Hepatitis B viral core proteins with an N-terminal extension can assemble into core-like particles but cannot be enveloped

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The structure of hepatitis B virus (HBV) nucleocapsids has been revealed in great detail by cryoelectron microscopy. How nucleocapsids interact with surface antigens to form enveloped virions remains unknown. In this study, core mutants with N-terminal additions were created to address two questions: (1) can these mutant core proteins still form nucleocapsids and (2) if so, can the mutant nucleocapsids interact with surface antigens to form virion-like particles. One plasmid encoding an extra stretch of 23 aa, including six histidine residues, fused to the N terminus of the core protein (designated HisC183) was expressed in Escherichia coli and detected by Western blot. CsCl gradient and electron microscopy analyses indicated that HisC183 could self-assemble into nucleocapsids. When HisC183 or another similar N-terminal fusion core protein (designated FlagC183) was co-expressed with a core-negative plasmid in human hepatoma cells, both mutant core proteins self-assembled into nucleocapsids. These particles also retained kinase activity. Using an endogenous polymerase assay, a fill-in HBV DNA labelled with isotope was obtained from intracellular nucleocapsids formed by mutant cores. In contrast, no such signal was detected from the transfection medium, which was consistent with PCR and Southern blot analyses. Results indicate that core mutants with N-terminal extensions can form nucleocapsids, but are blocked during the envelopment process and cannot form secreted virions. The mutant nucleocapsids generated from this work should facilitate further study on how nucleocapsids interact with surface antigens.

Introduction

Human hepatitis B virus (HBV) has been a focus of clinical and basic studies due to its infection being a worldwide health problem and its unique structure and life-cycle among animal DNA viruses (Szmuness, 1978; Beasley et al., 1981; Tiollais et al., 1981; Ganem & Varmus, 1987). HBV DNA is partially double-stranded and about 3-2 kb long. Although its genome is DNA, it replicates through an RNA intermediate (Summers & Mason, 1982), the so-called ‘pregenomic RNA’. The pregenomic RNA (pregenome) and viral polymerase, together with cellular kinase and heat shock proteins, are encapsidated by viral core proteins, which have 183 or 185 aa residues depending on sero-subtypes, to form nucleocapsids (Nassal & Schaller, 1993; Kann & Gerlich, 1994; Nassal, 1996; Hu & Seeger, 1997; Kau & Ting, 1998). Inside the nucleocapsids, the first strand of DNA is synthesized by reverse transcription (see review by Nassal, 1996). Thereafter, nucleocapsids interact with viral surface antigens that are embedded in a lipid bilayer of endoplasmic reticulum (ER) and bud into the ER lumen to form mature virions, termed Dane particles (Bruss & Ganem, 1991; Huovila et al., 1992; Wei et al., 1996). Recently, the interaction domains of capsid proteins and surface antigens were characterized and mapped (Dyson & Murray, 1995; Poisson et al., 1997; Bruss, 1997). However, the interaction mechanism between nucleocapsid proteins and surface antigens remains poorly understood.

A model of the three-dimensional structure of HBV nucleocapsid has been revealed to a resolution of 35–0-7-4 Å by cryoelectron microscopy (Crowther et al., 1994; Conway et al., 1997, 1998a; Zlotnick et al., 1996, 1997; Bottcher et al., 1997, 1998). The proposed models agree that three regions of core protein, as 78–82, 127–130 and 145–153, are exposed on the shell of nucleocapsids. Neither the N nor the C terminus of the core protein is on the external surface. The N-terminal
portion (aa 1–150) of core protein is capable of self-assembling into nucleocapsids even in the absence of the C terminus (Birnbaum & Nassal, 1990; Nassal, 1992; Halton et al., 1992). In contrast, core mutants bearing a small insertion, substitution or deletion in the N-terminal domain of HBV core protein (Beames & Lanford, 1995; Metzger & Bringas, 1998; Konig et al., 1998) or woodchuck hepatitis virus (WHV) core protein (Yu et al., 1996) fail to form nucleocapsids. However, studies on duck HBV show that the core protein N-terminal additions have various effects on capsid formation depending on the nature of the extension peptides (von Weiszacker et al., 1996; Kock et al., 1998). Therefore, the influence of the core protein N terminus on nucleocapsid assembly needs to be further investigated.

