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DNA-Binding Activity of Hepatitis B e Antigen Polypeptide Lacking the Protaminelike Sequence of Nucleocapsid Protein of Human Hepatitis B Virus

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The characteristics of binding of hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg) polypeptides to hepatitis B virus (HBV) DNA were analyzed. HBcAg polypeptide from recombinant HBV core particles and HBeAg polypeptide from partially purified serum HBeAg were prepared and verified to have molecular weights of 21,500 (P21.5) and of 17,000 (P17) and 18,000 (P18), respectively, by immunoblot analysis. By reaction of these proteins on a nitrocellulose membrane with cloned ^{32}P-HBV DNA, it was revealed that the HBeAg polypeptide, which lacks the C-terminal 34 amino acids of P21.5, as well as the HBcAg polypeptide, bound to the DNA. The secondary structures of nucleocapsid proteins of HBV, woodchuck hepatitis virus, and ground squirrel hepatitis virus were predicted by the Garnier algorithm. Amino acid sequences which, in addition to those of the C-terminal regions, may contribute to binding were proposed to be the 21-amino-acid residues located at amino acids 100 to 120 of the nucleocapsid proteins of these hepadnaviruses.

Human hepatitis B virus (HBV) replicates primarily in hepatocytes, in which it sometimes causes chronic infection. HBV is a 42-nm particle (the Dane particle) consisting of an outer coat, the hepatitis B surface antigen (HBsAg), and a 27-nm nucleocapsid containing the hepatitis B core antigen (HBcAg). The viral core contains (i) a partially double-stranded circular DNA of 3.2 kilobases with a full-length minus strand that has a protein covalently bound to its 5′ end and a plus strand that varies in length and (ii) the endogenous DNA polymerase (30).

The third antigen, hepatitis B e antigen (HBeAg) (12), was found in soluble form in the sera of patients when viruses were actively being produced (18, 28). HBeAg is now regarded as an integral component of viral cores (2, 16, 36). Later experiments showed that a nucleocapsid protein having a molecular weight (MW) of 21,500 (P21.5) had both HBcAg and HBeAg activities (11, 17, 35). Furthermore, recent studies have demonstrated that there are polypeptides with MWs of 17,000 (P17) and 18,000 (P18) in serum which also have HBeAg activity and that they are derivatives of P21.5 (K. Matsuda and H. Ohori, J. Immunol., in press). However, these polypeptides lack the 34-amino-acid sequence at the C-terminal region of P21.5 (29). It was recently demonstrated that HBeAg is more efficiently secreted into the culture medium from cells in which the mRNA containing the precore sequence is expressed than from cells lacking this sequence (20, 23). The absence of the precore DNA sequence results in the synthesis of HBcAg, which is predominantly localized in the nucleus (14).

However, the biological function of HBeAg is not fully understood because of complexities in molecular size (33, 34) and immunoreactivity (13). It was reported that core particles have protein kinase activity, which specifically phosphorylates serine residues (3, 8, 24). In later experiments, it was demonstrated that this enzyme activity was still conserved even in an HBeAg polypeptide with an MW of 14,000 (27). To determine the further biological functions of HBeAg, we examined the DNA-binding activity of HBeAg as well as of HBcAg. Here we demonstrate that HBeAg also binds to HBV DNA and propose the putative amino acid sequence involved in this activity in the nucleocapsid protein.

HBV-related antigens HBsAg, HBcAg, and HBeAg were semiquantitatively determined by the reversed passive hemagglutination method; Antihebscell for HBsAg was purchased from Green Cross Corp., Osaka, Japan, and reversed passive hemagglutination tests for HBcAg and HBeAg were prepared by methods described previously (13, 18). Antibodies to HBsAg, HBcAg, and HBeAg (anti-HBs, anti-HBc, and anti-HBe, respectively) were determined by the passive hemagglutination method (Hebsgencell, Corecell, and e-Cell, respectively; Green Cross Corp.).

