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Capsid Assembly and Involved Function Analysis of Twelve Core Protein Mutants of Duck Hepatitis B Virus

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The roles of different regions of the duck hepatitis B virus (DHBV) core protein on viral capsid assembly and related functions were examined. Twelve deletion and insertion mutations which covered 80% of the DHBV C open reading frame were constructed and expressed in *Escherichia coli*. The N-terminal region (amino acids 3 to 66) of DHBV core protein was important for its tertiary structure and function in *E. coli*. The expressed core mutants without this region apparently inhibited *E. coli* growth. The results of transmission electron microscopy of *E. coli* thin sections, capsid agarose gel, and sucrose gradient sedimentation demonstrated that a few DHBV core mutants with insertion in the N terminus and deletion in the C terminus retained the ability to form core-like particles in *E. coli*. However, other mutations in most of N-terminal and central regions strongly inhibited the self-assembly ability of DHBV core protein in *E. coli*. In addition, the mutant with a C-terminal region deletion (amino acids 181 to 228) lost most of the nucleic acid-binding activity of the DHBV core protein.

Hepadnaviruses are small DNA viruses and replicate through a pregenomic RNA intermediate (22), which, before the process of reverse transcription, is encapsidated into an icosahedral nucleocapsid (core particle) together with a viral polymerase (2, 10–12; for a review, see reference 7). The core gene of hepadnaviruses encodes distinctly different proteins, named the core, the precore, and a membrane-associated core gene product exhibiting e antigenicity (21). Initiation of translation at the 3′ start codon of the core gene leads to synthesis of the core protein, which assembles intracytoplasmically into the viral capsid.

Recent studies suggest that the core proteins of hepadnaviruses play both structural and functional roles in the replication process of the viruses (1, 3–6, 9, 16–18, 20, 21, 24, 27–29, 31, 32). Much attention was focused on the C-terminal region of this protein, in which several domains have been found to mediate different functions in the viral life cycle; e.g., the C-terminal region is dispensable for human hepatitis B virus (HBV) capsid assembly in *Escherichia coli* (3, 6) but is important for the nucleic acid binding activity of the HBV core protein and for the capsid stability (3, 6, 9). The arginine-rich sequence in the C-terminal region of the HBV core protein is involved in the nuclear localization of the capsid (5, 27), which is necessary for covalently closed circular DNA formation and amplification of hepadnaviruses (23). A C-terminal domain of the core protein of the duck hepatitis B virus (DHBV) is required for viral DNA maturation and assembly of nucleocapsid into the viral envelope (29). The C-terminal region of HBV (28) and DHBV (20) core protein contains phosphorylation sites which may be important for the intracellular transportation of the core gene products and for the viral replication process (20, 28).

Little is known about the roles of the N-terminal and central regions of hepadnavirus core protein in capsid assembly and other functions necessary for the viral life cycle. To obtain some clues to these questions, we have now carried out a series of mutational analysis of DHBV core protein. The results show that the N-terminal region was important for maintaining the tertiary structure of DHBV core protein. For the core particle assembly, mutations of small insertions at the N terminus and deletions at the C terminus can be tolerated. Deletion or insertion in most of the N-terminal and central regions largely changed the self-assembly ability of DHBV core protein in *E. coli*. In addition, using the mutants above, we located a nonspecific DNA-binding activity in the C-terminal region of DHBV core protein.

MATERIALS AND METHODS

Plasmids. All expression plasmids were constructed with a prokaryotic expression vector, pBV220 (30), which contained the bacteriophage p_L and p_R promoters and the phage λ c*I*857 gene, encoding a temperature-sensitive c*I*857 repressor.

Construction of deletion and insertion mutations in the DHBV core gene. Plasmid pDC12 containing DHBV 76 (25) core gene was generated by cloning the *Eco*RV-*Bgl*II fragment (nucleotides [nt] 2654 to 391 in the DHBV 76 genome) into *Eco*RV and *Bam*HI sites of pBV220. The DHBV core gene fragment was purified from plasmid pDD81, which contained a head-to-tail tandem *Eco*RI dimer of the DHBV 76 genome. According to the DNA sequences of DHBV 76 (25) and the expression vector pBV220 (30), this plasmid was expected to express a 256-amino-acid polypeptide identical in its primary sequence to that part of the natural product because there was a 3-amino-acid insertion (HKC) before the N-terminal aspartic acid residue (the second amino acid of the DHBV core protein) and a 5-amino-acid deletion (PSPRK) at the C terminus of the DHBV core protein, replaced by 11 amino acids (VDLQPSFCFGG) with the cloning strategy (Fig. 1). All plasmids expressing deleted and inserted DHBV core protein mutants were generated on the basis of plasmid pDC12.

To construct a series of deletion mutation which would cover most parts of the DHBV C open reading frame, we used different restriction sites scattering within this gene (Fig. 1)