Conway et al. (1998b) used an extraneous octapeptide to demonstrate that the core protein N terminus is localized at the spike near the entrance of the shell. In this study, we constructed and expressed N-terminal extension core proteins which were either rich in a positive charge of histidine (designated HisC183) or rich in a negative charge of glutamic acid (designated FlagC183) to test their effect on nucleocapsid assembly in either a prokaryotic or a eukaryotic system. We also tested similar constructs for their influence on virion formation by trans-supplementing a core-negative HBV clone in HuH-7 hepatoma cells. We have demonstrated that the N terminal extension of core proteins does not interfere with core protein dimerization (Zheng et al., 1992; Zhou & Standring, 1992) and nucleocapsid assembly. Since protein kinase activity has been demonstrated in the HBV nucleocapsid (Albin & Robinson, 1980; Gerlich et al., 1982), in which the serine residues located at the C terminus of core protein are the substrate of the HBV-associated kinase (Roossinck & Siddiqui, 1987; Yeh & Ou, 1991), we have also shown that the N-terminal core mutants do not interfere with kinase encapsidation. However, the result of blocking nucleocapsids from envelopment by surface antigens supported the hypothesis that the N terminus of the core protein is localized near the surface of the capsid shell.

Methods

**Plasmids and construction of mutants.** Standard techniques were used for DNA manipulation (Sambrook et al., 1989). Plasmids pMH 3/3097 (Junker et al., 1987) and pMH 3/3097mC (Lin & Lo, 1992) were used as described previously. pHisC183 (Fig. 1a, line 2) is a prokaryotic expression plasmid which encodes a N-terminal His-tagged core protein. The vector pET-15b (Novagen), containing a His-tag, was cleaved with Xhol (filled-in) and BamH1 and then ligated with the 1 kb Ncol (filled-in)/BamH1 fragment from pMH 3/3097mC to generate pHisC183. pHBVisC183 (Fig. 1a, line 3) is a eukaryotic expression plasmid which, like pHisC183, encodes an N-terminal His-tagged core protein. The Ncol/EcoRI fragment of pHisC183, containing the His-tag fused C gene, was ligated with the EcoRI/Ncol-digested pMH 3/3097mC to give pHBVisC183. pHBVFlagC183 (Fig. 1a, line 4) is another eukaryotic expression plasmid which encodes an N-terminal Flag-tagged core protein and was obtained from the insertion of Ncol (filled-in)/BamH1 fragment from pMH 3/3097mC into the HindIII (filled-in) and BamH1 sites of pFLAG-CMV2 vector (Eastman Kodak). The junctions of newly created plasmids were ascertainment by the dyeoxy termination method of DNA sequencing (Sanger et al., 1977). All plasmids used in this study are shown in Fig. 1(a) and the characteristics of the N-terminal extensions of the core protein are listed in Fig. 1(b).

**Core protein expression in Escherichia coli.** E. coli strains BL21(DE3) or JM109(DE3) harbouring various plasmids were grown to an OD_{600} of 1.0 in 800 ml LB medium containing 50 µg/ml ampicillin, followed by the addition of 1 mM IPTG; the culture was then shaken at 37°C for another 3 h. Bacteria were lysed in a French press (800 psi) and the cellular debris was removed by centrifugation at 18000 g for 15 min. The supernatant was collected and passed through a 0.45 µm filter.

**Purification of core protein.** E. coli lysates from the 800 ml culture described above were incubated with Ni^{2+}-chelated His-select® bind resin (Novagen) in binding buffer (20 mM Tris–HCl, pH 7.9, 0.5 M NaCl and 5 mM imidazole) at room temperature for 1 h. After three washes with binding buffer and washing buffer (20 mM Tris–HCl, pH 7.9, 0.5 M NaCl and 60 mM imidazole), the proteins were eluted with elute buffer (20 mM Tris–HCl, pH 7.9, 0.5 M NaCl and 1 M imidazole) at room temperature for 30 min. The eluate (5 ml) was collected (concentration 0.17 mg/ml) and aliquots (10 µl) were run on 15% SDS–PAGE (Laemmli, 1970) and stained with Coomassie brilliant blue for analysis of protein purity.

**CsCl gradient centrifugation.** E. coli lysates or nucleocapsids from media or cell lysate (see below) were resuspended in a high-salt TNE buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 0.1% sodium azide) and subjected to CsCl centrifugation (average density 1.24 g/ml) at 35000 r.p.m. in an SW41 rotor for 44 h. The gradients (final density 1.1–1.5 g/ml) were fractionated into 0.5 ml samples from the top to bottom and each fraction was subjected to ELISA for detection of core antigen and/or surface antigen (General Biologicals). The density of individual fractions was determined by the refractive index using a refractometer.

