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Functional Role of Hepatitis B Virus
Core Protein in Viral Replication

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A Dissertation Submitted to The Graduate School of
Ajou University in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy of Biomedical Sciences

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The Graduate School, Ajou University
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-ABSTRACT-

Functional Role of Hepatitis B Virus Core Protein in Viral Replication

PART I

Arginine-Rich $^{167}$RRRSQSPRR$^{175}$ Domain in C-Terminus of Core is Critical for HBV Replication

To investigate the contributions of carboxyl-terminal nucleic acid binding domain of HBV core protein for hepatitis B virus (HBV) replication, chimeric HBV core proteins were generated by substituting varying lengths of the carboxyl-terminus of duck hepatitis B virus (DHBV) core protein for the corresponding regions of HBV core protein. All chimeric core proteins formed core particles. A chimeric core protein with 221–262 amino acids of DHBV core protein, in place of 146–185 amino acids of the HBV core protein, supported HBV pregenomic RNA (pgRNA) encapsidation and DNA synthesis: 40% amino acid sequence identity or 45% homology in the nucleic-acid binding domain of HBV core protein was sufficient for pgRNA encapsidation and DNA synthesis, although we predominantly detected spliced DNA. A chimeric core protein with 221–241 and 251–262 amino acids of DHBV core, in place of HBV C 146–166 and 176–185 amino acids, respectively, could
rescue full-length DNA synthesis. However, a reciprocal core chimera with 242–250 of DHBV core (\textsuperscript{242}R\textsuperscript{AGSPPLPR}\textsuperscript{250}) introduced in place of 167–175 of HBV core (\textsuperscript{167}R\textsuperscript{RRSQPSPRR}\textsuperscript{175}) significantly decreased pgRNA encapsidation and DNA synthesis, and full-length DNA was not detected, demonstrating that the arginine-rich \textsuperscript{167}R\textsuperscript{RRSQPSPRR}\textsuperscript{175} domain may be critical for efficient viral replication. Five amino acids differing between viral species (underlined above) were tested for replication rescue; R169 and R175 were found to be important.

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Key words: Hepatitis B virus, Core protein, Chimeric core, Carboxyl-terminal domain of core protein, Encapsidation, Hepatitis B virus replication
PART II

Phosphorylation of the C-Terminal Domain of HBV Core Protein modulates Genome Replication

Phosphorylation of hepatitis B virus (HBV) core protein at Ser157, Ser164, and Ser172 residues by host serine/arginine protein-specific kinases (SRPK) or protein kinase C (PKC) has been demonstrated to modulate HBV replication. Also, three additional amino acid residues, Thr162, Ser170, and Ser178, of HBV core protein have been suggested as the putative protein kinase A (PKA) phosphorylation sites with the conserved RRXS/T motif. The in vivo phosphorylation assay reveals that Thr 162, Ser170, or Ser178 can be phosphorylated. In order to elucidate importance of these residues for HBV replication, each was mutated to Ala to mimic nonphosphorylated Ser or to Glu to mimic phosphorylated Ser. Thr 162 to Ala (T162A) mutation decreased replicative intermediate DNA significantly. To further investigate the importance of Thr 162 in conjunction with Ser170Ala and/or Ser178Ala mutations, more core protein mutants were constructed. In the presence of T162A mutation, the HBV DNA synthesis was decreased more dramatically, indicating that Thr 162 residue may be important for HBV DNA synthesis. Taken together, our results indicate that
the putative PKA phosphorylation sites, Thr 162, Ser170, or Ser178, is phosphorylated and can modulate DNA replication possibly through phosphorylation and dephosphorylation.

Key words: hepatitis B virus, phosphorylation of HBV core protein, cAMP depdent protein kinase
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PART II

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Functional Role of Hepatitis B Virus Core Protein in Viral Replication
PART I

Arginine-Rich $^{167}$RRRSQPRR$^{175}$ Domain in C-Terminus of Core is Critical for HBV Replication
I. INTRODUCTION

Hepadnaviruses are small, enveloped DNA viruses that replicate preferentially in liver cells and are associated with acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Brechot, 2004). Hepatitis B virus (HBV), a prototypic hepadnavirus, has a partially duplex relaxed circular (RC) DNA genome, which replicates by reverse transcription of a pregenomic RNA (pgRNA) to produce genomic DNA. The core protein of HBV consists of 183 or 185 amino acids that form core particles via dimeric intermediates (Zhou and Standring, 1992). Assembly of replication-competent HBV core particles requires interaction of pgRNA with the polymerase (P) and core proteins. The amino-terminus of core protein (amino acids 1–144) participates in core particle assembly through protein-protein interaction and is, by itself, assembly competent (Birnbaum and Nassal, 1990; Gallina et al, 1989). The carboxyl-terminus of core protein contains a protamine-like nucleic acid binding domain rich in arginine. Although this region is dispensable for core particle assembly, it is involved in hepadnaviral replication, including pgRNA encapsidation and DNA replication (Birnbaum and Nassal, 1990; Brechot, 2004; Certo et al, 1999; Daub et al, 2002; Enomoto et al, 2006). The importance of core protein’s carboxyl-terminus in hepadnavirus pgRNA encapsidation and DNA replication has been suggested through experiments with a series of carboxyl-terminal truncation mutants (Birnbaum and Nassal, 1990; Gallina et al, 1989; Beames and Lanford, 1993; Kock et al, 2004; Pogam et al, 2005; Nassal, 1992). The core protein amino-acid 164 variant deficient in 19 carboxyl-terminal amino acids (C164), corresponding to amino-acid 166 in our adw subtype, is competent for
pgRNA encapsidation, but not for synthesis of full-length RC DNA (Kock et al, 2004; Pogam et al, 2005; Nassal, 1992). DNA synthesized in variant C164 core particles is predominantly spliced (Kock et al, 2004; Pogam et al, 2005). However, a core variant containing 173 amino acids and lacking ten amino acids at the carboxyl-terminus (C173), corresponding to 175 amino acids in our adw subtype, was as competent for synthesis of full-length RC DNA as wild type (wt) core protein, suggesting that nine carboxyl-terminal amino acids (165–173) are sufficient for replication-competence (Pogam et al, 2005). Although it has been suggested that these residues are important for selective and/or productive viral RNA encapsidation in deletion- and site-directed mutants (Pogam et al, 2005), a direct demonstration of the amino acid residues or motif in the carboxyl-terminus of core protein critical for hepadnavirus pgRNA encapsidation or DNA replication has not yet been performed.

Heterologous complementation to generate chimeric proteins of related viruses is a way of identifying the viral-protein amino acid residues or motifs crucial for replication. Chimeric viruses or proteins have been used to identify viral cis-acting sequences and the functions of protein domains (Berkowitz et al, 1995; Certo et al, 1999; Kaye and Lever, 1998; Kim et al, 2000). Chimeras of duck HBV (DHBV) and heron HBV, another avian hepadnavirus sharing 79% nucleotide identity with DHBV, have been used to elucidate the functional interactions between cis-acting sequences and viral components for pgRNA encapsidation and plus-strand DNA synthesis (Mueller and Loeb, 2002; Ostrow and Loeb, 2004; Ostrow and Loeb, 2008). The genomes of HBV and woodchuck hepatitis virus (WHV) share approximately 60% identity (Kodama et al, 1985; Mandart, 1984); those of HBV and
DHBV share 40% homology (Mandart et al, 1984). However, heterologous complementation with related hapdnaviruses such as DHBV or WHV cannot be performed; while HBV replication is restored easily by complementation with WHV core and/or P proteins and vice versa, it cannot be complemented at all by DHBV core and/or P proteins and vice versa (Okamoto et al, 1990; Zierman and Ganem, 1996). Therefore, use of DHBV or WHV chimeric viruses or proteins to complement HBV replication has not been explored.