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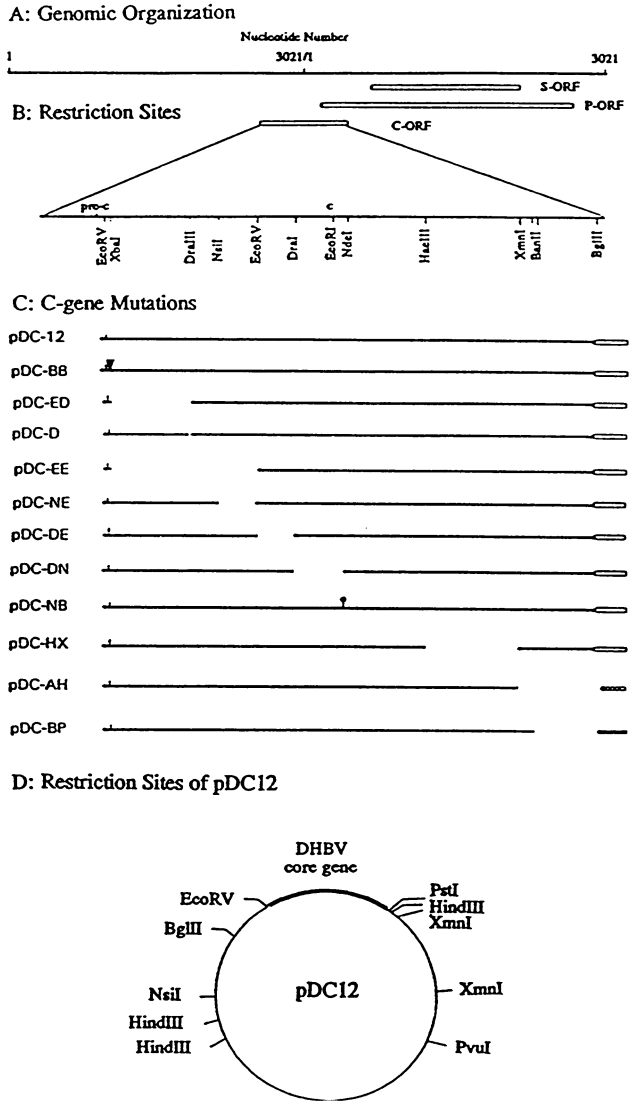

FIG. 1. Schematic view of the DHBV genomic organization and the mutations of the core gene constructed in this study. (A) The genomic organization of DHBV 76 (25) contained as a head-to-tail dimer in plasmid pDD81 (see Materials and Methods). Nucleotide numbers follow the numbering system of Mandart et al. (14). ORF, open reading frame. (B) Restriction sites in the core gene relevant for the construction of the mutations. (C) Construction of various core gene mutations. Symbols: |, insertion of three amino acids (HKC); ▼, insertion of four amino acids (SGSA); ▼, insertion of four amino acids (GIPY). The 5 amino acids at the C terminus (PSPRK) were replaced by a nonspecific 11 amino acids (VDLQPSFCFGG) □, by 6 amino acids (SFCFGG) (▥), or by seven amino acids (PSFCFGG) (■). Other positions of deleted amino acids in the deletion mutants and sequences encoded by linkers are listed in Table 1. (D) Restriction sites of pDC12.

and three types of linkers (10-nt *Bam*HI linker, CGGGATCCCG; 8-nt *Bam*HI linker, CGGATCCG; 8-nt *Xho*I linker, CCTCGAGG) in some mutations for maintaining in-frame deletion or insertion. All mutations were confirmed by DNA sequencing with Sequenase (United States Biochemical Corp., Cleveland, Ohio).

In pDC-ED, the *Eco*RV-*Dra*III fragment (nt 2654 to 2805 in DHBV 76) was deleted. This plasmid was constructed by cutting pDC12 with *Dra*III (nt 2805 in DHBV 76, the unique site in pDC12); removing protruding 3′ termini with T4 DNA polymerase (19); cutting with *Pvu*I (nt 969 in pBV220, the unique site in pDC12 [Fig. 1D]), purifying the *Dra*III-*Pvu*I fragment containing the central and C-terminal regions of the DHBV core gene, and subcloning into *Eco*RV (nt 2654 in DHBV) and *Pvu*I (nt 969 in pBV220) sites of pDC12.

In pDC-D, one amino acid (phenylalanine, amino acid 53 of the DHBV core protein) was deleted by cutting pDC12 with *Dra*III (nt 2805 in DHBV 76, the unique site in pDC12), removing protruding 3′ termini with T4 DNA polymerase, and recircularizating with T4 DNA ligase.

In pDC-EE, the *Eco*RV-*Eco*RV fragment (nt 2654 to 2913 in DHBV 76) was deleted. It was constructed by cutting pDC12 with *Eco*RV, purifying the large fragment, and inserting a 10-nt *Bam*HI linker sequence (CGGGATCCCG) in this site.

In pDC-NE, the *Nsi*I-*Eco*RV fragment (nt 2849 to 2913 in DHBV 76) was deleted. This plasmid was generated by partially cutting pDC12 with *Nsi*I (two sites in pDC12: nt 2894 in DHBV 76 and nt 3071 in pBV220), removing protruding 3′ termini with T4 DNA polymerase, cutting with *Pvu*I (nt 969 in pBV220), purifying the large vector-containing *Nsi*I-*Pvu*I fragment (nt 2849 in DHBV 76 to nt 969 in pBV220) acted as subclone vector, cutting pDC12 with *Eco*RV (two sites in pDC12: nt 2654 and 2913 in the DHBV 76 core gene) and *Pvu*I, purifying the *Eco*RV-*Pvu*I fragment (nt 2913 in DHBV 76 to nt 969 in pBV220) as the insert fragment, and subcloning into the *Nsi*I-*Pvu*I vector fragment.

In pDC-DE, the *Eco*RV-*Dra*I fragment (nt 2913 to 2961 in DHBV 76) was deleted. It was generated by a method involving bacteriophage M13 (26). Briefly, the pDC12 fragment of *Xba*I-*Pst*I (nt 2662 in DHBV 76 and nt 26 in pBV220; both were unique sites in pDC12) was cloned into the unique sites of *Xba*I and *Pst*I of M13mp18 and M13mp19, respectively. The single-stranded DNA of recombinant M13mp18 and M13mp19 was purified; it contained the complementary sequence between the two ends of polylinker including inserted *Xba*I-*Pst*I fragment. These two kinds of M13 single-stranded DNA were annealed in 0.2 M NaCl for 4 h at 62°C to form a partially double-stranded θ-form structure in which both *Eco*RV and *Dra*I were unique sites in this double-stranded DNA region. pDC-DE was constructed by digesting the θ-form structure with *Eco*RV and *Dra*I to remove the *Eco*RV-*Dra*I fragment (nt 2913 to 2961 in DHBV 76), inserting a *Xho*I linker (CCTCG AGG) to ensure that the right C open reading frame followed, recutting with *Xba*I and *Pst*I, and recloning into *Xba*I and *Pst*I sites of pDC12.