**Electron microscopy.** Core particles prepared from Ni^{2+}-chelated His-select® bind resin and CsCl fractions were collected and spotted onto Formvar-coated grids, then negatively stained with saturated uranyl acetate and visualized in a JEOL JEM-2000ExII transmission electron microscope as described previously (Chang et al., 1987).

**Cell culture and transfection.** Human hepatoma cells (HuH-7) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal bovine serum, 1 mM glutamine and 100 µg/ml streptomycin. HuH-7 cells were transfected with an appropriate amount of plasmid, either singly or in combinations, using the calcium phosphate co-precipitation procedure (Graham & van der Eb, 1973; Sambrook et al., 1989). At 3, 6 and 9 days post-transfection, media were harvested for viral particle analysis. At 9 days post-transfection, cells were harvested for analysis of nucleocapsid and viral DNA.

**Viral nucleocapsid isolation.** To collect nucleocapsids from intracellular extracts, transfected cells were detached from plates and incubated in PBS with 1% NP-40 at 4°C overnight (Beames & Lanford, 1995). After a low-speed centrifugation to remove nuclei and NP-40-insoluble cell debris, the cellular lysate was ready for a further isolation step. To collect nucleocapsids and virion-like particles from culture fluids, the media were incubated with or without detergent (NP-40 to a final concentration of 1%) at room temperature for 2 h before centrifugation. Both samples were clarified by centrifugation at 13000 r.p.m. for 30 min in a JA-20 rotor (Beckman Instruments). The nucleocapsids were then concentrated by further centrifugation at 45000 r.p.m. in a Ti55.2 rotor.
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Fig. 1. Diagrammatic representation of plasmids used in this study and characteristics of the N termini of core proteins.

(a) Plasmid designations are shown at the left of lines 1–7. Plasmids are shown by a partial sequence which contains the HBV DNA and the metallothionein or cytomegalovirus promoter (arrows marked MT and CMV, respectively). The boxes above the restriction enzyme map represent the HBV genes in a linear form, in which the shaded box marks the redundant core gene. Common restriction enzyme cutting sites used for cloning have also been indicated. The thick line on plasmids represents the HBV DNA sequence; ε indicates the packaging signal of HBV pregenome; the diamond symbol indicates the HBV polyadenylation signal. The thin line shown by an inverted V-shape is the region of HBV DNA deletion. The filled-in circle indicates the fusion epitope. (b) Comparison between the N-terminal amino acid sequences of the wild-type (C183) and mutants (HisC183 and FlagC183). The numbers indicate the positions of amino acids; the ‘1’ marks the first methionine residue of the core protein. Charged amino acid residues are indicated by ‘+’ and ‘−’.

for 2–5 h while the Dane-like particles were enriched under the same conditions for 5 h. The isolated viral particles were subsequently resuspended in a low-salt TNE buffer (10 mM Tris–HCl, pH 7–5, 100 mM NaCl and 1 mM EDTA).

**Immunoprecipitation and Western blotting analysis.** The precleared cell lysate was incubated with primary antibodies bound with Sepharose–protein A or G. Antibodies of rabbit polyclonal anti-core (Dako), mouse monoclonal anti-6His (Clontech) and anti-Flag (Kodak) were used. The antigen–antibody complexes were precipitated and washed three times with NET buffer (50 mM Tris–HCl, pH 7–5, 150 mM NaCl, 0–5 mM EDTA and 0–5% NP-40) and then boiled in sample buffer (Laemmli, 1970) and analysed by 15% SDS–PAGE under reducing (with DTT) or non-reducing (without DTT) conditions. Western blotting was performed by reacting with anti-core, anti-6His or anti-Flag antibodies followed by reacting with secondary antibodies, conjugated with horseradish peroxidase (Organ Teknika). The blot was developed with an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia).

**In vitro kinase assay.** The immunoprecipitated nucleocapsids were washed three times and then incubated in a kinase reaction buffer (50 mM Tris–HCl, pH 7–4, 10 mM MgCl₂ and 0–4% NP-40) containing 10 pmol [γ-³²P]ATP (7000 Ci/mmol; Amersham) as described previously (Jeng et al., 1991; Lin & Lo, 1992). After incubation at 37 °C for 1 h, the complex was washed five times with NET buffer and then subjected to 15% SDS–PAGE separation and autoradiographed.