In the present study, HBV chimeric core proteins were constructed by exchanging portions of the carboxyl-terminus of HBV core protein with the corresponding regions of DHBV core protein, while retaining wt HBV core protein amino-terminal sequence to investigate the critical regions for pgRNA encapsidation or HBV DNA synthesis. DHBV core protein, which consists of 262 amino acids, can form a three-dimensional core particle similar in structure to that of HBV (Kennery et al, 1995). Use of these chimeras demonstrated that some chimeric core particles are replication-competent, complementing HBV core proteins in C-deficient mutants to effect pgRNA encapsidation concomitant with reverse transcription. These results indicate that 40% amino acid sequence identity or 45% homology in the carboxyl-terminus of core protein is sufficient for HBV pgRNA encapsidation and DNA synthesis, even though predominantly spliced HBV DNA was synthesized. Serial substitutions of HBV core protein with the corresponding regions of DHBV core protein further allowed us to demonstrate that residues 167–175, 167RRRSQSPRR175, may be critical for full-length RC DNA synthesis as long as residues from 146–166 maintain 62% homology. Although the importance of residues R167, S170, P173, and R174 in the HBV core protein could not be examined due to the presence of
identical residues in the corresponding region of DHBV (167RRRSQPRR175 in HBV vs 242RAGSPLPRS250 in DHBV) in HHDH core chimera, in which HBV core 167-175 was replaced by DHBV core 242-250, we analyzed the importance of R168, R169, Q171, S172, and R175 residues using a series of point mutants. By analyzing the A168R, G169R, P171Q, L172S, and S175R mutants in HHDH core chimera, we further demonstrated that the R169 and R175 residues may be important for HBV replication and that S172 may be important for core particle formation, but not for pgRNA encapsidation or DNA synthesis. The importance of residues 167–175 in HBV core protein for replication in the context of neighboring amino acids or motifs is discussed.
II. MATERIALS AND METHODS

A. DNA construction

The partially redundant wt HBV subtype adw R9 plasmid construct pPB was used as a template for generation of HBV DNA constructs using PCR-based mutagenesis (Kim et al, 2004). An HBV wt core protein construct containing the HBV wt core open reading frame (ORF) and post-transcriptional regulatory element (PRE) (Huang and Liang, 1993) was generated as follows: pPB was digested with BstEII and EcoRV and then self-ligated to delete nt 1406–2848, generating pHCP. The sequence was additionally truncated by PCR-based mutagenesis, yielding pHCP. To generate a DHBV wt core protein construct containing the DHBV core ORF and HBV PRE, the DHBV core gene from pCMVDHBV (a gift from William Mason, Fox Chase Cancer Center) was cloned into pcDNA3 between the HindIII and EcoRV sites to yield pDC. The HBV PRE sequence was cloned into pDC to yield pDCP. Chimeric core protein variants were constructed by the PCR-derived recombination of HBV and DHBV core ORFs and the PCR-amplified fusion products cloned into the corresponding restriction sites of pHCP, yielding pHDI92-262, pHD221-262, pHDI92-220, pHD221-242, pHD242-262, pHHDH, and pHHDH. To generate pHCP145, a stop codon (TAG) was introduced at Thr146 (ACT) of HBV core protein by site-directed mutagenesis. To generate the assembly-deficient HBV variant (Konig et al, 1998), pHCP-R127Q, in which Arg127 (CGC) is modified to Gln (CAG) in pHCP by site-directed mutagenesis, was generated first; the HindIII- and BstEII-digested DNA fragment from pHCP-R127Q, which contains the Arg127→Gln mutation, was then cloned into pHCP145,
yielding the assembly-deficient pHCP145-R127Q variant. A C-deficient mutant that does not express core protein was generated by introducing a stop codon (TAA) at Glu8 (GAA) of the core protein by site-directed mutagenesis. This core-deficient mutant expresses pgRNA and all other HBV proteins except the core protein. To generate the c-deficient-RT-YMHA mutant, the EcoRI- and EcoRV-digested DNA fragment from a reverse transcriptase (RT) reaction deficient RT-YMHA mutant, wherein the conserved YMDD motif of the RT active site was modified to YMHA (Kim et al, 2004), was cloned into the C-deficient mutant. To further analyze the importance of Arg168, Arg169, Gln171, Ser172, and Arg175 residues (167RRRSQSPRR175 motif in HBV vs 242RAGSPLPRS250 motif in DHBV), A168R, G169R, P171Q, L172S, and S175R mutants were constructed in the HHDH core background by site-directed mutagenesis. To test trans-complementation of C-deficient-RT-YMHA or C-deficient mutants, a series of chimeric core proteins was used throughout the experiments for HBV encapsidation or HBV DNA replication. All constructs were sequenced to confirm the presence of specific mutations, and to ensure that no extraneous mutations were introduced during PCR.

B. Cell culture, transfection, and isolation of core particles

HuH7 hepatoma cells (Japan Health Sciences Foundation, Tokyo, Japan) were maintained as previously described (Kim et al, 2004). For expression of chimeric core protein variants and assessment of their core particle formation, 8 ug of pHCP plasmid or various chimeric core protein constructs were transfected into HuH7 cells as previously described (Kim et al, 2004). For analysis of pgRNA encapsidation or HBV DNA synthesis, 2
ug of C-deficient-RT-YMHA or C-deficient mutants and 6 ug of various chimeric core protein constructs were co-transfected into HuH7 cells as previously described. 1 ug of the Renilla luciferase expression plasmid phRL-CMV (Promega, Madison, WI, USA) was co-transfected into HuH7 cells as a transfection control. pcDNA3.1 was used in transfections to equalize total DNA transfected. Cytoplasmic core particles were precipitated from lysates of transfected cells with 6.5% polyethylene glycol as previously described (Kim et al, 2004). In brief, clarified lysate was adjusted with 10 mM MgCl₂ and 8 mM CaCl₂ solution, incubated overnight at 37 °C with 20 U DNase I (Sigma) and 60 U micrococcal nuclease (Calbiochem) to remove the transfected plasmid DNA and unencapsidated RNA, and precipitated with 6.5% polyethylene glycol. Transfection experiments were repeated more than three times.

C. RNase protection analysis (RPA)

To analyze encapsidated pgRNA, core particles were isolated as described above. pgRNA was extracted from core particles following digestion with proteinase K (100 ug/mL) and DNase I (20 U). To prepare riboprobe for RPA, nt 1805–2187 of the C-deficient mutant were cloned into pGEM3Zf(+) vector, generating pRPAFD-C-def. From this construct, 446 nt of radiolabeled anti-sense probe were synthesized in vitro using SP6 RNA polymerase with α-³²P-UTP (specific activity, 800 Ci/mmol). The RPA procedure was performed using the manufacturer’s protocol (RPA II, Ambion, Austin, TX, USA). Protected pgRNA was 369 nt in length. To discriminate encapsidated full-length pgRNA from spliced RNA, the spliced region containing probe, pRPA-PS, was also used (Park et al, 2008). The 470 nts of the HBV sequence was synthesized in vitro and the protected sequence, nt 2680-3092 of HBV
sequence, was 413 nts long (Park et al, 2008). The relative levels of encapsidated pgRNA from isolated core particles were measured with the Image Gauge V4.0 program (Fujifilm, Tokyo, Japan).

D. RNA encapsidation assay and Southern blotting

To analyze pgRNA in core particles with chimeric core protein variants, pellets of core particles isolated from HuH7 cells co-transfected with the C-deficient-RT-YMHA mutant and various chimeric core protein constructs were dissolved in 15μL Tris-acetate EDTA buffer and electrophoresed on 1% native agarose gels. Core particles were transferred to a nylon membrane and denatured with 0.2N NaOH in situ and neutralized. Nucleic acids from disrupted core particles were hybridized to a 32P-labeled random-primed probe specific for HBV sequence (Park et al, 2008). To analyze HBV DNA synthesis by Southern blotting, HBV DNA extracted from core particles was separated by agarose gel electrophoresis and hybridized to a 32P-labeled random-primed probe specific for HBV sequence (Kim et al, 2004). The relative levels of pgRNA and HBV DNA isolated from core particles were measured with the Image Gauge V4.0 program.

E. SDS-PAGE and Western blotting.

To analyze the core protein, total lysates were harvested and lysed in NP-40 containing lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40). The lysates were cleared by centrifugation and supernatants collected and 5% β-mercaptoethanol added; samples were then subjected to SDS-PAGE on 12% gels and the resolved proteins
transferred to polyvinylidene fluoride (PVDF) membranes. These membranes were incubated with polyclonal rabbit anti-HBc antibody, monoclonal mouse anti-tubulin (diluted 1:1000; Calbiochem, San Diego, CA, USA), or polyclonal rabbit anti-luciferase (diluted 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Immunoreactive bands were visualized by a horseradish-peroxidase conjugated secondary antibody (DAKO) using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA). Western blot analysis of core particles was performed as previously described (Kim et al, 2008). Isolated core particles were electrophoresed on a 1% native agarose gel and resolved core particles were transferred to PVDF membranes. Immunoblotting was performed using polyclonal rabbit anti-HBc antibody (diluted 1:1000). Horseradish-peroxidase conjugated anti-rabbit secondary antibody and enhanced chemiluminescence were employed to visualize HBV core particles.

F. PCR to detect HBV DNA from spliced RNA

HBV DNA was extracted from core particles isolated from HuH7 cells co-transfected with the C-deficient mutant and various core variant constructs. PCR was performed using primers HBV 155 (sense 5'-CTACTGTGGAGTTACTCTCG-3') and HBV 8, (antisense 5'-CACGATGCTGTACAGACTTG -3'), which correspond to position nt 1935-1954 and nt 706-725 of the HBV genome, respectively. PCR amplified products were separated by agarose gel electrophoresis, gel-purified, and sequenced.
A. Chimeric core protein expression and core particle formation.