In pDC-DN, the *Dra*I-*Nde*I fragment (nt 2961 to 18 in DHBV 76) was deleted. This plasmid was generated by cutting pDC12 with *Nde*I (unique site in pDC12), filling in the overhangs with Klenow enzyme, recutting with *Bgl*II (nt 3402 in pBV220; unique site in pDC12), purifying the larger *Nde*I-*Bgl*II fragment which acted as vector (see Fig. 1B and D for positions of *Nde*I and *Bgl*II), purifying the small 673-bp fragment for recutting with *Dra*I (nt 2961; unique site in this 673-bp fragment), and recloning the *Dra*I-*Bgl*II fragment into the *Nde*I-*Bgl*II large fragment, resulting in an in-frame deletion of 78-bp.

In pDC-HX, the *Hae*III-*Xmn*I fragment (nt 164 and 309 in DHBV 76) was deleted. It was generated as pDC-DE, that is, by first generating the M13 θ-form structure containing the double-stranded *Xba*I-*Pst*I fragment of pDC12, digesting this form with *Hae*III and *Xmn*I (both were unique sites in this double-stranded DNA region), inserting a 10-nt *Bam*HI linker (CGGGATCCCG), and recloning the deleted *Xba*I-*Pst*I frag-

ment into the corresponding position of pDC12, resulting in an in-frame deletion of 135 bp.

In pDC-AH, the *Xmn*I-*Hin*dIII fragment (nt 309 in DHBV 76 to nt 30 in pBV220) in pDC12 was deleted. It was constructed by purifying *Xmn*I-*Pvu*I (nt 309 in DHBV 76 to nt 969 in pBV220), the large fragment of pDC12 which acted as vector (see Fig. 1B and D for positions of *Xmn*I and *Pvu*I), purifying the *Hin*dIII-*Pvu*I fragment (nt 30 to 969 in pBV220; the overhangs of *Hin*dIII have been filled with Klenow enzyme), and recloning into the *Xmn*I-*Pvu*I large fragment of pDC12, resulting in an in-frame deletion with 6 (SFCFGG) of 11 nonspecific amino acids coming from the vector at the C terminus of this mutant.

In pDC-BP, the *Ban*II-*Pst*I fragment (nt 336 in DHBV 76 to nt 26 in pBV220) was deleted. pDC12 was digested with *Ban*II (nt 336 and 358 in DHBV 76; no site in pBV220) and *Pst*I (nt 26 in pBV220; unique site in pDC12), protruding 3′ termini were removed with T4 DNA polymerase, and the plasmid was recircularizated with T4 DNA ligase resulting in an in-frame deletion with seven nonspecific amino acids (PSFCFGG) deriving from the vector at the C terminus of this mutant.

Two insertion mutations, pDC-BB and pDC-NB, of DHBV core gene were constructed by digesting pDC12 with *Xba*I or *Nde*I, respectively (both of which were unique sites in plasmid pDC12), filling in the overhangs with Klenow enzyme, and inserting an 8- or 10-nt *Bam*HI linker (CGGATCCG, CGGG ATCCCG), respectively, resulting in insertion of four amino acids, SGSA or GIPY in the core protein mutants encoded by pDC-BB or pDC-NB, respectively.

Expression of DHBV core gene mutants and sucrose gradient sedimentation analysis. Transformed *E. coli* DH5α was inoculated into LB medium in the presence of 50 μg of ampicillin per ml, grown at 32°C overnight, diluted with high-expression medium (3.2% tryptone, 2% yeast extract, 1× M9 salt, 0.1 mM $MgSO_4$, 0.001 mM $FeCI_3$, 50 μg of ampicillin per ml), and grown as above until the optical density at 600 nm (OD_{600}) was 0.6. The cultures were induced by shifting the temperature to 42°C for 4 to 5 h for expression. The cell pellets were lysed with lysis buffer (50 mM Tris hydrochloride [pH 8.0], 1 mM disodium EDTA, 100 mM NaCI, 1% Triton X-100, 1 mg of lysozyme per ml) at 0°C for 30 min, and DNase I, RNase A, and $MgCI_2$ were added to final concentrations of 50 U/ml, 10 μg/ml, and 50 mM, respectively. The mixture was incubated at room temperature for 15 min. After the cellular debris had been removed by centrifugation, the supernatant was subjected to a sedimentation in a linear 10 to 50% sucrose gradient (SW27 rotor at 100,000 × *g* for 3 h at 4°C). Fractions were monitored for DHBV core protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting (see below).

Polyclonal rabbit antibody to DHBV core particles. DHBV core particles were purified from DHBV-infected duck liver by the gradient centrifugation method described previously (8). Briefly, the infected duck liver was homogenized in buffer (10 mM Tris hydrochloride [pH 7.6], 1 mM disodium EDTA, 0.3% [vol/vol] β-mercaptoethanol). The cell debris were removed by two centrifugation steps at 12,000 × *g* for 30 min at 4°C. The core particles were collected by ultracentrifugation (SW40 rotor; 100,000 × *g* for 4 h at 4°C), suspended in buffer (10 mM Tris hydrochloride [pH 7.6], 100 mM NaCI, 1 mM disodium EDTA, 0.3% [vol/vol] β-mercaptoethanol, 1% Nonidet P-40), and then applied to a 5 to 45% linear sucrose gradient. After being centrifuged for 4 h at 50,000 × *g* in an SW 40 rotor, fractions were monitored by endogenous DNA polymerase assay. Peak fractions were pooled and precipitated by a third centrifugation (SW40 rotor; 100,000 × *g* for 10 h at 4°C).

Purified DHBV core particles (200 μg) were emulsified with complete Freund's adjuvant and injected intradermally at several sites on the back of a New Zealand White rabbit. After being given two intramuscular boosters with emulsion of DHBV core particles (200 μg each time) and incomplete Freund's adjuvant, the animal was bled to obtain antiserum.