**Endogenous DNA polymerase assay.** Endogenous DNA polymerase activity was assayed as described previously (Junker et al., 1987) with some modification (Chiang et al., 1990; Lin & Lo, 1992). Briefly, one-fifth of the partially purified viral particle samples was incubated with a
Fig. 2. Detection of HisC183 expression and nucleocapsid formation in *E. coli*. (a) *E. coli* strains BL21(DE3) (lanes 1–4) or JM109(DE3) (lanes 5–8) were transfected with plasmid pHisC183. After incubation with IPTG (lanes 2, 4, 6 and 8) or without IPTG (lanes 1, 3, 5 and 7), the total cell lysates were analysed by 15% SDS–PAGE and then stained by Coomassie brilliant blue (upper panel) or probed with anti-core antibody (lower panel). Numbers on the right indicate the positions of the protein molecular mass markers (lane M) in kDa. (b) Density gradient analysis of nucleocapsids in soluble fractions from *E. coli* harbouring pHisC183 after IPTG induction. Lysates were obtained in a French press and subjected to CsCl gradient density centrifugation. Twenty fractions (500 µl each) were collected and analysed for the presence of core protein by ELISA. The density (g/ml) of individual fractions is shown by a dotted line. The filled-in circles show the OD₄₉₀ in the ELISA of each fraction. The inset shows the particle-like structure from the HBCAg-positive fraction (fractions 5–8); bar, 50 nm. (c) Cell lysates were passed through an Ni²⁺ affinity chromatography column, the eluate was analysed by 15% SDS–PAGE and stained by Coomassie brilliant blue. Numbers on the left (lane 1) indicate the position of the protein molecular mass markers in kDa. The arrow on the right indicates core protein. (d) Electron micrograph showing core-like particles from the eluate sample; bar, 50 nm.

pol-mix buffer (50 mM Tris–HCl pH 7.4, 40 mM NH₄Cl, 5 mM MgCl₂, 0.5% NP-40, 0.2% 2-mercaptoethanol and 25 µM each of dATP, dGTP and dTTP) at 37 °C for 2 h in the presence of [α-³²P]dCTP (5000 mCi/mmol; Amersham). Subsequently, a chase was performed for 2 h by adding unlabelled dCTP (25 µM final concentration) at 37 °C. Unwanted nucleic acids present outside of particles were digested by micrococcal
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Results

Expression of HBV core proteins with an N-terminal extension in E. coli

In this study, pHisC183 was constructed for the expression of HBV core proteins with an N-terminal extension, designated HisC183. HisC183 has 23 extra amino acids at the N terminus, including a stretch of six histidine residues (Fig. 1b). Expression of HisC183 was examined in two different strains of E. coli, BL21(DE3) and JM109(DE3), by SDS–PAGE and Western blot analyses. The Coomassie brilliant blue-stained gel showed no extra band (Fig. 2a, upper panel); however, numerous bands were detected by anti-core antibodies in Western blots (Fig. 2a, lower panel) when plasmid-harbouring bacteria were treated with IPTG. Although a few core-positive bands were also detected in the same plasmid-harbouring bacteria without IPTG treatment, this was most likely due to leaky transcription of the T7 promoter. Nevertheless, the molecular mass of the highest and dominant band is as expected, suggesting that fusion core proteins were expressed and some of them were degraded into small fragments.

Mutant core with an N-terminal His-tag can self-assemble in E. coli

Since HisC183 was expressed in E. coli and easily extracted, it was of great interest to know whether HisC183 could self-assemble into nucleocapsids spontaneously as previous reports have suggested (Pasek et al., 1979; Cohen & Richmond, 1982; Stahl et al., 1982). Soluble lysates from bacteria expressing HisC183 were centrifuged through CsCl density gradients and each fraction was subjected to ELISA for the presence of HBV core antigen (HBcAg). Several fractions of cell lysates from IPTG-induced bacteria exhibited a positive signal for HBcAg, i.e. peaks at densities of 1.18–1.20 and 1.26–1.31 g/ml (Fig. 2b). This result strongly indicated that HisC183 could self-assemble into nucleocapsids. Electron
Fig. 4. For legend see facing page.
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Fig. 5. Particle-associated protein kinase assay. HuH-7 cells were transfected with pUC-MT (lane 1), pMH 3/3097 plus pUC-MT (lane 2), pHBVC183 plus pHBVΔC (lane 3) or pHBVFlagC183 plus pHBVΔC (lane 4). Particles were isolated from intracellular extracts (upper panel) or extracellular media (lower panel) and were subjected to an in vitro phosphorylation assay. The expected core proteins from transfections are indicated at the top of the gel. Numbers on the right indicate the position of the protein molecular mass markers in kDa.

