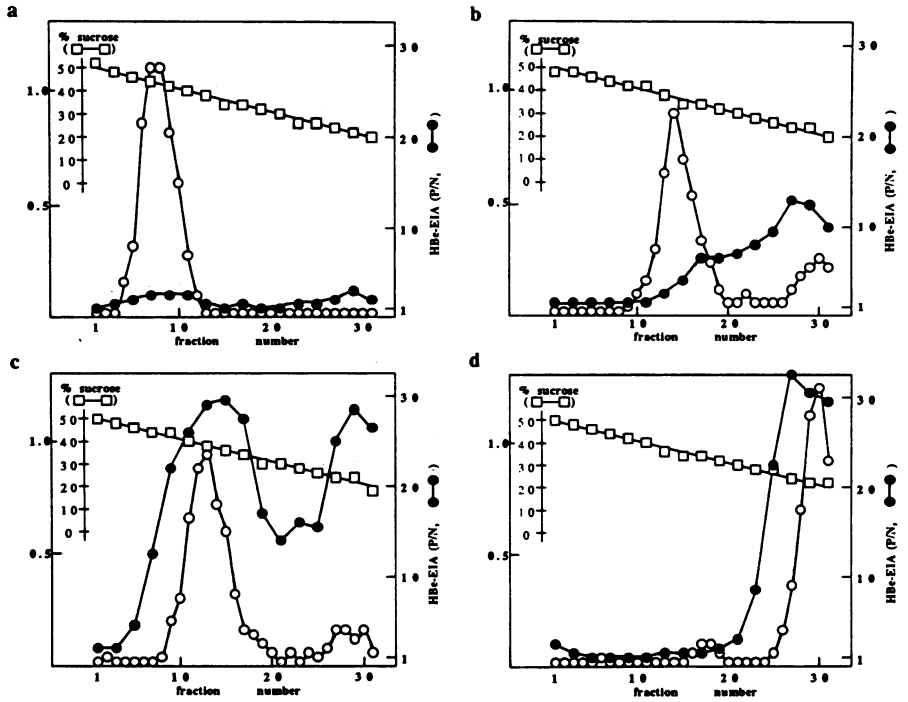

FIG. 3. Sucrose gradient analysis of size and HBeAg immunoreactivity in r-p22 and r-p16 purified preparations. Samples (200 μg of protein in 0.4 ml) of r-p22 (a and c) or r-p16 (b and d) were layered onto a 20-to-50% (wt/vol) sucrose gradient (□) prepared in column buffer (described in Materials and Methods). In denaturing gradients (c and d), 1 M urea was included in buffer, and samples were pretreated in 8 M urea. Centrifugation was carried out at 35,000 rpm for 13 h at 4°C (SW40 rotor, Beckman Instruments), and gradients were collected in 0.4-ml fractions. Protein concentrations (○) were determined by the Coomassie brilliant blue method (absorbance measured at 595 nm); HBe immune reactivity (●) was tested by the ETI-EBK enzyme immunoassay (SORIN Biomedica). β-Galactosidase and ferritin (data not shown) were included in native gradients as sedimentation standards.

weight material trailing to the top of the gradient (Fig. 3b). HBe immune reactivity found in the gradient mostly comigrated with these lighter forms. Under the same conditions, r-p22 sedimented as a single, isolated peak of approximately 110S, with very little associated HBe reactivity (Fig. 3a). Thus, HBe-specific immunoreactivity appeared to be due mainly to dissociated polypeptides or small complexes present in r-p16 preparations. This suggests that r-p16 core-like particles are less stable than the corresponding r-p22 particles, possibly because the protamine-like carboxy-terminal domain, absent in r-p16, plays a relevant role in stabilizing the particle structure.