Transmission electron microscopy. The induced *E. coli* cells expressing different DHBV core protein mutants were fixed overnight at 4°C in 2.5% glutaraldehyde–2% paraformaldehyde. The fixed bacteria were rinsed with 0.1 M phosphate (pH 7.3), postfixed with 1% OsO_4, rinsed again, dehydrated in a graded series of ethanol, cleared in propylene oxide, and embedded in Epon 812. Thin sections (silver grey) were cut, stained with uranyl acetate and lead citrate, and examined with a H-800 transmission electron microscope.

SDS-PAGE and Western blotting. Bacterial suspensions or partially purified recombinant proteins were solubilized with an equal volume of 2× sample buffer (100 mM Tris hydrochloride [pH 6.8], 5% SDS, 10% [vol/vol] β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue). Samples were heated for 5 min at 100°C and centrifuged, and the supernatant was applied to the gel. SDS-PAGE was performed as previously described (13) on a 15% resolving gel. For Western blotting, the proteins on the SDS-PAGE gel were transferred electrophoretically to a nitrocellulose (NC) membrane and examined for DHBV core antigen by using a polyclonal rabbit antibody raised against the purified DHBV core particles (see above).

Agarose gel electrophoresis of DHBV core particles. The supernatant of the bacterial lysate containing DHBV core protein mutants was analyzed on a capsid agarose gel (3, 21a). The samples were mixed with 5× loading buffer (50% glycerol, 0.02% bromophenol blue) and loaded onto a 1% agarose gel with ethidium bromide (0.5 μg/ml). The gel was run in 1× TAE buffer (19) or in 10 mM sodium phosphate buffer (pH 7.5), and the gel was detected under UV light. For immunostaining, the protein was capillary transferred to NC and detected with DHBV core protein antibody.

Nucleic acid binding assays. Nucleic acid binding activities of the expressed DHBV core protein mutants were assayed by the Southwestern (DNA-protein) blotting method (6) with minor modification. Bacterial suspensions were incubated in SDS sample buffer plus β-mercaptoethanol for 10 min at 60°C and then separated by SDS-PAGE (15% running gel). After incubation of the gel for 4 to 6 h at room temperature in refolding buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM disodium EDTA, 50 mM NaCI, 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. NC membranes were presaturated in binding buffer (10 mM Tris hydrochloride [pH 7.5], 0.1 mM disodium EDTA, 1 mM dithiothreitol, 125 mM NaCI, 2× Denhardt's solution) overnight at 4°C and then incubated for 1 h at 30°C in binding buffer containing 20 ng of nick-translated plasmid pDD81, which contained a dimer DHBV 76 genome, or pUC18 (approximately 10^8 cpm/μg) per ml. After incubation, the NC membranes were extensively washed in washing solution (10 mM Tris hydrochloride [pH 7.5], 0.1 mM disodium EDTA, 50 mM NaCI, 0.5× Denhardt's solution) at 37°C, dried, and exposed to X-ray film.

RESULTS

Expression of the DHBV core protein mutants in *E. coli*. In this study, 12 plasmids with various deletion and insertion mutations in the DHBV C open reading frame were constructed (Fig. 1). The deletion mutations covered 80% of this

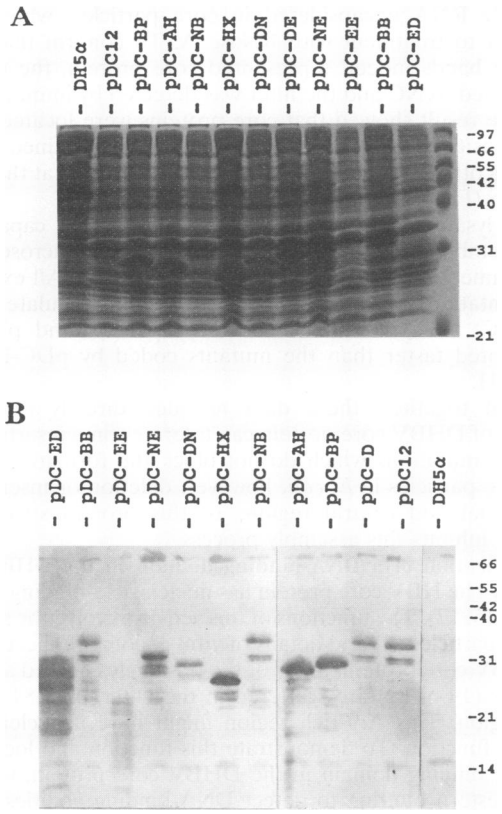

FIG. 2. Expression of different DHBV core protein mutants in *E. coli*. Freshly induced *E. coli* cells containing the individual plasmid with the core gene mutation were lysed with SDS buffer and analyzed by SDS-PAGE (15% polyacrylamide) followed by staining with Coomassie blue (A) or transferred onto an NC filter and reacted with a polyclonal rabbit anti-DHBV core particle antibody (see Materials and Methods) for Western blot analysis (B). Sizes are shown in kilodaltons.

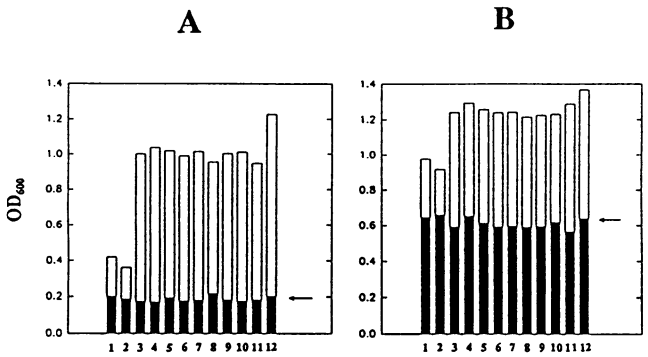

FIG. 3. The expressed DHBV core protein mutants encoded by pDC-ED and pDC-EE inhibited the growth of *E. coli*. The transformed cells were grown overnight at 32°C in LB containing ampicillin (50 μg/ml). The culture medium was diluted 1:100 into 20 ml of fresh LB-ampicillin broth and grown at 32°C. The OD_{600} was determined every 1 h. When the OD_{600} was around 0.2 (A) or 0.6 (B), 2 ml of the cultures was removed, added to one-third volume of LB-ampicillin broth at 65°C, and grown at 42°C for 5 h. Then the OD_{600} was measured. Symbols: ■, OD_{600} before induction; □, OD_{600} after 5 h of induction; ←, beginning of induction. Bars: 1, pDC-ED; 2, pDC-EE; 3, pDC-NE; 4, pDC-DE; 5, pDC-DN; 6, pDC-HX; 7, pDC-AH; 8, pDC-BP; 9, pDC-BB; 10, pDC-NB; 11, pDC-12; 12, *E. coli* DH5α.