Fig. 6. Endogenous polymerase reaction products. HuH-7 cells were transfected with various plasmids as specified at the top of the gel. Intracellular nucleocapsids (a) and Dane-like particles from extracellular media (b) were collected. Dane-like particles were treated with NP-40 and, together with nucleocapsids, were analysed by endogenous polymerase reaction and the products were separated on an agarose gel and visualized by autoradiography of the dried gel. Positions of the 32P-labelled λ HindIII markers (bp) are shown on the right (lane M). Arrowheads indicate the positions of the repaired HBV DNA signals.

microscopic examination of negatively stained samples from the CsCl density peak (fractions 5–8) further confirmed that the nucleocapsids indeed formed in E. coli (inset of Fig. 2b).

To clarify whether or not the His-tag of HisC183 is exposed at the surface of nucleocapsids, we used an affinity column to purify HisC183. Results of Ni²⁺-affinity binding resin showed a single band in a 15% SDS–PAGE gel stained with Coomassie brilliant blue (Fig. 2c), indicating that the His-tag of HisC183 was accessible by nickel resins. Protein concentration analyses revealed that this band comprised approximately 8.5 mg HisC183, which was recovered from the 800 ml overnight culture. However, HisC183 bound on nickel resins could be in monomeric, dimeric or particulate form. Electron microscopic examination revealed that at least some HisC183 proteins, if not all of them, retained the integrity of nucleocapsids as those separated by CsCl gradient (Fig. 2d).

Dimerization of HBV core proteins with an N-terminal extension in hepatoma cells

Since the core-like particles obtained from E. coli lack several features of nucleocapsids, e.g. encapsidation of viral
Fig. 7. For legend see facing page.
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pregenome and cellular kinases, pHBVHisC183 (Fig. 1a, line 3) was constructed for expression of HisC183 in HuH-7 hepatoma cells. Another plasmid, pHBVFlagC183 (Fig. 1a, line 4), was also constructed for expression of FlagC183, which has 10 extra amino acid residues, including five negatively charged glutamic acid residues, at the N terminus of the core protein (see Fig. 1b). In addition to having a different tag sequence to pHVC183, pHBVFlagC183 is driven by the cytomegalovirus (CMV) promoter to express FlagC183 and the HBV sequence within it lacks the polyadenylation signal. To characterize and test the expression of the terminal extension mutants, pHBVHisC183 or pHBVFlagC183 were co-transfected with a core-negative plasmid (pHBVΔC; Fig. 1a, line 6) (Hui et al., 1999) into HuH-7 hepatoma cells. In addition, a full-length HBV-containing plasmid, pMH 3/3097 (Fig. 1a, line 1), co-transfected with a vector plasmid, pUC-MT (Fig. 1a, line 7), was used as a positive control. Western blot analysis showed that wild-type and two mutant cores were detected by anti-core antibodies (Fig. 3a). Migration of wild-type C183, and mutants HisC183 and FlagC183 was as expected, i.e. molecular masses of 21.5, ~ 24 and ~ 22 kDa, respectively. With a His-tag or Flag-epitope at the N terminus, HisC183 and FlagC183 could be recognized by anti-His or anti-Flag antibodies, respectively, while wild-type HisC183 could not (Fig. 3a, xHis and xFlag panels). To test whether the His-tagging and Flag-tagging could affect dimer formation of core proteins (Zheng et al., 1992; Zhou & Staudinger, 1992), samples were analysed under non-reducing conditions. Results showed that a dimer was present in the wild-type C183 as well as in HisC183 and FlagC183 (Fig. 3b), indicating that formation of intermolecular disulfide bonds was not inhibited by the extensions of amino acid residues located at the N terminus.