Carboxyl-terminal amino acid sequences of HBV and DHBV core proteins exhibited 40% identity or 45% homology (Fig 1A), while full-length core protein sequences of HBV and DHBV were approximately 27% homologous. DHBV core protein contains an additional 29 amino acids that are absent in HBV core protein (Fig1A). To investigate residues in the carboxyl-terminal nucleic acid binding domain of HBV core protein required for HBV replication, various chimeric core proteins were constructed by substituting the corresponding regions of DHBV core protein for the carboxyl-terminus of HBV core protein (Fig 1A). As positive and negative controls, the HBV and DHBV core protein expression plasmids pHCP and pDCP were first constructed and used as template for chimeric core protein construction. The HD221-262 core protein chimera was designed to substitute the carboxyl-terminal region from residues 221–262 of the DHBV core protein for the corresponding region from residues 146–185 of the HBV core protein, while the amino-terminal 145 amino acids of the HBV core protein were unchanged. The HD192-262 core protein chimera contains the amino-terminal 145 amino acids of HBV core protein and the carboxyl-terminus of DHBV C from residues 192–262 to include an additional 29 amino acids. The HD192-220 core protein variant has the entire HBV core protein sequence but an additional 29 amino acids which are part of DHBV core flexible linker region are inserted between residues 145–146 of the HBV core protein. The HCP145 construct was generated as a positive control for core particle assembly, but as a negative control for pgRNA
encapsidation (Beams and Lanford, 1993). HCP145-R127Q, the assembly-deficient variant, was constructed as a negative control for core particle assembly (Konig et al, 1998). Construct transcription was controlled by the cytomegalovirus immediate early (CMV IE) promoter, and nuclear export of RNAs facilitated by the HBV PRE sequence (Figure 1A). The C-deficient mutant that does not express core protein by the introduced stop codon (TAA) at Glu8 (GAA) (Fig 2A) and pHCP were used as control and/or reference. The Renilla luciferase expression plasmid phRL-CMV was co-transfected into HuH7 cells as a transfection control.

Following transfection of the core protein variants or C-deficient mutant indicated into HuH7 cells, core proteins from HBV wt and chimeric, mutated, and/or truncated variant constructs migrated as expected after SDS-PAGE and Western blotting with polyclonal anti-HBc antibody, but not the C-deficient mutant, as expected (Fig 1B, top panel). To exclude the possibility that the existence of HBV components such as pgRNA and P protein could affect assembly and/or stability of core particles, we transfected core protein variants alone, without the pgRNA expressing construct, into HuH7 cells. Most core protein chimeras were expressed similarly to or, occasionally, at higher levels than the HBV wt core protein from pHCP, except the core protein chimera from HD192-262 (Fig 1B, top panel, lane 3). Native agarose gel electrophoresis followed by Western blotting with polyclonal anti-HBc antibody revealed that core particles formed by chimeric core variants produced slightly different migration patterns (Fig 1B and 2B, second panel, lanes 3–6), suggesting that carboxyl-terminal nucleic acid binding domain sequence might affect core particle formation to some extent, even though the amino-terminal assembly domain remained intact in these chimeric
core variants. DHBV core protein and core particles could not be detected with anti-HBc antibody (Fig 1B and C, lane 2). Also, the assembly-deficient mutant HCP145-R127Q could not form core particles (Konig et al, 1998), even though HCP145-R127Q core protein was compatible with HCP145 core protein (Fig 1B and C, lanes 6 and 7). When levels of core particle formation were compared with core protein expression by normalization to the phRL-CMV transfection control, all variants exhibited similar patterns except the assembly-deficient mutant (Figure 1C). The very inefficient core particle formation by HD192-262 may have been due to poor core protein expression (Fig 1B and C, lane 3). Furthermore, the migration pattern displayed by core particles formed with the HD192-262 core chimera was slightly slower than those of other core particles (Fig 1B, 2B, 5A second panels, and 6 bottom panel), suggesting that HD192-262 core particles may be less stable (Newman et al, 2003). Alternatively, it might be caused by the differences in net charges.
Fig. 1. Core protein expression and core particle assembly by chimeric Core protein variants. (A) Schematic diagrams of HBV, DHBV, and chimeric core protein variant constructs aligned with amino acid sequences of HBV and DHBV core protein carboxy-terminal domains. Amino acids in bold are identical or homologous. SRPK and PKA phosphorylation sites of HBV are marked with asterisks and arrowheads, respectively. Phosphorylation sites of DHBV (Yu and Summers, 1994; Yeh et al, 1993) are marked with open arrowheads. Amino acid sequences of the HBV and DHBV core proteins are presented as open and closed boxes, respectively. The cytomegalovirus immediate early (CMV IE) promoter is represented as an open arrow. PRE, post-transcriptional regulatory element. (B) Identification of core protein and core particles by chimeric core protein variants. To examine expression of core protein variants, lysates from HuH7 cells transfected with a pHCP, pDCP, pHD192-262, pHD192-220, pHD221-262, pHCP145, pHCP145-R127Q, or
C-deficient mutant were electrophoresed on 12% SDS-PAGE gels and protein levels visualized by Western blotting using polyclonal rabbit anti-HBc antibody (top panel). Core protein variants (arrowheads) with expected molecular weights are indicated. The C-deficient mutant lacks core protein due to the introduction of a stop codon at Glu 8 in the C ORF. The pHCP and the C-deficient mutant constituted positive and negative controls, respectively. Transfection experiments were repeated four times. To detect core particles formed by core protein variants from native agarose gels, isolated core particles were transferred to PVDF membranes and incubated with polyclonal rabbit anti-HBc antibody (second panel). The Renilla luciferase expression plasmid pHRL-CMV was co-transfected into HuH7 cells as a transfection control (third panel). Luciferase and α-tubulin (bottom panel) levels were determined by Western blotting using polyclonal rabbit anti-luciferase and monoclonal mouse anti-tubulin antibodies as transfection and loading controls, respectively. HRP-conjugated secondary antibody and enhanced chemiluminescence were used to visualize C, α-tubulin, and luciferase proteins and core particles. (C) Relative levels of core protein expression and core particle assembly by chimeric core protein variants. Relative levels of core proteins, core particles, and luciferase were measured with the Fujifilm Image Gauge V4.0 program. Relative levels of core protein variant expression and core particle assembly were compared to normalized transfection efficiencies. The data represent the mean ± standard deviation (SD) from four independent experiments.
B. HBV RNA encapsidation in core particles with core protein chimeras.

To examine RNA encapsidation by assembly-competent chimeric core variants, various core protein chimeras were co-transfected into HuH7 cells with the C-deficient-RT-YMHA mutant (Fig 2A). To ensure that the nucleic acids within core particles hybridized in situ are encapsidated RNA, not synthesized HBV DNA, the C-deficient-RT-YMHA mutant was used for co-transfection experiments. The conserved YMDD reverse transcriptase motif was modified to YMHA (Kim et al, 2004) in the C-deficient mutant background in the C-deficient-RT-YMHA mutant; thus, core protein deficient and RT reaction-deficient. In this system, core proteins were supplied in trans from core protein chimeras to trans-complement C-deficient-RT-YMHA mutant, and pgRNA and HBV P protein for pgRNA encapsidation were provided from C-deficient-RT-YMHA mutant to trans-complement core protein chimera. HCP and C-deficient-RT-YMHA co-transfected cells were used as a positive control that complements one another. Core particles from co-transfected cells (Fig 2B, second panel, and 2C) were assembled with efficiency and stability similar to those from singly transfected cells (Fig 1B and C), indicating that core particle stability might not be affected by the existence of pgRNA and P protein. We designated HCP145 as the encapsidation-negative control based on previous reports (Birnbaum and Nassal, 1990; Hatton et al, 1992). Encapsidated RNAs were not detected from HD192-262 and HD192-220 core variant co-transfected cells (Fig 2B, top panel, and 2C). Encapsidated RNAs were detected only from HD221-262 core variant and C-deficient-RT-YMHA co-transfected cells (Fig 2B, top panel, and 2C; see lane 5 in each), indicating that substituting part of HBV is involved in pgRNA encapsidation. To confirm encapsidation efficiency, RNase Protection
assay (RPA) was also performed with 5’-end specific probe to show encapsidation of HBV RNA by chimeric core variants. Consistent with encapsidation assays (Fig. 2B, top panel), encapsidation of HBV RNA was only detected in HD221-262 core variant and C-deficient-RT-YMHA co-transfected cells (Fig 2D, lane 5). RPA and encapsidation assay results indicated that 40% identity or 45% homology in the carboxyl-terminus of HBV core protein was sufficient for pgRNA encapsidation.
Fig. 2. HBV pgRNA encapsidation in core particles with chimeric core protein variants.