gene. Expression of these 12 DHBV core protein mutants was induced by raising the temperature of the culture, which caused inactivation of a temperature-sensitive repressor encoded by the phage λ *c*I857 gene. The predicted sizes of the expressed DHBV core protein mutants, according to deduced amino acid sequences, were detected in an SDS-PAGE gel stained with Coomassie blue (Fig. 2A) and confirmed by a Western blot (Fig. 2B) with polyclonal rabbit antibody raised against the core particles purified from DHBV-infected duck liver (see Materials and Methods).

It was interesting to find that there were no apparent expressed protein bands for plasmids pDC-ED and pDC-EE in Coomassie blue-stained SDS-PAGE gels (Fig. 2A). However, the results of Western blot (Fig. 2B) and DNA-binding (see Fig. 6A) studies demonstrated that the DHBV core protein mutants were indeed expressed in the bacteria harboring pDC-ED and pDC-EE, respectively, but were in a state of degradation compared with the other DHBV core protein mutants. On the other hand, as shown in Fig. 2A, the amounts of total proteins in the bacterial lysates containing pDC-ED or pDC-EE were smaller than those in the other strains. This indicated that the mutated DHBV core gene in pDC-ED and pDC-EE or expressed products would be toxic to the host cells. By using an assay of bacterial growth rate, we demonstrated that it was the expressed products encoded by pDC-ED or pDC-EE which were responsible for this effect, because the growth rate of *E. coli* carrying these two plasmids began to decrease just after induction at 42°C (Fig. 3). This inhibition by expressed products was more apparent at the early exponential growth phase of the host cells (compare Fig. 3A and B). In pDC-ED and pDC-EE, most of the N-terminal sequence of the DHBV core protein was deleted; that is, the regions coding for amino acids 3 to 53 and 3 to 89 of the core protein were deleted in pDC-ED and pDC-EE, respectively (Fig. 1; Table 1). However, deletion of the region coding for amino acids 67 to 89 in pDC-NE did not result in either degradation of the core products or inhibitory effects on bacterial growth as in pDC-ED and pDC-EE (Fig. 2 and 3; also see Fig. 6A). These data suggested that the existence of the N-terminal sequence, probably within amino acids 3 to 66, was necessary to maintain the basic structure and function of the expressed DHBV core protein in *E. coli* and presumably was important for the self-assembly process of the DHBV core particle.

Transmission electron micrographs of thin sections of *E. coli* expressing different DHBV core protein mutants. In a preliminary experiment, we noted that the DHBV core-like

TABLE 1. Deleted amino acid sequences and insertions at deletion sites caused by deletion or inserted linker

Plasmid	Position of deletion (amino acid)	Insertion at deletion site
pDC-ED	3–53	No insertion
pDC-EE	3–89	RDPD
pDC-D	53	No insertion
pDC-NE	67–89	N
pDC-DE	89–105	SSR
pDC-DN	106–131	No insertion
pDC-HX	181–228	GIP
pDC-AH	229–262	Nonspecific[a]
pDC-BP	237–262	Nonspecific[b]

[a] Six nonspecific amino acids (SFCFGG) at the C terminus.
[b] Seven nonspecific amino acids (PSFCFGG) at the C terminus.

particles can be seen in the transmission electron micrograph of *E. coli* thin sections harboring pDC12. These spherical particles with uniform morphology arranged in a crystalline model, located almost at the ends of *E. coli*, are easy to recognize. This unique feature would undoubtedly facilitate the studies of the self-assembly ability of the DHBV core protein mutants. For this purpose, the pellets of freshly induced bacteria expressing different DHBV core protein mutants were directly fixed, dehydrated, and embedded in Epon 812. After being stained with uranyl acetate and lead citrate, the thin sections of *E. coli* were examined under a transmission electron microscope.

Among 12 DHBV core protein mutants, 3 (pDC12, pDC-BB, and pDC-BP) self-assembled to form the core-like particles in *E. coli*. (Fig. 4, panels 2, 3, and 13). The core particles assembled by the different mutants showed similar size, averaging 28 nm in diameter. Unlike pDC12 and pDC-BB, pDC-BP lost the crystalline array ability in *E. coli*. The mutations in these plasmids were located at both 5′ and 3′ ends of the C open reading frame (Fig. 1). It was notable for pDC-AH, in which the deletion of an additional 8 amino acids based on pDC-BP (amino acids 237 to 262 deleted in pDC-BP) (Fig. 1; Table 1) largely changed the morphology of assembled particles. As shown in Fig. 4, panel 12, many small particles with a diameter of ca. 20 nm accumulated randomly at the ends of *E. coli*.

In contrast, other DHBV core protein mutants could not all form the typical core particles, which were replaced by inclusion bodies in *E. coli* (Fig. 4, panels 4 to 11). These core protein mutants contained deletion mutations in the N-terminal and central regions (pDC-ED, pDC-D, pDC-EE, pDC-NE, pDC-DE, pDC-DN, and pDC-HX) or an insertion mutation in the central region (pDC-NB) (Fig. 1; Table 1). Compared with pDC12, whose products have the self-assembly ability in *E. coli*, there was only one amino acid deleted (phenylalanine, residue 53 of the DHBV core protein) or four amino acids inserted (GIPY inserted between residues 132 and 133 of this protein) in the mutants encoded by pDC-D and pDC-NB, respectively. However, these small mutations resulted in the failure of the formation of the core particles in *E. coli*.