Self-assembly into nucleocapsids by mutant cores in hepatoma cells

To verify whether the HisC183 and FlagC183 proteins can self-assemble into core-like particles in HuH-7 cells as in E. coli, we performed CsCl gradient analyses. Both intracellular extracts and detergent-treated extracellular media from cells transfected with the two plasmids in various combinations (as described above) were subjected to CsCl banding and then to ELISA analysis for the presence of core proteins. For another positive control of wild-type core expression, plasmid pHBVΔPSX, in which the P, S and X genes are deleted and the C gene remains intact (Fig. 1a, line 5), was also used as a comparison. Results showed a similar profile of core protein distribution obtained from the transfection of wild-type C183 (Fig. 4a), mutants HisC183 (Fig. 4b) and FlagC183 (Fig. 4c) as well as from the co-transfection of pHBVΔC and pHBVΔPSX (Fig. 4d). The core proteins were present at the peak of 1.18–1.20 g/ml or at the peak of 1.25–1.30 g/ml, which correspond to the density of empty core particles (light core) and nucleic acid-containing particles (heavy core), respectively. These results suggest that the N-terminal fusion core proteins do not significantly disrupt the structure of an assembled particle in HuH-7 cells as observed in E. coli (Fig. 2b).

Fusion core proteins can be phosphorylated by in vitro kinase reaction

Protein kinase activity has been demonstrated inside the HBV nucleocapsid (Albin & Robinson, 1980; Gerlich et al., 1982), which is an indicator of intact particles. In order to obtain more evidence concerning the integrity of nucleocapsids assembled by HisC183 and FlagC183, we performed an in vitro kinase assay for nucleocapsids. Nucleocapsids were collected using immunoprecipitation with anti-core antibodies from both intracellular extracts and extracellular (NP-40-treated) media. The results of the in vitro phosphorylation showed that not only the wild-type C183 but also the HisC183 and FlagC183 mutant core proteins can be phosphorylated in both intracellular nucleocapsids (Fig. 5, upper panel) and extracellular nucleocapsids (Fig. 5, lower panel). However, no sign of phosphorylation occurred in the FlagC151 mutant, which lacks the C-terminal serine residues, although nucleocapsids can be formed by FlagC151 (Hui et al., 1999). These data suggest that mutant core proteins with an N-terminal extension do not interfere with the kinase packaging into nucleocapsids and particles remain intact.

Intracellular mutant nucleocapsids retain DNA repairing activity

One prominent property of nucleocapsids is the formation of partially double-stranded HBV DNA from the encapsidated pregenome by reverse transcriptase/DNA polymerase. This feature can be demonstrated by an endogenous polymerase activity assay (HBV DNA gap fill-in assay), in which a specific

Fig. 7. Detection of HBV DNA from secreted Dane-like particles by PCR and Southern blot. (a) The expected sizes of PCR product from pregenomes are given on the left. The pregenomes derived from plasmids are shown by thick lines. The boxes shown above the thick lines are as described in Fig. 1(a). Locations of primers, C401F and S1764R, for PCR amplification are indicated by arrowheads. The BamH1/EcoRI fragment, indicated by arrows, was used as a probe for Southern hybridization. (b) HuH-7 cells were transfected with various plasmids as specified at the top of each graph. Extracellular media were collected and analysed for the presence of HBsAg and core protein by ELISA. The density (g/ml) of individual fractions is shown by dotted lines. Filled-in (HBsAg) and open (HbsAg) circles show the OD_{260} in the ELISA of each fraction. Fractions were pooled into < D, D and > D tubes and subjected to PCR amplification. PCR products from tubes 1–12 were separated on a 1% agarose gel and probed by the HBV fragment as indicated in (a). Lane numbers on the gel correspond to the tube numbers collected from the CsCl fractions. Lane 13 is the negative control for PCR. Arrows on the left of the gel indicate the expected sizes of fragments (1363 and 1046 bp, respectively). Numbers on the right are the DNA markers in bp.
length of HBV DNA is filled-in by isotope-labelled nucleotides and is shown by autoradiography. To characterize the integrity of nucleocapsids assembled by HisC183 and FlagC183, an endogenous polymerase activity assay was applied to the samples of complementation experiments as used in the CsCl gradient assay (Fig. 4b, c). A band of 3–0 kb HBV DNA, which is presumably derived from the pregenome transcribed by pHBVAC, was present in intracellular extracts from both co-transfections with pHBVHisC183 or pHBVFlagC183, together with a core-negative plasmid (pHBVAC) (Fig. 6a, lanes 3 and 4). As expected, two bands of 3–0 kb and 2–1 kb were observed in the co-transfection of pHBVA1PSX and pHBVAC and a single band of 3–2 kb was seen in pMH 3/3097 and pUC-MT co-transfection (Fig. 6a, lanes 1 and 2). Although the signal was weaker in the sample of co-transfection with pHBVFlagC183 and pHBVAC (lane 4), data support the notion that intracellular nucleocapsids formed by HisC183 and FlagC183 remain functional in reverse transcription of the pregenome.