(A) Schematic diagram of HBV wt (Kim et al, 2004), C-deficient mutant, and C-deficient-RT-YMHA mutant. The C-deficient-RT-YMHA mutant is RT- and core protein deficient due to mutation of the YMDD motif to YMHA, in addition to the presence of a stop codon at Glu 8 in the C ORF. The positions of point mutations are indicated as closed arrowheads. Four ORFs of HBV are shown at the top as open boxes. The CMV promoter is
denoted by an open arrow. (B) Encapsidation assay to detect HBV nucleic acid in situ from disrupted core particles. To examine encapsidation by chimeric core protein variants, the C-deficient-RT-YMHA mutant was co-transfected into HuH7 cells with the pHCP, pDCP, pHD192-262, pHD192-220, pHD221-262, or pHCP145. HBV wt core protein from pHCP served as a positive control. Isolated core particles were electrophoresed through a 1% native agarose gel and transferred to nylon membrane. A $^{32}$P-labeled HBV DNA probe was hybridized to HBV nucleic acids in core particles after disruption of the particles in situ. Core particles were also detected as described for Figure 1B. (C) Relative levels of RNA encapsidation and core particle assembly by chimeric core protein variants. Relative levels of encapsidated RNA and core particles were measured with the Fujifilm Image Gauge V4.0 program. Relative levels of encapsidated RNA and core particles were compared with normalized transfection efficiencies (n=3). (D) RNase protection assay (RPA) to detect encapsidated pgRNA. In vitro transcribed radiolabeled antisense RNA probe (446 nt) was hybridized overnight at 50°C with pgRNA from isolated core particles. Following RNase digestion, the protected pgRNA (369 nt), nt 1819-2187 of the HBV sequence, was run on a 5% polyacrylamide–8 M urea gel and visualized by autoradiography. Relative levels of encapsidated pgRNA were measured with the Fujifilm Image Gauge V4.0 program. Transfection experiments were repeated three times. The Renilla luciferase expression plasmid phRL-CMV was used as a transfection control and pcDNA3.1 was used to equalize the total amount of DNA transfected. The data represent the mean ± SD from three independent experiments.
C. HBV DNA is synthesized in core particles by the HD221-262 core variant.

To further investigate whether this chimeric core protein could support HBV DNA synthesis, Southern blot analysis was performed. As expected, HBV DNA was detected only from HuH7 cells co-transfected with the HD221-262 chimera and C-deficient mutant, and migrated faster than major replicative intermediate (RI) HBV DNAs (Fig 3, lane 6, asterisk), which includes RC, double-stranded linear (DL), and single-stranded (SS) DNA. A shorter exposure to visualize HBV full-length RI DNA from HCP and C-deficient mutant co-transfected cells clearly revealed production of smaller DNA species from HuH7 cells co-transfected with the HD221-262 chimera and C-deficient mutant (Fig 3, middle panel). A faint band, potentially corresponding to one of major RI DNA, was also detected by longer exposure (Fig 3, lane 6, arrowhead). However, full-length RC HBV DNA was not detected (Fig 3). This result demonstrated that 40% identity or 45% homology at the carboxyl-terminus of the core protein was sufficient to support HBV DNA synthesis, but not that of full-length HBV DNA.
Fig. 3. HBV DNA synthesis in core particles with chimeric core protein variants. To examine HBV DNA synthesis in core particles with chimeric core variants, the C-deficient mutant and the pHCP, pDCP, pH192-262, pH192-220, or pH221-262 were co-transfected into HuH7 cells. HBV DNA was extracted from isolated core particles and Southern blot analysis performed. Briefly, HBV DNA was separated, transferred to nylon membranes, hybridized with a random-primed $^{32}$P-labeled HBV specific probe, and subjected to autoradiography. Transfection experiments were repeated more than three times. The HBV replicative intermediate (RI) DNA is marked with arrows. Core particle formation (bottom panel) was determined as described for Figure 1B. Symbol (*) represents smaller DNA species than full length DNA.
D. Core particle formation and RNA encapsidation by chimeric C variants

The detection of fast-migrating, smaller than full-length RI HBV DNA, as a major DNA species from HD221-262 co-transfected cells led us to attempt identification of the motif critical for full-length HBV DNA synthesis. To accomplish this, we constructed and analyzed new chimeric core variants with varying lengths of the DHBV core protein carboxyl-terminus. Since it has been reported that a carboxyl-terminal deleted C164 variant (C166 in adw) can support pgRNA encapsidation and DNA synthesis, even though it is predominantly spliced, we constructed the HD221-241 core variant, with residues 221–241 of DHBV core substituted for residues 146–166 of HBV core, as well as the HD242-262 core variant, with residues 242–262 of DHBV C in place of residues 167–185 of HBV core (Fig 4A). Also, prompted by the suggestion that residues 165–173 (167–175 in adw) of the HBV core protein were important for selective and/or productive viral RNA encapsidation (Pogam et al, 2005), we further constructed the HDHD core chimeric variant with residues 221–241 and 251–262 of DHBV core in the position of residues 146–166 and 176–185 of HBV core, respectively, and the reciprocal HHDH chimeric core variant with residues 242–250 of DHBV core in the position of residues 167–175 of HBV core (Fig 4A). Expression of chimeric core proteins and assembly of core particles were analyzed from core variants transfected HuH7 cells (Fig 4B, top and second panels, and 4C). All core protein variants were expressed and core particles assembled (Fig 4B and C). Similar to the core particles of the HD192-262 core variant, the core particles of the HD242-262 and HHDH core variants seemed to migrate slightly more slowly than core particles of pHCP core protein (Fig 4B, second panel, lanes 5,7).
Fig. 4. Expression and core particle assembly of additional core protein variants. (A) Aligned amino acid sequences of carboxyl-terminal domains of HBV and DHBV core proteins and schematic diagrams of additional chimeric core protein variant constructs. Amino acids in bold are identical or homologous. SRPK and PKA phosphorylation sites in the HBV genome are marked with asterisks and arrowheads, respectively. DHBV phosphorylation sites are marked with open arrowheads. The amino acid sequences of HBV and DHBV core protein are presented as open and closed boxes, respectively. The cytomegalovirus immediate early (CMV IE) promoter is represented as an open arrow. PRE, post-transcriptional regulatory element. (B) Expression of chimeric core proteins and core particle assembly by additional chimeric core protein variants. To examine core protein expression by HBV variants with chimeric core sequence, Western blotting was performed on lysates from HuH7 cells and HuH7 cells transfected with pHCP, pDCP, pHD221-262,
pHD221-241, pHD242-262, pHDHD, or pHHDH variants, as described for Fig 1B (top panel). Core particle formation by core protein variants was detected as described for Fig 1B (second panel). Transfection experiments were repeated four times. As the respective transfection and loading controls, luciferase (third panel) and α-tubulin (bottom panel) levels were determined as described for Fig 1B. (C) Relative levels of core protein expression and core particle assembly by additional chimeric core protein variants. Relative levels of core proteins, core particles, and luciferase were measured with the Fujifilm Image Gauge V4.0 program. Relative levels of core protein variant expression and core particle assembly were compared with normalized transfection efficiencies. The data represent the mean ± SD from four independent experiments.
To examine RNA encapsidation by these core variants, RPAs (Fig 5) was performed in chimeric core variants and C-deficient-RT-YMHA mutant co-transfected HuH7 cells. Core particles from these co-transfections exhibited similar assembly efficiency and migration patterns as those of singly transfected cells (see Figure 4B; Figure 5A, second panel). We could not detect significantly increased RNA encapsidation from cells co-transfected with these additional core variants compared to that from HD221-262 co-transfected cells (Fig 5A and B). pgRNA from cells co-transfected with the HDHD core variant and C-deficient-RT-YMHA mutant displayed slightly increased RNA encapsidation (Fig 5, lane 8). Since RPA with a 5'-end specific probe could not distinguish spliced encapsidated RNA from unspliced full-length pgRNA, encapsidation efficiency determined by RPA represented total encapsidated HBV RNA rather than full-length pgRNA incorporated into core particles. Consistent with RPA, RNAs encapsidated within core particles in situ were also detected from cells co-transfected with HD221-262, HD221-241, HD242-262, HDHD, or HHDH core variant and the C-deficient-RT-YMHA mutant (data not shown).