To support the results of electron-microscopic observation, we tried to partially purify expressed DHBV core protein mutants from *E. coli* for the purpose of performing capsid agarose gel and sucrose gradient sedimentation analysis. We found that lysozyme-detergent treatment cannot release expressed products into the soluble supernatant of bacterial lysate; however, after the lysate was digested with DNase I and RNase A, the expressed products were released from the insoluble materials. A Western blot of SDS-PAGE gels showed lower molecular weights, compared with predicted sizes, for the core mutants which did not form core-like particles under electron-microscopic observation; the products encoded by N-terminally deleted mutants, pDC-ED and pDC-EE, nearly disappeared (data not shown). The results indicated that proteolysis had occurred during the nuclease treatment. On the other hand, a large proportion of released core mutants encoded by pDC-12, pDC-BB, pDC-BP, and pDC-AH, which all formed electron microscopically observable core-like particles in *E. coli*, still located at the positions with predicted molecular weights after nuclease digestion (data not shown), suggesting that the assembled state of these proteins may be responsible for this resistance to proteolysis.

For capsid agarose gel analysis, the lysate supernatant of different core mutants was used. Ethidium bromide-stained bands were seen only in the samples of bacteria harboring pDC12, pDC-BP, pDC-BB, and pDC-AH (Fig. 5A). According to the data reported previously (3), these bands were most probably RNA encapsidated in core particles, which were resistant to treatment with RNase A. To confirm that these staining bands indeed represented core protein, the gel was transferred to NC and the filter was detected by immunostaining. The result showed that core proteins were located at the same positions as that of ethidium bromide-stained bands, whereas other mutants displayed the smear bands at the lower position (Fig. 5B).

The lysate supernatants of the four strains capable of self-assembling core mutants were subjected to sucrose gradient sedimentation (see Materials and Methods). All exhibited sedimentation velocities indicative of their particulate nature (Fig. 5C). The mutants encoded by pDC12 and pDC-BB sedimented faster than the mutants coded by pDC-BP and pDC-AH.

Taken together, these data revealed directly that both termini of DHBV core protein can tolerate some insertion and deletion mutations which do not block the formation of the core-like particles in *E. coli*; however, deletion or insertion in N-terminal and central regions of this protein strongly or entirely inhibits this assembly process.

Localization of a DNA-binding domain in the DHBV core protein. The HBV core protein has nucleic acid-binding activity (3, 6, 9, 15, 17). The functions of this activity involve the stability of core particles (3) and details of viral genome replication (9). In DHBV core protein, many of the positively charged arginine residues (19 of 29) are scattered in the C-terminal 84-amino-acid region. This Arg-rich region might have a nucleic acid-binding function. To demonstrate this function and locate the possible binding domain in the DHBV core protein, we used Southwestern blotting to detect DNA-binding abilities of the different core protein mutants. As shown in Fig. 6A, all DHBV core protein mutants bound effectively to the denatured pDD81 DNA probe (see Materials and Methods), except the mutant encoded by pDC-HX, in which amino acids 181 to 228 were deleted (Fig. 1; Table 1). The result suggested that amino acids 181 to 228 of DHBV core protein contained, at least in part, a DNA-binding domain.

The binding of the expressed HBV core protein by cellular nucleic acid (3) indicates that this binding ability of hepadnavirus core protein was not specific to viral nucleic acid itself. The results shown in Fig. 6B, lane 1, demonstrate that the expressed DHBV core protein (encoded by pDC12) can bind a nonspecific DNA probe, pUC18, as effectively as pDD81, which contained a head-to-tail double-copied DHBV DNA. The binding with pUC18 can be blocked completely by denatured salmon sperm DNA (Fig. 6B, lane 2).

DISCUSSION

This article presents the consequences of different DHBV core protein mutants on the self-assembly and nucleic acid binding of this virus.

The fact that core-like particles can be assembled in *E. coli* (3, 4, 6) indicates that the core protein itself contains all information necessary for the self-assembly process. As the basis of the assembly which would involve intermolecular recognition and interaction, intramolecular folding of individual molecules must happen first to form a suitable tertiary structure. The results reported here suggest that the N-terminal region of the DHBV core protein is important for its tertiary structure. The DHBV core mutants with a deleted N-terminal region apparently inhibited the growth of the host cells more than did the other core mutants with central or C-terminal deletions. This functional difference caused by the expressed products indicates that a structural variation proba-