**Impairment of envelopment in mutant nucleocapsids to form virion-like particles**

Since intracellular nucleocapsids assembled by mutant core proteins contain functional components, we were interested to know whether these intracellular nucleocapsids can still interact with surface antigens to form secretable virion-like particles. Because a small amount of naked nucleocapsids can be found in the media from transfected HuH-7 cells (Figs 4 and 5; Gerelsaikhan et al., 1996), we performed immunoprecipitation twice using excess anti-core antibodies to eliminate extracellular nucleocapsids. The HBV DNA gap fill-in assay for those media precleared by anti-core antibodies showed no sign of isotope-labelled HBV DNA in co-transfections of pHBVHisC183 or pHBVFlagC183 with pHBVAC (Fig. 6b, lanes 3 and 4). As expected, two bands of 3–0 kb and 2–1 kb and a single band of 3–2 kb were observed in the positive groups, respectively (Fig. 6b, lanes 1 and 2). The results suggest that intracellular nucleocapsids assembled by HisC183 and FlagC183 could not be enveloped to form mature virions.

To further confirm that the observation of envelopment impairment occurred in mutant nucleocapsids, a more sensitive assay was carried out. Cell culture media precleared by anti-core antibodies as described above were fractionated by CsCl gradient centrifugation and divided into three tubes: <D, lighter density than Dane particles; D, equal density to Dane particles; and >D, heavier density than Dane particles. Each tube was subjected to PCR amplification followed by Southern blot analysis for detection of HBV DNA present in the fraction. ELISA analyses of CsCl fractions showed that no naked nucleocapsids were present in any preparations (Fig. 7b). PCR and Southern blot results showed two bands of 1363 bp and 1046 bp in the sample co-transfected with pHBVAC and pHBVA1PSX and a single band of 1363 bp in the sample co-transfected with pMH 3/3097 and pUC-MT (Fig. 7b), indicating that they were indeed derived from Dane-like particles instead of nucleocapsids in media. Lighter bands present in both <D and >D tubes of positive control groups might result from the incomplete separation and the over-sensitive detection by PCR amplification plus Southern hybridization. Consistent with the DNA repairing assay results, no PCR product was detected in the tubes of <D, D and >D from samples which were co-transfected with mutant core plasmids and pHBVAC (Fig. 7b, lanes 7–12); this strongly suggests that no detectable virion was secreted by these transfected cells. Taking together all results from this study, we conclude that HBV core mutants with an N-terminal extension, HisC183 and FlagC183, can form a functional nucleocapsid intracellularly but are incapable of forming a mature and secretable Dane-like particle.

**Discussion**

In order to elucidate the role of the N terminus of the HBV core protein in virion maturation, we constructed core mutants with an N-terminal extension to test their ability to assemble nucleocapsids. Using CsCl gradient separation and electron microscopy analyses, we demonstrated that HisC183 could form nucleocapsids in both prokaryotic and eukaryotic systems and that FlagC183 could do so in eukaryotic cells (Figs 2 and 4). In addition, nucleocapsids assembled by HisC183 and FlagC183 in hepatoma cells retained a kinase activity and an endogenous polymerase activity (Figs 5 and 6a). However, these particles could not form secretable virions (Figs 6b and 7b), suggesting that either the additional amino acids at the N terminus interfere directly with envelopment by surface antigens or block the signals for the process of envelopment.