For the purpose of verifying the identities of the polypeptides that had HBcAg and HBeAg activities, purified recombinant core particles produced by *Escherichia coli* cells (purchased from Green Cross Corp.) and partially purified serum HBeAg prepared by a method described previously (13) were analyzed by the immunoblotting method (17). For detection of HBcAg and HBeAg polypeptides, human anti-HBc immunoglobulin G (IgG) (anti-HBc titer, $1:2^{15}$) and mouse monoclonal anti-HBe IgG (anti-HBe titer, $1:2^{13}$) (13) were used as the first reactants. Peroxidase-labeled protein A (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was used as the second reactant. Only one polypeptide band migrating at the position of MW 21,500 was found in the sample of core particles (Fig. 1). This polypeptide (P21.5) was detectable by anti-HBc (lane 1) and anti-HBe (lane 2), indicating that it has epitopes of both HBcAg and HBeAg. On the other hand, a major polypeptide band with an MW of 17,000 (P17) and a minor polypeptide with an MW of 18,000 (P18) were detectable when partially purified HBeAg prepared from 12 different serum specimens having various

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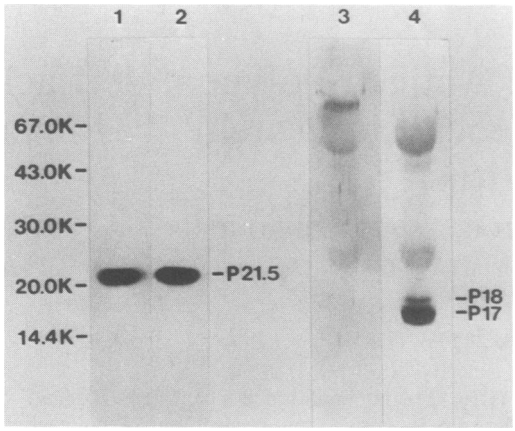

FIG. 1. Identification by immunoblotting of HBcAg and HBeAg polypeptides. Samples (12 μl) of recombinant HBV core particles produced by *E. coli* cells (HBcAg titer, $1:2^{13}$; lanes 1 and 2) and partially purified serum HBeAg (HBeAg titer, $1:2^{15}$; lanes 3 and 4) were probed with human anti-HBc IgG (anti-HBc titer, $1:2^{15}$; lanes 1 and 3) and mouse monoclonal anti-HBe IgG (anti-HBe titer, $1:2^{13}$; lanes 2 and 4). All of the bands found in the region above MW 20,000 (20.0K) in lanes 3 and 4 reacted nonspecifically.

titers of HBeAg was analyzed with anti-HBe IgG (lane 4). However, no detectable band corresponding to the regions of P17 and P18 was found when anti-HBc IgG was used (lane 3). These results indicate that unit polypeptides sharing HBeAg activity are uniform in having MWs of 17,000 and 18,000 irrespective of the HBeAg activity in serum and that they have no HBcAg epitope.

Figure 2 shows a comparison of DNA-binding activities among intact cores and disrupted cores which were treated with 2% sodium dodecyl sulfate–5% 2-mercaptoethanol, calf thymus histone H-1, and bovine serum albumin (BSA). Various amounts of these proteins were blotted on a nitrocellulose membrane (NCM) and examined for their DNA-binding activities. The NCM was immersed in a standard binding buffer (0.01 M Tris hydrochloride [pH 7.0], 1 mM sodium EDTA, 0.02% BSA, 0.2% Ficoll, and 0.02% polyvinyl pyrrolidone containing 0.05 M NaCl) overnight at 4°C to reduce background binding. The NCM was then incubated with the cloned ^{32}P-labeled HBV DNA (subtype adr; supplied by K. Matsubara, University of Osaka, Osaka, Japan) for 1 h at room temperature to permit DNA binding. The DNA probe was labeled by nick translation to a specific activity of 2×10^8 to 4×10^8 cpm/μg. After incubation, the NCM was extensively washed in the standard binding buffer. The NCM was then dried and exposed to X-ray film. Intact core particles and BSA in any amounts did not bind to HBV DNA. On the other hand, disrupted core particles and histone protein bound to HBV DNA according to their amounts on the NCM. These results indicate that the amino acid sequence bearing the HBcAg epitope arranged on the surface of the intact core particle does not have DNA-binding activity; however, when the intact core particles are disintegrated into the nucleocapsid protein P21.5, DNA-binding activity does appear.