To further investigate this point, sedimentation experiments were repeated under dissociating conditions. Purified r-p22 and r-p16 were treated with 8 M urea (as described in Materials and Methods) before they were applied to 20 to 50% sucrose gradients in 1 M urea. Under these conditions, the bulk of r-p22 sedimented again as a high-molecular-weight peak, although some lower-molecular-weight material was generated (Fig. 3c). Both heavy and light forms possessed HBe immune reactivity, which was stronger in the low-molecular-weight material. In contrast, r-p16 was completely dissociated by the urea treatment and sedimented as a low-molecular-weight peak, to which a very high HBe immune reactivity was associated (Fig. 3d). When these HBeAg-positive fractions were tested in the Ouchterlony immune diffusion assay with monoclonal antibody 905, a clear immunoprecipitation line was seen (data not shown). Since monoclonal antibodies cannot precipitate a monovalent antigen, the latter result indicates that the low-molecular-weight forms generated by urea treatment of r-p16 are, at least in part, divalent for an HBeAg epitope.

The simplest interpretation of the above-mentioned results is that in r-p22 particles covalent bonds prevent disruption by chaotropic agents and that similar bonds cannot be formed in r-p16 particles. On the other hand, the structural units released by urea treatment of r-p16 particles contain dimers or oligomers of r-p16 polypeptide.

Electrophoretic behavior of r-p22 and r-p16 under reducing and nonreducing conditions. The arrangement of covalent bonds inside r-p22 and r-p16 particles was further analyzed by SDS-PAGE. As already shown (Fig. 1A), under reducing conditions both recombinant products give a band of the expected size for the monomer (Fig. 4A, lanes 1 and 5).

When the run was repeated under nonreducing conditions, r-p16 showed a band of about 32 kilodaltons, corresponding to a dimer of the r-p16 polypeptide (Fig. 4A, lane 6). Under the same conditions, r-p22 did not enter the running gel (Fig. 4A, lane 2). Taken together with the sedimentation analysis, these results suggest that the basic structural units forming r-p16 particles are dimers formed by two r-p16 polypeptides linked by disulfide bonds. In r-p22 particles, additional disulfide bonds, presumably between the carboxy-terminal cysteines of the protamine-like regions (see Discussion), further stabilize the particle structure, making it resistant to treatment with chaotropic agents.

Limited proteolysis of r-p22 particles. r-p22 particles were incubated with 5 μg of pronase per ml for 45 min at 30°C, and the digestion mixture was analyzed by SDS-PAGE under either reducing or nonreducing conditions. In the former

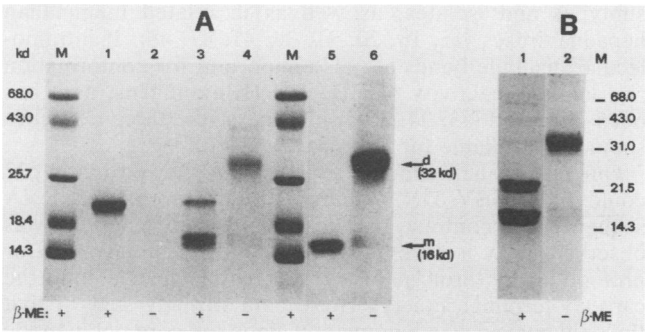

FIG. 4. Analysis of disulfide bond arrangement in r-p22 and r-p16 particles. (A) Electrophoretic mobility of purified r-p22 and r-p16 preparations in a 0.1% SDS-15% polyacrylamide gel, under reducing (+) or nonreducing (−) conditions, were compared. For a full denaturation-reduction, samples were heated for 5 min at 95°C, after being mixed with an equal volume of 2× sample buffer including 6% (vol/vol) β-ME. For fractionation under nonreducing conditions, β-ME was omitted and samples were heated for 20 min at 60°C. Lanes 1 through 4, r-p22; lanes 5 and 6, r-p16. In lanes 3 and 4, r-p22 particles were digested for 45 min at 30°C with 5 μg of pronase (Boehringer) per ml before electrophoretic separation. (B) Tryptic digestion of r-p22 particles. Purified r-p22 capsids (0.5 mg/ml) were incubated at 37°C for 2 h with 2.5 μg of trypsin (Boehringer, chymotrypsin-free) per ml in 20 mM Tris hydrochloride (pH 8.0)–0.5 mM EDTA. After incubation, portions were taken and mixed with equal volumes of 2× sample buffer for reducing or nonreducing treatment, as described above. M, Molecular mass markers; kd, kilodalton. The positions of monomers (m) and dimers (d) of r-p16 or proteolyzed r-p22 are indicated by arrows.