In the past, HBV core proteins have been successfully used to express heterologous epitopes in many prokaryotic and eukaryotic systems because of their ability to form particles (see review by Pumpens & Grens, 1999). In addition, HBV nucleocapsids are exceptionally potent antigens that induce both T-cell-dependent and T-cell-independent responses (see review by Schoel et al., 1996). Several insertion sites have been tested to improve the immunogenicity of foreign peptides (Clarke et al., 1990; Pumpens et al., 1995; Borisova et al., 1996; Ulrich et al., 1998). Three insertions, located at aa 1–3, 74–93 and 141–183 of core proteins, are found to be regions that are dispensable for nucleocapsid assembly (Pumpens & Grens, 1999). In this study, the nucleocapsid assembled by HisC183 is consistent with previous studies and gives a new example that additional amino acids at the N terminus do not disturb formation of the nucleocapsid. Furthermore, it shows that the His-tag of HisC183 is possibly located at the surface of nucleocapsids, since the particles can be purified on a nickel column (Fig. 2c and d). It is noted that three bands of HisC183 were observed on the Western blot as compared with one single band of purified HisC183 on the gel stained by Coomassie brilliant blue (Fig. 2a, lane 8 vs c, lane 2). This could
incorporation. Although we did not produce FlagC183 in 
additions to the core protein do not interfere in cellular kinase 
Our current finding provides evidence that the N-terminal 
encapsidated by HBV core particles remains poorly defined. 
polymerase activities. To date, the role of cellular kinase 
reduced, or (ii) degradation of HisC183 occurs inside bacteria 
be because (i) the HisC183 protein inside the bacteria is not 
completely reduced by DTT while the purified HisC183 is fully 
reduced, or (ii) degradation of HisC183 occurs inside bacteria 
while those degraded forms cannot be recovered from the 
nickel column.

In addition to demonstrating that nucleocapsids are 
assembled from HisC183 in E. coli, we also showed that 
nucleocapsids are able to assemble using HisC183 in human 
hepatoma cells and that they retained kinase and DNA 
polymerase activities. To date, the role of cellular kinase 
capsidated by HBV core particles remains poorly defined. 

Our current finding provides evidence that the N-terminal 
additions to the core protein do not interfere in cellular kinase 
incorporation. Although we did not produce FlagC183 in E. 
coli to test for nucleocapsid formation, we showed that 
FlagC183 has all the characteristics of HisC183 in hepatoma 
cells. Basically, the additional amino acids present in HisC183 
or FlagC183 do not disrupt the formation of core dimer, which 
provides grounds for further polymerization to form particles 
(Zhou & Standring, 1992). If the intermolecular Cys-61–Cys-
61 disulfide bond of core proteins is disrupted, no particles are 
produced (Conway et al., 1998b).

In this study, we provide additional information on the 
envelopment of mutant nucleocapsids, since envelopment is 
one of the critical steps in HBV maturation. Three surface 
proteins (L-HBsAg, M-HBsAg and S-HBsAg) on the envelope 
are important for virus maturation and infection (Ueda 
et al., 1991; Bruss & Ganem, 1991; Le Seyec et al., 1998). 
The molecular nature of the HBV envelopment signal is still 
unknown. However, previous reports have suggested that 
triggering the envelopment signal is linked to genomic 
replication in the interior of nucleocapsids (Gerelsaikhan et al., 
1996; Wei et al., 1996). Such a signal may result in a 
conformational change in the core proteins and allow 
interaction between the core proteins and surface proteins to 
occur. Bottcher et al. (1998) have demonstrated that L-HBsAg 
may bind to Glu-77 and Asp-78 of the core protein. Therefore, 
the impairment of envelopment in nucleocapsids assembled 
from HisC183 and FlagC183 (Fig. 7) is possibly influenced by 
the six histidines or the five glutamic acids at the N terminus 
of the core protein (Fig. 1b). These N-terminal extensions 
could either block the envelopment signal or interfere with 
surface antigen binding to core particles. Based on results of 
the accessibility of the His-tag and Flag-tag by antibodies (data 
not shown) or nickel resins (Fig. 2c, d), we favour the second 
hypothesis. Nevertheless, further cryoelectron microscopy is 
required to reveal the structure of HisC183 particles isolated 
from E. coli, which are easily obtained from the pHisC183-
harbouring cells. The structure of HisC183 particles will 
definitely provide a better conclusion.

We gratefully acknowledge Drs Yan-Hwa Wu Lee and Shu-Der Tsen 
for their critical review of this manuscript and Kathy Sun for English 
editing. Thanks are also due to Dr Heinz Schaller for providing us the 
plasmid pH3/3097. The assistance of Miss Y.-Y. Yu with electron 
microscopy is greatly appreciated. This study was supported by grants 
of NSC86-2315-B010-004-MH and NSC87-2314-B010-043 from the 
National Science Council. S.J.L. was awarded by the National Science 
Council and the Medical Research and Advancement Foundation in 
Memory of Dr Chi-Shuen Tsou.

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Received 18 May 1999; Accepted 24 June 1999