It is obvious from the results shown in Fig. 2 that the amino acid sequence corresponding to the HBcAg epitope has no DNA-binding activity when it exists in a particulate form. Considering the fact that HBV DNA binds to the nucleocapsid protein in the inner parts of core particles (19), an amino acid sequence other than that possessing HBcAg activity may be involved in the binding to HBV DNA. To search for this sequence, we examined the HBeAg polypeptide, which lacks an HBcAg-bearing sequence, for DNA-binding activity. The DNA-binding activity was determined by the method described by Bowen et al. (1). Figure 3 shows the profiles of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (panel A), immunoblot analysis (panel B), and DNA binding (panel C) of HBV-related antigens. Small particles of HBsAg in serum were purified by the method described by Mishiro et al. (15). Although many protein bands were found in the sample of partially purified serum HBeAg (Fig. 3A, lane 3), two clear bands corresponding to MWs of 18,000 (P18) and 17,000 (P17) were again verified to have HBeAg activity (Fig. 3B, lane 3). As has been reported elsewhere (30), three typical HBsAg-positive proteins having MWs of 24,000 (P24), 27,000 (P27), and 49,000 (P49) were also demonstrated (Fig. 3B, lane 4) with goat anti-HBs IgG (anti-HBs titer, $1:2^{11}$). The HBcAg polypeptide (P21.5; Fig. 3C, lane 2) and the HBeAg polypeptide (P17; Fig. 3C, lane 3) had DNA-binding activity. However, no clear band was detected in samples of marker proteins, including BSA (MW, 67,000), ovalbumin (MW, 43,000), bovine erythrocyte carbonic anhydrase (MW, 30,000), soybean trypsin inhibitor (MW, 20,000), and bovine milk α-lactalbumin (MW, 14,400) (Fig. 3C, lane 1), and purified HBsAg (Fig. 3C, lane 4). P18 was present in amounts too small for its DNA-binding activity to be detectable (Fig. 3C, lane 3). These results strongly suggest that an amino acid sequence other than that bearing HBcAg activity can also bind to HBV DNA.

The C-terminal region of P21.5 has been considered to be remarkable for its wealth of arginine, serine, and proline residues. Presumably, the arginine-rich sequence, which resembles that found in protamine, is involved in electrostatic interactions with the HBV genome in the nucleocapsid. However, our results (Fig. 3) suggest that another sequence lying in the HBeAg polypeptide also interacts with the HBV genome. In the present study, we examined the amino acid sequences putatively having DNA-binding activity by analyzing the secondary structures predicted for the nucleocapsid proteins of HBV (subtype adr) (4), woodchuck hepatitis virus (5), and ground squirrel hepatitis virus (26) by the method developed by Garnier et al. (7). The HBcAg-coding sequence, the "C-gene," contains two in-phase initiation codons. However, the sequence between these two initiation codons, "the precore region," is not required for the expression of HBcAg in procaryotic (21) or mammalian