To our surprise, pgRNA encapsidation from HHDH core variant and C-deficient-RT-YMHA mutant co-transfections was less efficient than from HD221-262 core variant and C-deficient-RT-YMHA mutant co-transfections (Fig 5, and data not shown). The HHDH core variant contains most HBV core protein sequences, with the substitution only of a nine amino-acid motif from residues 242–250 (242RAGSPLPRS250) of DHBV core for residues 167–175 (167RRRSQSPRR175) of HBV core. A closer inspection of the HHDH core
variant revealed that only five amino acids (in italics and underlined) differed from the HBV core protein sequence. These results indicated that amino acid residues 167–175 of the HBV core protein were critical for efficient pgRNA encapsidation, and amino acid residues 150–166 and 176–185 of HBV core protein were not essential, as long as the 40% amino-acid identity or 45% homology or the several critical residues, presumably within the conserved region, were maintained.
FIG. 5. pgRNA encapsidation in core particles by additional core protein variants. (A) RPA to detect encapsidated pgRNA. To detect the pgRNA encapsidated by chimeric core protein variants, the C-deficient-RT-YMHA mutant and the core protein chimeras pHCP, pDCP, pH192-262, pH192-220, pH221-262, pH221-241, pH242-262, pH242-241, or pHHD were co-transfected into HuH7 cells. RPA (top panel) was performed as described for Figure 2D. Core particle formation (second panel) and luciferase levels (bottom panel) were determined as described for Figure 1B. Transfection experiments were repeated three times. (B) Relative levels of encapsidated pgRNA and core particle assembly by additional chimeric core protein variants. Relative levels of encapsidated pgRNA, core particles, and luciferase were measured with the Fujifilm Image Gauge V4.0 program. Relative levels of encapsidated pgRNA and core particle assembly were compared to normalized transfection efficiencies. The data represent the mean ± SD from three independent experiments.
E. Full-length HBV DNA is synthesized in core particles by the HDHD core variants

To identify the motif required for full-length HBV DNA synthesis, we analyzed DNA from HuH7 cells co-transfected with these additional chimeric core variants and C-deficient mutant. We clearly detected full-length DL DNA in HD221-241, HD242-262, and HDHD co-transfected HuH7 cells (Fig 6A and 6B, lanes 6–8). RC DNA was detected only from HDHD core variant co-transfected HuH7 cells (Fig 6A, lane 8). Full-length DNA was barely detectable from HHDH co-transfected cells. Collectively, these results further suggest that the amino acid residues 167–175 of the HBV core protein (167RRRSQSPRR175) are important for full-length DNA synthesis, while residues 150–166 and 176–185 are not. Since the HHDH core variant encapsidated pgRNA less efficiently (Fig 5 and 7C), the low level of DNA produced by cells co-transfected with the HHDH core variant and C-deficient mutant likely reflects this low encapsidation efficiency (Figs 5 and 7D). Similar to co-transfection with HD221-262 (Fig 3), we also detected small-sized DNA from all cells co-transfected with core variants (Figure 6A, asterisks). We speculate that these small-sized DNAs were synthesized from spliced RNA, since HBV DNAs produced by the carboxyl-terminally deleted C164 variant are predominantly from spliced RNA (Kock et al, 2004; Pogam et al, 2005). When we used a probe corresponding to the region most frequently removed during splicing (Park et al, 2008; Gunther et al, 1997), the intensities of these small DNA forms were significantly decreased in core variant co-transfected cells (data not shown) and were barely detectable in core particles from HD221-262 and HHDH co-transfected cells (data not shown); this is consistent with the forms synthesized by HD221-241, HD242-262, and HDHD variant core proteins primarily comprising DNA from spliced RNA. We
also used a minus-strand RNA probe to detect plus-stranded HBV DNA (data not shown) and a plus-strand RNA probe to detect minus-stranded HBV DNA (data not shown). Both probes detected small-sized DNA, indicating that small DNA was double-stranded DNA synthesized from spliced RNA (data not shown). To further analyze small-sized DNA in detail, (Fig 6A, asterisks), polymerase chain reaction (PCR) was performed from core particles isolated from C-deficient mutant and various core variants co-transfected HuH7 cells. Consistent with the results from Southern blotting using strand-specific probes (data not shown) and spliced-out region specific probe (data not shown), small-sized DNA was amplified from HCP, HD221-262, HD221-241, HD242-262, HDHD, HHDH core variants co-transfected cells (Fig 6C, lanes 1, 5–9), further indicating that small DNA was from spliced RNA. Consistent with Southern blotting (Figure 6A), full-length DNA was also amplified from HCP, HD221-241, HD242-262, and HDHD core variants co-transfected cells (Fig 6C, lanes 1, 6–8), but not or hardly detectable from DCP, HD192-262, HD192-220, HD221-262, HHDH core variant co-transfected cells (Fig 6C, lanes 2–4, and 9). Sequence analysis revealed that the PCR-amplified small DNA was generated from encapsidated RNA which is spliced from nucleotides (nt) 2455–491, one of the most spliced sites, deleting 1257 nt (Figure 6C). Since the intensity of the small-sized DNA from cells co-transfected with HHDH was even weaker than those from cells co-transfected with HD221-241 and HD242-262 (Figure 6A and C, lanes 6 and 7 vs 9), we further speculate that the HHDH may be less competent to encapsidate spliced RNA also (Fig 7F)
FIG. 6. Full-length HBV DNA synthesis in core particles with additional chimeric core protein variants. (A) To examine synthesis of HBV DNA in core particles with chimeric core variants, the C-deficient mutant and the core protein chimeras pHCP, pDCP, pH192-262, pH192-220, pH221-262, pH221-241, pH242-262, pHHD, or pHHDH were co-transfected into HuH7 cells. HBV DNA was extracted from isolated core particles and Southern blot analysis performed as described for Figure 3. Transfection experiments were repeated five times. The HBV replicative intermediate (RI) DNA is marked. Core particle formation (bottom panel) was determined as described for Figure 1B. (B) Relative levels of
HBV double-stranded linear (DL) DNA from isolated core particles were measured with the Fujifilm Image Gauge V4.0 program and compared after normalization to transfection efficiencies (top right panel). The data represent the mean ± SD from five independent experiments. * p < 0.001, ** p < 0.01, *** p < 0.05 (n=5). (C) PCR and sequence alignment of the spliced junction of DNAs from isolated core particles. HBV DNA was extracted from isolated core particles and PCR was performed. Primers were shown as dotted arrows. The 814 base-pair (bp) DNA that was 1257 nt smaller than 2,071 bp of full-length HBV DNA and full-length HBV DNA were amplified (arrowheads).
F. Residues R169 and R175 are important for HBV replication

To identify the amino acid residues from 167–175 of HBV core protein (167RRRSQSPRR175) that are important for rescue of full-length HBV DNA synthesis from HHDH core variant co-transfected cells, we singly altered amino acids in the HHDH background motif, comprising 167RAGSPLPRS175, to the corresponding residues in HBV, resulting in HHDH-A168R, HHDH-G169R, HHDH-P171Q, HHDH-L172S, and HHDH-S175R core variants (Fig 7A). Core particle formation was examined by particle Western blotting from HuH7 cells co-transfected with HHDH-A168R, HHDH-G169R, HHDH-P171Q, HHDH-L172S, or HHDH-S175R core variants and the C-deficient mutant (Fig 7B). Core particle assembly efficiency of the HHDH-A168R, HHDH-G169R, and HHDH-171Q was not restored to the level of HBV wt core protein and, although not significant, was slightly less efficient than that of the HDHD core variant (Fig 7B, n=5). However, core particle assembly efficiency of the HHDH-L172S (p<0.001, n=5) and HHDH-S175R (p<0.0001, n=5) was restored and was more efficient than that of the HDHD core variant and HBV wt core protein (Fig 7B).

RNA encapsidation was examined by RPA in HuH7 cells co-transfected with additional core variants and the C-deficient-RT-YMHA mutant (Fig 7C, n=4). Consistent with Figure 5, RNA encapsidation by the HHDH C variant was markedly reduced compared to HBV wt core protein or HDHD core variants (Fig 7C, lanes 1 and 2 vs lane 3). HHDH-A168R, HHDH-G169R, and HHDH-P171Q C variants could rescue pgRNA encapsidation, although less efficiently than HBV wt core protein or the HDHD C variant (Fig 7C).
Fig 7. HBV core particle formation, pgRNA encapsidation, and HBV DNA synthesis by core variants. (A) Aligned amino acid sequences of HDHD and HHDH and amino acid substitutions in the HHDH-derived core variants HHDH-A168R, HHDH-G169R, HHDH-P170Q, HHDH-L172S, and HHDH-S175R. Amino acids in bold are identical or homologous. SRPK and PKA phosphorylation sites of HBV are marked with asterisks and arrowheads, respectively. Phosphorylation sites of DHBV are marked with open arrowheads. Amino acid sequences of HBV and DHBV core proteins are presented as black and white letters, respectively, on contrasting background. (B-D) HBV core particle formation, pgRNA encapsidation, and HBV DNA synthesis by core variants. To examine HBV core particle formation (B), pgRNA encapsidation (C), and HBV DNA synthesis (D) in core particles with the C-deficient or C-deficient-RT-YMHA mutants and pHCP or the core protein chimeras, pHDHD, pHHDH, HHDH-A168R, HHDH-G169R, HHDH-P170Q, HHDH-L172S, or HHDH-S175R, were co-transfected into HuH7 cells. pcDNA3.1 was used to
equalize the amount of DNA transfected. (B) Core particle formation and luciferase levels (data not shown) were determined as described for Figure 1B. The data represent the mean ± SD (n=5). * p < 0.001, ** p < 0.01, and * p < 0.05 (n=5). (C) To examine pgRNA encapsidation, RPA was performed as described for Figure 2D. The data represent the mean ± SD from four independent experiments. (D) HBV DNA was extracted from isolated core particles and Southern blot analysis performed as described for Figure 3. The HBV replicative intermediate (RI) DNA is marked. DNAs from spliced RNAs are indicated by asterisks. Relative levels of core particles and encapsidated pgRNA and HBV DL DNA from isolated core particles were measured with the Fujifilm Image Gauge V4.0 program and compared after normalization to transfection efficiencies. The data represent the mean ± SD from five independent experiments. * p < 0.001 HHDH vs HHDH-G169R, ** p < 0.01 HHDH vs HHDH-S175R, p=0.21 HDHD vs HHDH-G169R, or p=0.24 HDHD vs HHDH-S175R (n=5). (E) PCR and sequence alignment of the spliced junction. HBV DNA was extracted from isolated core particles and PCR was performed as described for Figure 6C. The 814 base-pair (bp) DNA that was 1257 nt smaller than 2,071 bp of full-length HBV DNA and full-length HBV DNA were amplified (arrowheads). (F) RPA to discriminate encapsidated full-length pgRNA and spliced RNA. To detect the pgRNA encapsidated by chimeric core protein variants, the C-deficient-RT-YMHA mutant and the core protein chimeras were co-transfected into HuH7 cells as described for Fig7. RPA was performed as described for Fig 2D using spliced-region probe. The 470 nts of the HBV sequence was synthesized in vitro and the protected sequence, nt 2680-3092 of HBV sequence, was 413 nts long.
The HHDH-S175R could encapsidate pgRNA more efficiently than HBV wt core protein (Fig 7C, lanes 1 vs 8). However, the HHDH-L172S encapsidated pgRNA very poorly (Fig 7C, lane 7), even though core particles by HHDH-L172S were assembled more efficiently than HBV wt core protein or the HDHD core variant (Fig 7B and C, lanes 7).