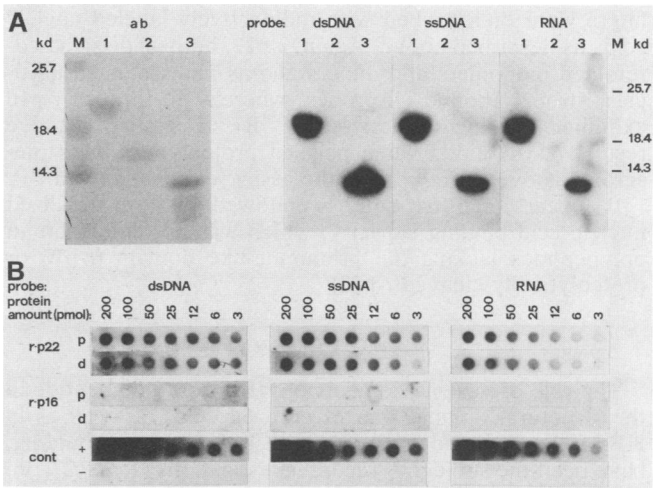

FIG. 5. Nucleic acid binding capacity of r-p22 and r-p16. (A) Protein electroblotting and incubation with HBV-specific nucleic acid probes. Samples were mixed with an equal volume of 2× sample buffer (containing β-mercaptoethanol), heated for 30 min at 60°C, and applied to a 0.1% SDS-13% polyacrylamide gel. After incubation of the gel in renaturing buffer, the fractionated proteins were electroblotted onto an NC membrane and either stained with amido black (ab) or incubated with ^{32}P-labeled probes (ca. 5×10^5 cpm/ml). dsDNA, native nick-translated plasmid pAM6; ssDNA, denatured native, nick-translated plasmid pAM6; RNA, HBV genome-sized RNA transcribed in vitro from plasmid pBSadw. Lanes 1 through 3 contained equimolar amounts of r-p22, r-p16, or histone H4 (Boehringer), respectively. M, Molecular mass markers; kd, kilodalton. (B) NC dot tests. Twofold dilutions of equimolar amounts of r-p22 or r-p16, before (p) or after (d) a cycle of denaturation-reduction-refolding (described in Materials and Methods), were dotted onto NC sheets and incubated with HBV-specific probes as described above. Controls were salmon sperm protamine (+) and bovine serum albumin (−).

case (Fig. 4A, lane 3), together with the uncleaved r-p22 monomer, a band roughly comigrating with r-p16 appeared. (A slower band also in the 16-kilodalton region was probably the result of incomplete pronase digestion of repetitive arginine stretches in the protamine-like region). This result was expected, since other authors have reported that limited proteolysis of natural or recombinant HBcAg with pronase induces HBc-to-HBe antigenic conversion (5, 22, 43) and that the enzyme digests the arginine-rich protamine-like domain up to the carboxy terminus of HBeAg (44). If sample reduction after digestion was omitted, only a smeared band approximately comigrating with the r-p16 dimer was observed (Fig. 4A, lane 4). This proteolytic dimer was purified by ion-exchange high-performance liquid chromatography and was found to exhibit a high HBe immune reactivity in enzyme immunoassay (data not shown). When it was further proteolyzed by splitting Asp-Pro bonds with acetic acid at pH 2.5, it generated the correct peptide fingerprint for the amino-terminal moiety (data not shown).