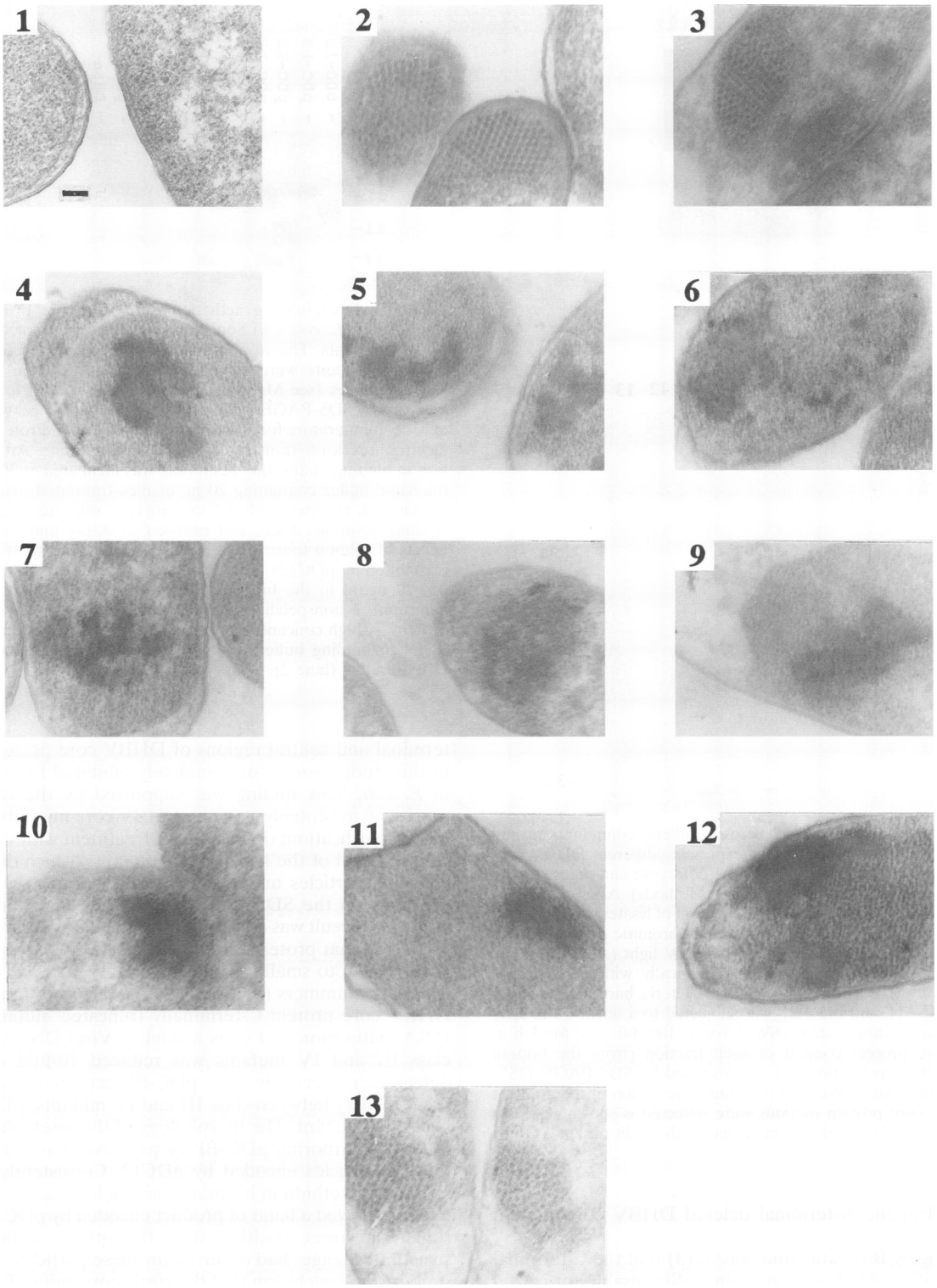

FIG. 4. Transmission electron micrographs of thin sections of *E. coli* expressing the different mutants of DHBV core protein. Freshly induced *E. coli* cells were fixed overnight at 4°C in 2.5% glutaraldehyde–2% paraformaldehyde. The fixed cells were rinsed in phosphate (pH 7.3), postfixed in 1% osmic acid at room temperature for 2 h, rinsed in phosphate (pH 7.3) again, dehydrated in a graded series of ethanol, cleared in propylene oxide, and embedded in Epon 812. Thin sections (silver grey) were cut, stained with uranyl acetate and lead citrate, and examined under a H-800 transmission electron microscope. Numbers represent *E. coli* DH5α transformed with different recombinant plasmids: 1, DH5α only; 2, pDC12; 3, pDC-BB; 4, pDC-ED; 5, pDC-EE; 6, pDC-D; 7, pDC-NE; 8, pDC-DE; 9, pDC-DN; 10, pDC-NB; 11, pDC-HX; 12, pDC-AH; 13, pDC-BP. Magnification, ×30,000. Bar, 100 nm.

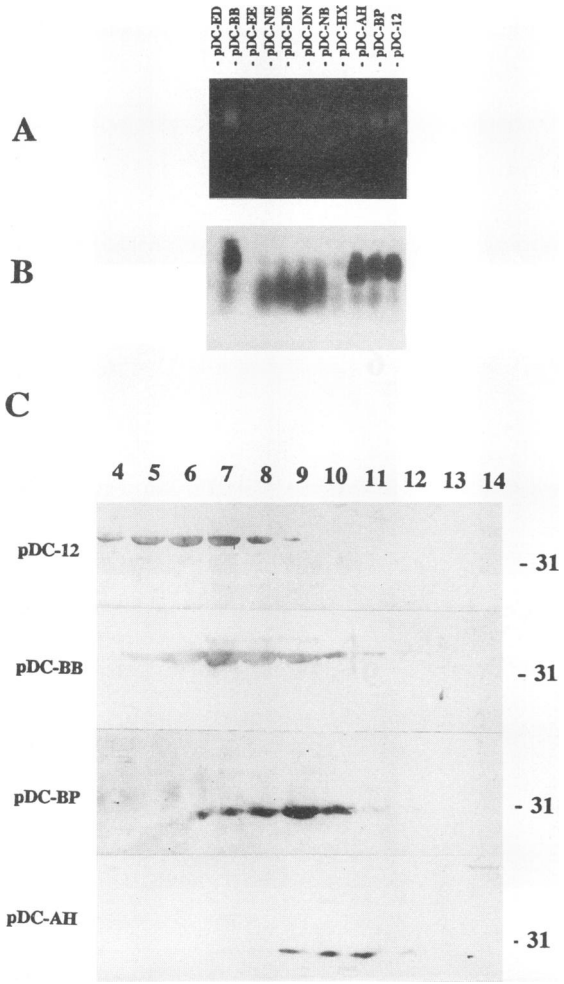

FIG. 5. Capsid agarose gel and sucrose gradient sedimentation analyses of bacterial lysates. The bacteria expressing different DHBV core protein mutants were lysed with lysozyme-detergent and digested with DNase I and RNase A (see Materials and Methods). After centrifugation to pellet the cellular debris, the supernatant of lysates was loaded on 1% agarose gel containing 0.5 μg of ethidium bromide per ml and run in TAE buffer. The gel was detected under UV light (A), or capillary transferred to NC for detection immunologically with DHBV core protein antibody (B). The supernatant of bacteria harboring pDC12, pDC-BB, pDC-AH, and pDC-BP was subjected to a linear 10 to 50% sucrose gradient sedimentation (SW27 rotor; 100,000 × *g* for 3 h at 4°C). The core protein content of each fraction (from the bottom fraction 1 to the top fraction 16) was analyzed by SDS-PAGE (15% running gel) followed by Western blot. Only the region of the gradient where DHBV core protein mutants were detected is shown (C). The position of the 31-kDa marker is indicated to the right of panel C.