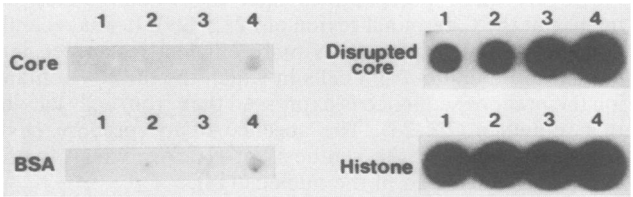

FIG. 2. Binding of HBV intact and disrupted core particles to HBV DNA. Samples (5 μl) were dot blotted on NCM and assayed for binding to ^{32}P-HBV DNA (specific activity, 2×10^8 cpm/μg). Different amounts of recombinant HBV core particles having HBcAg titers of $1:2^{14}$, $1:2^{15}$, $1:2^{16}$, and $1:2^{17}$ and disrupted core particles in the same amounts were blotted at positions 1, 2, 3, and 4, respectively. Four different amounts, 0.2, 0.4, 0.8, and 1.6 μg, of histone and BSA were separately blotted at positions 1, 2, 3, and 4, respectively.

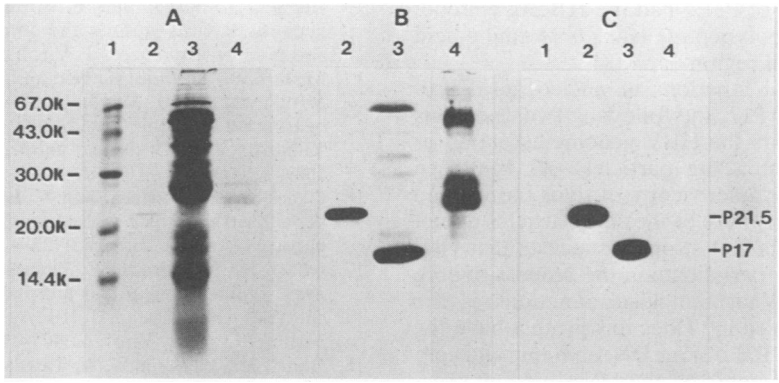

FIG. 3. Binding of HBcAg and HBeAg polypeptides to HBV DNA. Samples (12 μl) of marker proteins (0.75 mg/ml; lane 1), recombinant HBV core particles (HBcAg titer, $1:2^{13}$; lane 2), partially purified serum HBeAg (HBeAg titer, $1:2^{15}$; lane 3), and purified HBsAg (HBsAg titer, $1:2^{13}$; lane 4) were run on sodium dodecyl sulfate-polyacrylamide gels and detected by staining with Coomassie brilliant blue (A), immunoblotting by probing with human serum IgG containing both anti-HBc and anti-HBe (anti-HBc titer, $1:2^{13}$; anti-HBe titer, $1:2^{7}$; lanes 2 and 3) and goat anti-HBs IgG (anti-HBs titer, $1:2^{11}$; lane 4) (B), and a DNA-binding assay with ^{32}P-HBV DNA (specific activity, 2×10^8 cpm/μg) (C). k, 10^3.

(32) cells. Therefore, the predicted secondary structures of HBV, woodchuck hepatitis virus, and ground squirrel hepatitis virus nucleocapsid proteins translated from the second initiation codon of the C-gene were compared. The nucleocapsid proteins of all hepadnaviruses examined were characterized as having a high content of turn structures, especially in the regions from amino acid residue 150 to the C terminus (Fig. 4). These sequences are probably involved in DNA binding. As to the DNA-binding regions before amino acid residue 149, which is presumed to be the C terminus of the HBeAg polypeptide, a 21-amino-acid sequence located between residues 100 and 120 was present in the HBV nucleocapsid protein. This sequence had an α-helix-turn-α-helix structure and resembled DNA-binding polypeptides, such as the oncogene product, *jun*, or GCN4, a yeast transcriptional activator protein, which were predicted to bind to DNA in a sequence-specific manner (9, 31). Furthermore, similar structures are highly conserved in the nucleocapsid proteins of other hepadnaviruses in the same region as in the nucleocapsid protein of HBV. No other probable sequence for DNA binding could be found in the region corresponding to the HBeAg polypeptide.