Digestion of r-p22 particles with trypsin, which, in contrast to pronase, is strictly specific for arginine and lysine residues, produced both under reducing and nonreducing conditions an electrophoretic pattern resembling that generated by pronase (Fig. 4B). In contrast to pronase treatment, in which longer incubation periods led to extensive proteolysis also of the amino-terminal moiety, the tryptic pattern remained substantially unchanged after the first 10 min in a time course experiment (data not shown).

Thus, selective proteolysis of the protamine-like domain of r-p22 in capsid-like particles allowed subsequent dissociation of the particles into dimers by SDS treatment, confirming the role of the carboxy-terminal region in particle stabilization. Preferential degradation of the arginine-rich domain also indicates that the protamine-like carboxy terminus is more accessible to proteolytic enzymes than the rest of the polypeptide in the organized particle.

Comparison of nucleic acid binding properties of r-p22 and r-p16. Purified r-p22 and r-p16 were analyzed for their ability to ligate nucleic acids by the southwestern or northwestern blotting technique (3, 36). Equimolar amounts of r-p22, r-p16, and a positive control (histone H4) were separated by SDS-PAGE, electrophoretically transferred to NC membranes after partial in-gel renaturation, and incubated with ^{32}P-labeled probes. These consisted of native or heat-denatured nick-translated plasmid pAM6, containing a complete HBV genome, or of a genome-long HBV RNA obtained by in vitro transcription of plasmid pBSadw (plasmid described in Materials and Methods). In all cases, r-p22 and the histone control gave strong autoradiographic signals, whereas r-p16 gave no detectable signal, even after prolonged exposure (Fig. 5A).

Since SDS treatment could interfere with nucleic acid binding, r-p22 and r-p16 were probed also under different conditions. Pure preparations of either protein were subjected to a cycle of total denaturation-reduction-renaturation in solution (described in Materials and Methods). After this treatment, both preparations were converted to a mixture of free monomers and dimers (as judged from nonreducing SDS-PAGE or sedimentation through nondissociating sucrose gradients), all sharing a strong HBe immune reactivity (data not shown). Serial twofold dilutions of refolded r-p22 and r-p16, in parallel with equal amounts of untreated particles, were dotted directly onto NC membranes. The

filters were then probed with radioactively labeled nucleic acids as described above. Again, r-p22, both as dissociated-refolded monomers and dimers and as undissociated particles, strongly bound all probes, whereas no form of r-p16 exhibited binding capacity (Fig. 5B). A similar negative result was obtained when purified proteolytic dimers (described above) were used in the assay (data not shown).

It appears then that r-p22 is endowed with a generalized nucleic acid binding capacity, which was not detectable in polypeptides lacking the protamine-like domain (r-p16 or proteolytically cleaved r-p22).

DISCUSSION

The role of various domains of HBV core polypeptide in the structural organization of core particles, as well as in HBc-HBe antigenic reactivity and in nucleic acid binding, have been the subject of recent investigations (1, 25, 27, 36, 50). Although *E. coli*-made HBV polypeptides are not processed like their natural counterparts, comparison of structural properties of r-p22 and r-p16 particles described here provided information that might be relevant to viral structure and assembly. Our results demonstrate that genetic deletion of the carboxy-terminal domain does not hamper the ability of a bacterially produced core polypeptide to self-assemble into a highly ordered, capside-like particle. This suggests that the upstream moiety of core polypeptide bears sufficient structural information for the assembly to take place, and that deletion of the carboxy-terminal moiety does not induce, per se, major conformational rearrangements. A similar result with an HBeAg-like polypeptide produced in *E. coli* has been recently described by Mimms et al. (27).