HBV DNA synthesis was also examined by Southern blotting, using a probe specific for full-length HBV, of cells co-transfected with HHDH-A168R, HHDH-G169R, HHDH-P171Q, HHDH-L172S, or HHDH-S175R core variants and the C-deficient mutant (Fig 7D). Full-length RC HBV DNA synthesis was observed from cells co-transfected with HCP, HDHD, HHDH-G169R, or HHDH-S175R core variants and the C-deficient mutant, indicating the importance of the R169 and R175 residues for full-length RC HBV DNA synthesis (Fig 7D, lanes 1, 2, 5, and 8). However, HBV DNA synthesis was not rescued by HHDH-A168R, HHDH-P171Q, or HHDH-L172S core variants (Fig 7D, lanes 4, 6, and 7). The low level of HBV DNA synthesis by the HHDH-L172S C variant was due to its very inefficient pgRNA encapsidation (Fig 7C and D, lanes 7). Since residues R167, S170, P173, and R174 are identical to the corresponding regions from DHBV (242RAGSPLPRS250 in DHBV vs 167RRRSQSPRR175 in HBV), their importance remains to be determined.

Polymerase chain reaction (PCR) was also performed as in Figure 6C to further examine the small-sized DNA (see Fig 7D, asterisks). Consistent with Figure 6C and the result from spliced-out region specific probe (data not shown), small-sized DNA was amplified from core variants co-transfected cells (Fig 7E, lanes 1–8), further indicating that small-sized DNA was from spliced RNA. In consistent with Southern blotting (Fig 7D), full-length DNA was also amplified from HCP, HDHD, HHDH-G169R, and HHDH-S175R co-
transfected cells (Fig 7E, lanes 1, 2, 5, and 8), but not from HHDH, HHDH-A168R, HHDH-P171Q, or HHDH-L172S co-transfected cells (Fig 7E, lanes 3, 4, 6, and 7). Consistent with the result from Figure 6C, sequence analysis revealed that one of the most spliced sites, nucleotides (nt) 2455–491, was deleted by splicing (Fig 7E).

To further confirm this result, RPA was also performed to discriminate encapsidated full-length pgRNA from spliced RNA using a spliced-out region probe which encompasses nt 2689-3092 of HBV sequence (Fig 7F). If chimeric core variants encapsidated spliced RNA more efficiently than full-length pgRNA, the encapsidation level by spliced-out region probe would be low compare to that by 5'-end specific probe. As shown in Figure 7F, the encapsidation efficiencies did not differ significantly between spliced-out region and 5'-end specific probes (Figure 7C vs F), we could conclude that the low level of HBV DNA synthesis by the HHDH and HHDH-L172S core variant was due to very inefficient pgRNA encapsidation (Fig 7C-D and Fig 7F, lanes 3 and 7).
IV. DISCUSSION

In this study, chimeric core variants by substituting the carboxyl-terminal regions of HBV core protein with the corresponding regions of DHBV core protein were generated and core particle assembly, pgRNA encapsidation, and HBV DNA synthesis were examined. Unlike DHBV core protein, which fails to trans-complement HBV core protein (Okamoto et al, 1990), various chimeric core variants could trans-complement HBV replication, including the HD221-262 with carboxyl-terminal 42 amino acids of DHBV core protein for those in HBV. We therefore hypothesize that the amino-terminus of HBV core protein may interact with viral or host components to form a tertiary structure and/or to support HBV replication. Core particle assembly for HBV replication. All chimeric core variants can assemble into core particles as long as the N-terminal 145 residues of HBV core proteins are intact (Fig 1B). However, core particles formed by chimeric core variants migrated slowly and demonstrated differing assembly competencies (Fig 1B, 2B, 4B, 5A, and 6A), suggesting that the carboxyl-terminal nucleic acid binding domain may affect core particle formation to some extent or interact with the amino-terminal assembly domain for particle stability. The presence of slowly migrating core particles (HD192-262, HD242-262, and HHDH core chimeras) suggests that these core particles might be less stable or differ in net charge, thus affecting HBV replication (Pogam et al, 2005; Newman et al, 2003; Kim et al, 2004). HHDH core particles migrated more slowly than HDHD, providing evidence of inefficient pgRNA encapsidation and DNA synthesis (Fig 5–7). However, this speculation did not apply to all C
chimeras, since pgRNA encapsidation and HBV DNA synthesis by HD221-242 and HD242-262 were similar, even though core particle migration patterns were different (Fig 5 and 6).

Core particles assembled from truncated core proteins or those with insertions are unstable (Kock et al, 2004; Pogam et al, 2005; Kock et al, 1998); destabilization through insertional mutagenesis may explain the failure of trans-complementation by HD192-262 and HD192-220. It is still possible to speculate that the insertion of 29 residues in HD192-220 destabilizes core particles, thus preventing protection of encapsidated pgRNA from nuclease treatment during core particle isolation. Since HD192-262 has more extensive alterations, these alterations may cause the destabilizations of chimeric core protein and/or core particles or reduce the efficiency of core particle assembly. Sequence conservation in the carboxyl-terminal domain of hepadnavirus core protein. Using a series of core protein carboxyl-terminal deletion mutants, Le Pogam et al. (Pogam et al, 2005) suggested that residues 167–175 (165–173 of ayw) of HBV core protein are important for selective and/or productive viral RNA encapsidation by charge balance and core particle stability through the arginine-rich domain. The carboxyl-terminal 10 amino acids of HBV core protein are dispensable for HBV DNA replication (Kock et al, 2004; Pogam et al, 2005). The present study extends these results by showing that residues 167–175 (165–173 of ayw), as well as the 62% homologous residues from 146–166, of HBV core protein are sufficient for full-length HBV DNA synthesis using the HDHD core variant (Fig 6A and 6B). From the 27% homology between HBV and DHBV core proteins, the carboxyl-terminus is 45% homologous or 40% identical, and residues 146–166 are 62% homologous, suggesting that several critical residues from 146–166 may be conserved or have coevolved to encapsidate
pgRNA and subsequently synthesize DNA. Even though our results also demonstrated that residues 167–175 (165–173 of ayw) of HBV core protein are important for HBV replication, several questions are still unanswered. First, if these residues are solely essential for HBV replication as long as residues 146–166 are at least 62% homologous, the replication efficiencies of HD221-241 and HDHD should be similar, and the former should engage in full-length RC DNA synthesis, as does the HDHD. Second, replication of the HD242-262 should be inefficient, similar to that of the HD221-262 and/or HDHD. However, HD221-241 and HD242-262 exhibited similar replication efficiencies; replication efficiency was improved relative to HD221-262. Although not significant (p=0.065, n=5), HBV DNA synthesis was little more efficient by HD242-262 than HD221-241 (Fig 6B).