It has been presumed that the C-terminal region of P21.5 has DNA-binding activity because of the resemblance of its amino acid sequence to those of protamine and galline of rooster sperm (6, 21). This concept was recently confirmed by Petit and Pillot (22). P21.5 was detectable by anti-HBc and anti-HBe, but HBeAg polypeptides (P17 and P18) were detectable only by anti-HBe (Fig. 1). These results, as well as those reported by Takahashi et al. (29), who demonstrated

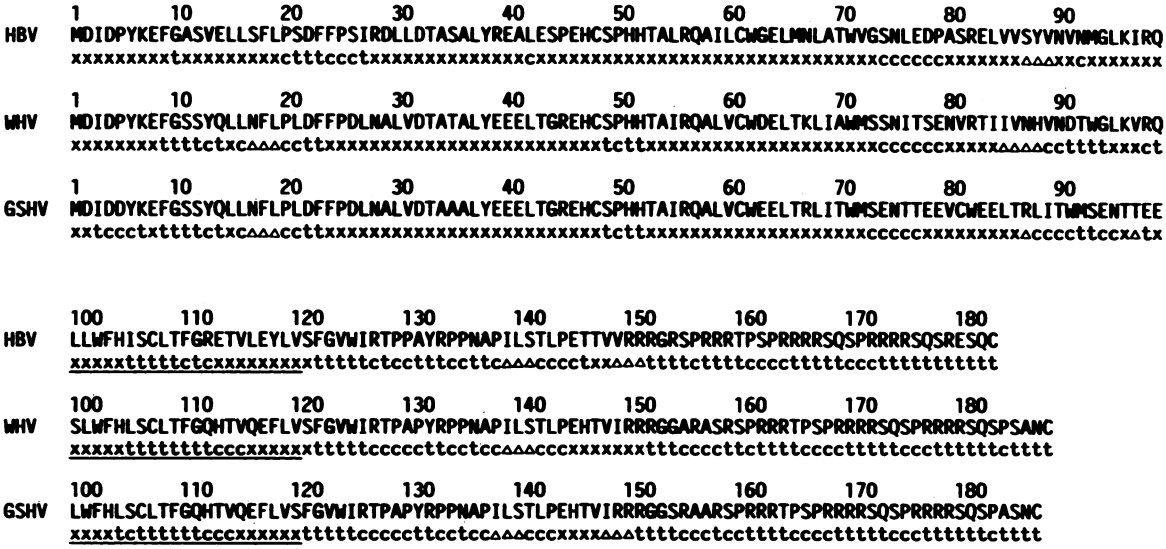

FIG. 4. Predicted secondary structures of the nucleocapsid proteins of HBV, woodchuck hepatitis virus (WHV), and ground squirrel hepatitis virus (GSHV). x, α-helix-permissive structure; t, turn; c, coil; Δ, β-sheet. The most predictable site for DNA binding is underlined. The upper line indicates the positions of amino acid residues. Position 1 is the amino terminus of the nucleocapsid protein. Amino acid sequence alignments are shown in the middle line. The standard single-letter code is used. The lower line of conformation prediction was generated by the program of Garnier et al. (7).

that the C-terminal region of P21.5 had the HBcAg epitope, indicate that the HBeAg polypeptide lacks a 34-amino-acid sequence in the C-terminal region.

Regarding the biological functions of nucleocapsid proteins, including P21.5 and P17, the following two questions remain to be resolved. (i) Is the HBV genome essential for the assembly of P21.5 into core particles? As has been reported from the electron microscopy of liver tissues and sera of HBV carriers, nearly 90% of the core particles do not contain DNA (10, 25). This fact strongly suggests that viral DNA does not necessarily participate in the process of core particle formation. (ii) What biological functions is the HBeAg polypeptide involved in? Does this protein have the same functions as those of P21.5, i.e., DNA binding and core particle formation? Experiments on the DNA-binding specificities of P21.5 and P17 are now in progress in an attempt to answer these questions.

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