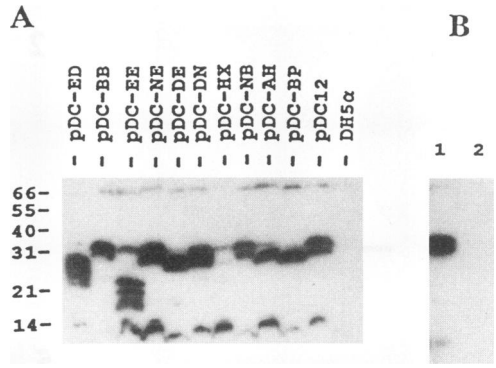

FIG. 6. DNA-binding activities of DHBV core protein mutants expressed in *E. coli*. (A) Southwestern blot results for different core protein mutants. The suspensions of bacteria induced for expressing different mutants were lysed with an equal volume of 2× SDS reducing buffer (see Materials and Methods) at 60°C for 10 min and analyzed by SDS-PAGE (15% running gel), and the gel was incubated at room temperature for 4 to 6 h in refolding buffer. Proteins were then electrophoretically transferred to an NC membrane. After presaturation in binding buffer, the membrane was incubated at 30°C for 1 h in the same buffer containing 20 ng of nick-translated plasmid pDD81 (denatured, approximately 10^8 cpm/μg) per ml, extensively washed in washing solution, dried, and exposed to X-ray film. (B) Inhibitory effects of salmon sperm DNA on the binding activity of core protein (expressed by pDC12). The denatured plasmid pUC18 (ca. 10^8 cpm/μg, 20 ng/ml in the binding buffer) was used in lanes 1 and 2 to determine a nonspecific nucleic acid-binding ability of DHBV core protein. A high concentration of denatured salmon sperm DNA (500 μg/ml in binding buffer) was added during both presaturation and DNA binding (lane 2).

bly occurred in the N-terminal deleted DHBV core protein mutants.

The finding by Birnbaum and Nassal (3) that the C-terminal 39 residues of the HBV core protein is dispensable for capsid formation in *E. coli* prompted us to address whether DHBV core protein can tolerate an N-terminal or central mutation for assembling the capsid. The results of electron microscopy, capsid gel, and sucrose gradient centrifugation demonstrate that a small-fragment insertion at the N terminus as well as a deletion in the C-terminal region can be tolerated in the assembly process. Deletion or insertion in most of the N-terminal and central regions of DHBV core protein reported in this study strongly or completely inhibited its self-assembly in *E. coli*. This finding was supported by the data on the sensitivity to proteolysis of the DHBV core mutants during the partial purification; that is, during treatment with DNase I and RNase A, all of the DHBV core mutants, which did not show core-like particles under electron microscopy, degradated to positions on the SDS-PAGE gel smaller than the predicted sizes. This result was consistent with the data reported by Zhou et al. (32) that protease treatment cleaved unassembled HBV core protein to smaller products.

Yu and Summers (29) reported the effects of four classes of DHBV core protein C-terminally truncated mutants on viral DNA maturation and virus assembly. Viral DNA synthesis in class III and IV mutants was reduced 10-fold or severely defective, respectively (29). pDC-BP reported here fell near the boundary between class III and IV mutants; pDC-AH was a class IV mutant. The morphology of the assembled particles in *E. coli* harboring pDC-BP or pDC-AH was different from that of particles encoded by pDC12. Consistently, immunostaining and ethidium bromide staining in a capsid agarose gel (Fig. 5) showed a band of product encoded by pDC-AH with a somewhat lower position than the others, indicating that structural changes had occurred for these particles. Our results support the conclusion that the steric constraint of the mutant capsids is one of the reasons responsible for the inhibition of viral DNA synthesis.

In HBV, identification of the possible amino acid sequences responsible for the binding between the core protein and nucleic acid was controversial (6, 15). Most studies (3, 6, 9) suggest that the C-terminal arginine-rich region is a nucleic acid-binding domain and that this binding contributes impor-

tantly to HBV capsid stability (3) and viral DNA replication (9). Here, we detected a nucleic acid-binding activity of DHBV core protein by the Southwestern blot method (Fig. 6). On the basis of the results that most of this activity was lost in the mutant encoded by pDC-HX, whereas no other mutant showed a detectable change of the nucleic acid-binding activity, we suggest that at least part of the nucleic acid-binding domain of DHBV core protein is located in the deletion region of pDC-HX (amino acids 181 to 228). The high content of positively charged amino acids (10 arginine residues and 5 lysine residues in this region of 48 residues) indicates that it may be a nonspecific binding secondary to interactions with negatively charged nucleic acids. The results of the binding studies with pUC18 were consistent with this prediction (Fig. 6B). It was interesting that the C terminus of DHBV core protein does not involve this nucleic acid-binding activity, although eight arginine residues, two lysine residues, and two histidine residues are located in this 34-amino-acid region. These data might be interpreted to indicate that the position of a fragment in the core protein molecule (for example, on the surface or in the inner part) or some unknown secondary structure in the fragment may determine its role on the nucleic acid binding of DHBV core protein. Moreover, the results of nonspecific nucleic acid binding of core protein in this study cannot eliminate the possibility that some different but highly specific binding between the core protein and viral genome is involved in the encapsidation of the viral RNA pregenome.

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