Putative phosphorylation sites in the carboxyl-terminal domain of HBV core protein. Hepadnavirus core proteins are heavily phosphorylated (Gazina et al, 2000; Lan et al, 1999; Machida et al, 1991; Liao et al, 1995; Yeh et al, 1991). The core protein of DHBV is phosphorylated at six sites (S230, S232, T239, S245, S257, and S259) on an S/TP motif within the carboxyl-terminal domain (Perlman et al, 2005; Yu and Summers, 1994). Three phosphorylation sites (S155, S162, and S170 in subtype ayw and S157, S164, and S172 in subtype adw) in the carboxyl-terminal domain of HBV core protein have been identified as having an SPRRR motif (Liao and Ou, 1995). Several intracellular protein kinases such as protein kinase C (Kann and Gerlich, 1994), the cyclin-dependent kinase cdc2 (Yeh et al, 1993), the 46 kDa serine protein kinase (Kau and Ting, 1998), and serine/arginine protein-specific kinases 1 (95 kDa SRPK1) and 2 (105 kDa SRPK2) (Daub et al, 2002) have been shown to phosphorylate these serine residues in vitro. The synthesis of smaller than full-
length DNA was also demonstrated for major phosphorylation-site mutants (S155E, S162E, and S170E) (Kock et al, 2004). Phosphorylation at these sites is important for pgRNA encapsidation and HBV replication (Kock et al, 2004; Gazina et al, 2000; Lan et al, 1999; Melegari et al, 2005) and these serine phospho-acceptor sites contribute pleiotropically toward modulating HBV replication (Lewellyn and Loeb, 2011b). Three additional putative cAMP-dependent protein kinase A (PKA) phosphorylation sites (RRXS/T: T162, S170, S178) have been identified, and two α-type CK2-activated PKAs (PKAIα and PKAIIα) phosphorylate both S170 and S178 in vitro in the absence of cAMP (Enomoto et al, 2006).

Phosphorylation and dephosphorylation states may not be drastically altered in the core protein chimeras, since both HBV and DHBV core proteins have six phospho-acceptor sites (Fig 1A and 4A). However, for the HHDH, the S172L and R175S substitutions may have reduced the number of putative phosphorylation sites to four (Fig 4A). Also, even though S178 was retained in the HHDH, the 175SRRS178 could have disrupted 175RRRS178 motif, the putative PKA phosphorylation site at S178, contributing to inefficient pgRNA encapsidation and HBV replication (Fig 5–7). HDHD, however, have seven putative phosphorylation sites, ensuing efficient pgRNA encapsidation and HBV replication.

Arginine-rich domains in the carboxyl-terminus of HBV core protein. The carboxyl-terminal domain of HBV core protein has 16 (ayw) or 17 (adw) arginine residues with four clusters (150RRR152, 159RRR161, 166RRRR169, and 174RRRR177) comprising arginine-rich domains (ARD) I–IV and conferring a net positive charge (Fig 4A). The carboxyl-terminal domain of DHBV core protein, in contrast, has 12 positively charged
amino acids (arginine or lysine) but does not conserve the four ARDs, although the 227RRR229 and 233RERR236 motifs may be equivalent to ARD-I and -II (Fig 4A). Recently, mutagenesis of the ARDs of HBV core protein demonstrated their pleiotropic contribution to HBV replication (Lewellyn and Loeb, 2011a). R to A (RRRR→AAAA) mutation in ARD-III impaired in pgRNA encapsidation and minus-strand DNA template switching most strikingly (Lewellyn and Loeb 2011a).

Since ARD-I and -II remained intact in HHDH, inefficient pgRNA encapsidation and subsequent poor replication might be attributed to the 166RRAG169 (ARD-III) and 174RSRR177 (ARD-IV) changes. In HD242-262, ARD-III and ARD-IV were disrupted to 166RRAG169 and 174RSSS177, respectively, but HBV replication was more efficient than HHDH. HDHD has 158RERR161 (ARD-II), 166QRRR169 (ARD-III), and 174RRSS177 (ARD-IV), indicating that these changes may be tolerated to maintain full-length DNA synthesis. Also, HD221-241, HD242-262, HHDH, and HDHD have 15, 14, 15, and 14 positively charged amino acids (arginine or lysine) respectively, in their carboxyl-terminal domains (Fig 4A).

Important amino acids in the carboxyl-terminal domain of HBV core protein for HBV replication. The HHDH-A168R rescued pgRNA encapsidation, even though HBV DNA synthesis was not fully rescued (Fig 7C and 7D, lane 4), suggesting that R168 itself, partial restoration of ARD-III (166RRR168), and/or S170 in 167RRGS170 motif (a putative PKA phosphorylation site) may be important for encapsidation, but are not sufficient to support HBV DNA synthesis. HHDH-G169R restored pgRNA encapsidation and HBV DNA synthesis, suggesting that the 167RARSPRLPRS175 motif might be sufficient to form a
replication-competent tertiary structure or protein-protein and/or protein-nucleic acid interactions (Fig 7). The HHDH-P171Q showed a similar phenotype to the HHDH-A168R, suggesting that Q171 itself may not be important for HBV DNA synthesis. The HHDH-L172S failed to rescue pgRNA encapsidation and HBV DNA synthesis, which is not consistent with previous reports, showing that S172 (S170 for ayw), the putative SRPK phosphorylation site, is important for HBV replication at various stages (Kock et al, 2004; Lewellyn and Loeb, 2011b; Gazina et al, 2000, Lan et al, 1999; Melegari et al, 2005). We hypothesize that S172 in 172SP173 may not be critical to enhance impaired HBV replication, or that 170SP171 compensates such that either 172SP173 is not necessary or the SP motif repeated in 170SPSP173 has a negative effect. The HHDH-S175R restored pgRNA encapsidation and HBV DNA synthesis, suggesting that R175 itself, restoration of ARD-IV (174RRRR177), S178 phosphorylation site (comprising a putative PKA in the 175RRRS178 motif), or the positively charged 174RR175 (Pogam et al, 2005) may be important. Alternatively, the 167RAGSPLPRR175 motif might be sufficient. To note, R169 and R175 are conserved in all HBV strains. Cumulatively, ARDs, phosphorylation sites, and 167RRRSQSPRR175 motif in the carboxyl-terminal domain of HBV core protein may interact to influence each other’s conformation, or that of the amino-terminus, to form a replication-competent tertiary structure or to support intra-molecular or inter-molecular protein interactions, protein-nucleic acid interactions, and/or interactions with host proteins promoting efficient HBV replication, with pleiotropic contributions at various stages of replication. We were unable to examine whether the R167, S170, P173, and R174 residues
were critical for full-length RC DNA synthesis because the corresponding residues of DHBV (242RAGSPLPRS250) are identical. Their contribution will be the subject of future study.
V. CONCLUSION

To identify residues and motifs critical for hepatitis B virus (HBV) replication in the carboxyl-terminal nucleic acid binding domain of HBV core protein, chimeric HBV core proteins were generated by substituting varying lengths of the carboxyl-terminus of duck hepatitis B virus (DHBV) core protein for the corresponding regions of HBV core protein. A core protein with carboxyl-terminal amino acids 221–262 of DHBV core protein substituted for residues 146–185 of the HBV core protein supported HBV pgRNA encapsidation and DNA synthesis. A chimeric core protein with residues 221–241 and 251–262 of DHBV core substituted for HBV core residues 146–166 and 176–185, respectively, could rescue full-length DNA synthesis. However, a reciprocal core chimera with residues 242–250 of DHBV core (\textsuperscript{242}RGASPLPR\textsuperscript{250}) introduced in place of 167–175 of HBV core (\textsuperscript{167}RRRSQPRR\textsuperscript{175}) significantly decreased pgRNA encapsidation and DNA synthesis, and full-length DNA was not detected, demonstrating that the \textsuperscript{167}RRRSQPRR\textsuperscript{175} motif may be critical to efficient viral replication. Five amino acids differing between viral species (underlined above) were tested for replication rescue; R169 and R175 were found to be necessary.
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PART I

B형 간염 바이러스 Core 단백질 C-말단의 167RRRSQSPRR175
이 복제에 미치는 영향

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B형 간염 바이러스(HBV) core 단백질 C-말단의 핵산 결합 도메인이 HBV 복제에 미치는 영향을 알아보기 위하여, HBV의 core 단백질 C-말단의 일부를 오리 B형 간염바이러스 (DHBV)의 core 단백질과 치환하여 키메라 캡시드 단백질을 제작하였다. HBV의 core 단백질의 146-185 아미노산을 DHBV core 단백질의 221-262 아미노산과 치환해주면 HBV pgRNA의 인캡시테이션과 DNA 복제를 할 수 있는 것으로 확인되었다. Core 단백질의 C-말단 중 40%의 아미노산 동일성 혹은 45%의 아미노산 상동성만으로도 HBV pgRNA의
인캡시데이션과 DNA 복제를 하는데 충분한 것으로 확인되었다. DHBV 221-241 과 251-262 아미노산을 HBV의 146-166과 176-185 아미노산으로 치환하여 만든 키메라 core 단백질은 완전체 길이의 DNA를 합성할 수 있는 것으로 확인되었지만 DHBV의 (242RAGSP256)를 HBV의 (167RRRSO255)로 치환하게 되면 HBV pgRNA의 인캡시데이션과 DNA 복제가 상당히 감소하는 것으로 나타났다. 이 결과를 통해서 C-말단의 (167RRRSO255) 도메인이 바이러스의 복제에 중요한 것이라고 추측할 수 있다. 이 도메인에서 두 바이러스간의 다른 아미노산을 분석하여 추가의 키메라 단백질을 제작하여 확인해본 결과, 알지닌169 와 알지닌175가 중요한 것으로 확인되었다.