On the other hand, results from sedimentation and electrophoretic migration under dissociating or nondissociating conditions strongly suggest that the protamine-like domain is involved in capsid stabilization via disulfide bonds. In fact, we found that r-p16 particles could be dissociated into dimers of r-p16 polypeptide by 8 M urea or by SDS, in the absence of reduction; r-p22 particles, on the contrary, could not be dissociated without reduction. However, if the protamine-like domain was selectively cleaved by proteolysis, then also r-p22 particles could be dissociated by urea or SDS into dimers of the core amino-terminal moiety. Both r-p16 dimers and proteolytic dimers were shifted to monomers by reduction. This suggests a model for capsid structure, in which the central domains of the polypeptide are joined intermolecularly by at least one disulfide bond to give dimers. The carboxy-terminal domains in turn stabilize the particle by binding to each other two by two through their carboxy-terminal cysteine, thus linking neighboring dimers and converting the entire structure into a topologically closed net of disulfide bonds. The lack of this covalent closure in r-p16 particles might explain their slightly larger diameter and their different hydrodynamic properties. Interestingly, dimerization via disulfide bonds has been reported also for natural circulating HBeAg (30, 45).

The importance of disulfide bonds for HBV capsid stabilization and HBe antigenic reactivity has been repeatedly invoked. Feitelson has already reported that natural core particles cannot be resolved into lower-molecular-weight forms by SDS-PAGE in the absence of a reducing agent, and suggested that the carboxy-terminal cysteine is directly involved in this disulfide-bond-mediated reinforcement (13). Inspection of core amino acid sequence shows three cysteines upstream from the protamine-like domain, at positions 48, 61, and 107, all strictly conserved among various HBV subtypes and isolates, as well as in related mammalian hepadnaviruses (15, 16, 20, 31, 34, 41, 47, 48). If intramolecular disulfide bonds are also important for conformation and immunoreactivity of HBc and HBe antigens, as it has been proposed (13, 24, 35), only one of the three cysteines should be available for intermolecular binding.

Our nucleic acid binding experiments showed that r-p22 could bind HBV DNA (native or denatured) and RNA sequences. In contrast, no nucleic acid binding activity was observed with a core polypeptide genetically (r-p16) or proteolytically (pronase or trypsin treatment) deleted of the carboxy-terminal moiety. These data strongly suggest that the only nucleic acid binding domain in the core polypeptide maps in the protamine-like carboxy-terminal moiety. That the protamine-like domain is indeed a nucleic acid binding region was confirmed directly by recent experiments with a recombinant β-galactosidase–protamine region fusion polypeptide (A. Gallina et al., manuscript in preparation). These findings are in apparent contrast to those reported by Matsuda et al. (25) showing that also circulating HBeAg is a DNA binding protein. It is possible that some of the differences in amino acid sequence between natural HBeAg and r-p16 can account for this discordance. On the other hand, r-p16 completely includes the region of positions 100 to 120, which the cited authors hypothesize as being involved in DNA binding. A direct comparison of highly purified natural HBe polypeptide and its recombinant equivalent will be necessary in order to resolve this issue.

Our results from limited proteolysis and nucleic acid binding experiments suggest also that the protamine-like domain may be at least partly exposed at the surface of core particles. The basic carboxy terminus was preferentially attacked by pronase or trypsin, in spite of 10 potential tryptic sites in the upstream moiety. On the other hand, undissociated r-p22 particles bound viral nucleic acids, whereas r-p16 particles did not. These results can be correlated with a previous observation by Takahashi et al. (44), who described a monoclonal antibody capable of recognizing denatured core polypeptide as well as capsid particles but no form of natural or proteolytic HBeAg. A tantalizing interpretation of these data may be that an HBc-specific epitope maps in the carboxy-terminal domain of the core polypeptide and that such a domain is available for antibody or nucleic acid binding, as well as selective proteolysis, on the surface of the core particle. Mention should be made, however, of the fact that particles synthesized in *E. coli* lack the viral genome as well as the phosphorylation which seems selectively targeted against the protamine moiety (39).

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