핵심어: B형 간염 바이러스, 키메라 Core 단백질, Core 단백질의 C-말단, 인캡시데이션, B형 간염 바이러스 복제
PART II

Phosphorylation of the C-Terminal Domain of HBV Core Protein modulates Genome Replication
I. INTRODUCTION

Hepadnaviruses are small, enveloped DNA viruses that replicate preferentially in liver cells and are involved in acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). Hepatitis B virus (HBV), a prototype hepadnavirus, has a partially duplexed relaxed circular DNA genome, which is replicated by reverse transcription of a pregenomic (pg) RNA within cytoplasmic core particle composed of viral core protein (Ganem, 2001).

HBV replication cycle starts when N-terminal domain of the large envelope protein (L protein) of the virus binds to the unidentified receptor of hepatocyte (Ganem, 2001; Hourioux et al, 2002). Fusion of the viral envelope with a cellular membrane liberates the subviral core particle into the cell cytoplasm. Core particle releases the partially double-stranded relaxed circular (RC) DNA to the nucleus and RC DNA is then converted to covalently closed circular DNA (cccDNA). This cccDNA serves as a template for transcription via host RNA polymerase II (Yao et al, 2000). The viral mRNAs are exported to the cytoplasm. pgRNA serves as mRNA for core and polymerase (P) proteins. After translation, core proteins form core particles via dimeric intermediates (core particle assembly) and package its pgRNA and P protein into core particles (encapsidation). To accomplish encapsidation, pgRNA having an epsilon (ε) sequence is associated with viral P protein to generate RNA and P protein complex (RNP complex). After encapsidation, when hydroxyl group of tyrosine 63 (for ayw or 65 for adw) of P protein, a protein primer, is covalently linked to newly synthesized nucleotide without a nucleic acid primer, minus-
strand DNA synthesis is initiated at a bulge region of epsilon (ε), the encapsidation signal, synthesizing the TGAA or GAA, the short oligomer (Tavis et al, 1994; Wang et al, 1994; Nassal and Rieger, 1996; Kim et al, 2004). The short, nascent DNA then switches template to a complementary sequence near the 3’-end of the pgRNA where minus-strand DNA synthesis resumes (template switch) (Loeb et al, 1991). The specificity of this template switch is determined by base pairing between nucleotides in ε and nucleotides flanking the acceptor site (Staprans et al, 1991). Subsequently, minus-strand DNA synthesis continues until the 5’-end of pgRNA, while RNA template is degraded by the RNase H activity of HBV P protein except the short stretch of RNA (Loeb et al, 1991). In a majority of cases, this RNA fragment undergoes a template switch from the 3’-end of the minus strand to the near the 5’-end, termed DR2, anneal to a complementary 11-nucleotide sequence and serves as primer for plus-strand DNA synthesis at DR2 (primer translocation) (Seeger and Maragos, 1989; Loeb et al, 1991). Once synthesizing plus-strand DNA has reached at the 5’-end of minus-strand DNA, a final translocation to the 3’-end of minus-strand DNA occurs (circularization), resulting in partially double-stranded RC DNA genome. In a minority of capsids, primer translocation does not occur, and plus-strand DNA primes from DR1 (in situ priming) to produce a duplex-linear genome (DL) DNA (Staprans et al, 1991).

The HBV core protein consists of 183 or 185 amino acids. The amino-terminus of core protein (amino acids 1–144) participates in core particle assembly through protein-protein interaction and is, by itself, assembly competent (Gallina et al, 1989) and . The carboxyl-terminus of core protein contains a protamine-like nucleic acid binding domain rich in arginine. Fourteen of the sixteen arginines are grouped into four clusters with three or
four arginines (Lewellyn and Loeb, 2011a) and are conserved among mammalian hepadnaviruses, suggesting the importance in HBV replication. In addition to arginine-rich domain, many studies have reported phosphorylation sites located in the arginine-rich carboxyl-terminal domain of the core protein (Machida, 1991; Yeh, 1991; Lan et al, 1999; Köck et al, 2004; Pogam et al, 2005). Three phosphorylation sites, serine 155, serine 162, and serine 170 residues in the SPRRR motif of subtype ayw (equivalent to serine 157, serine 164, and serine 172 of subtype adw) in the arginine-rich carboxyl-terminal domain of core protein have been identified to be phosphorylated by serine/arginine rich protein kinase (SRPK) and/or other intracellular protein kinases (Lan, 1999; Gazina et al, 2000; Melegari et al, 2005). In addition to serine 157, serine 164, and serine 172 (in adw subtype) residues, threonine 162, serine 170, and serine 178 residues (adw) in the conserved RRXS/T motif have been suggested to be the putative phosphorylation sites. Enomoto and his colleague have demonstrated that serine 170 and serine 178 residues are phosphorylated by cAMP-dependent protein kinase A (PKA) in vitro (Enomoto, 2006).

Phosphorylation of core protein may play a major role in regulating nucleocytoplasmic transport of the core protein. (Liao and Ou, 1995; Kann, 1999; Perlman, 2005; Basagoudanavar, 2007). While agreeing on the importance of phosphorylation, previous studies are controversial in detail: mutational analysis of phosphorylation target sites in the transfected cells indicated the inhibitory roles by phosphorylation (Liao and Ou, 1995), while nuclear localization signal (NLS) activation through phosphorylation was suggested by the nuclear transport of core particles (Kann, 1999; Kann 2003). Unlike Ser157, Ser164, and Ser172 (in adw subtype) residues that contribute pgRNA encapsidation and
HBV DNA replication, the importance of putative PKA phosphorylation site for HBV replication has never been explored.

The goals of the present study are to understand whether the putative PKA phosphorylation sites are indeed phosphorylated and can contribute to HBV replication. First, in vivo phosphorylation assay were performed. Then, the role of phosphorylation at the PKA phosphorylation sites for virus replication were examined by analyzing the phenotypes of a series of glutamic acid or alanine substitutions in the putative phosphorylation sites of core protein. HBV RNA encapsidation, DNA synthesis, endogenous polymerase activity, and intracellular localization of the core protein were analyzed. Finally, HBV replication was investigated by phosphorylation and dephosphorylation by PKA. HBV DNA synthesis was decreased when PKA up and down-regulated by PKA over-expressing vector or siRNA transfection. Further studies are required to examine how PKA regulates HBV replication. In addition to the SRPK phosphorylation and dephosphorylation of core protein, putative phosphorylation and dephosphorylation by PKA may modulate HBV replication.
II. MATERIALS AND METHODS

A. DNA construction

The partially redundant HBV wild type (wt) construct designated pPB from the 1.2 length of the HBV subtype adw R9 had been subcloned into pcDNA3 (Invitrogen™) to express HBV under the cytomegalovirus immediate early (CMV IE) promoter (Kim et al, 2004). P protein-deficient mutant was prepared, in which polymerase expression was abolished by modifying AUG start codon to ACG with frame-shifting the polymerase ORF with a silent mutation in core ORF. Phosphorylation site point and combination mutants of HBV core protein were generated from P-deficient mutant background by PCR mediated mutagenesis. To supply HBV polymerase in trans, we constructed the HBV P protein supplier, HBV pol ΔPS by deleting the 5’end signal, from which HBV polymerase (P) protein was provided but encapsidation were prohibited. The P gene of HBV was amplified by PCR from plasmid pPB, which contains full length HBV genome, and cloned into HindIII / BstEII sites in of pPB. To generate the RT-YMHA pol ΔPS mutant, the EcoRI- and EcoRV-digested DNA fragment from a reverse transcriptase (RT) reaction deficient RT-YMHA mutant, wherein the conserved YMDD motif of the RT active site was modified to YMHA (Kim et al, 2004), was cloned into the pHBV pol construct. A plasmid encoding PKAα was created by inserting a fragment of the PKAα into the multicloning region of the pcDNA3 vector (Promega).

B. Cell culture and transfection
HuH7 cells were grown in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Gibco-BRL), 1% penicillin/streptomycin (PS) under a humidified atmosphere at 37°C in 5% CO₂ and were passaged every third day. Cells were transfected using polyethylenimine (PEI) (Poly-science) according to the manufacturer’s instructions. Transfection experiments were repeated more than three times. For cotransfections, 5 µg of plasmid HBV pol was mixed with 5 µg of plasmid encoding core protein which are generate P-deficient backbone.

C. siRNA transfection

The siRNA oligonucleotides for PKAα were purchased from santacruz. Nontarget-siRNA (Santacruz, CA, U.S.A) was used as a control. Transfection was conducted using Lipofectamin RNAiMAX (Invitrogen™, Gaithersburg, Maryland, U.S.A), in accordance with the manufacturer's instructions. In brief, 20 nM of siRNA was mixed with Lipofectamin RNAiMAX in serum-free media. While the complexes were forming, the cells were diluted in complete growth medium without antibiotics, resulting in an 50~70% confluence at 24 h after plating, after which they were mixed with the siRNA-Lipofectatin RNAiMAX complexes. Forty-eight hours after transfection, the lysates were prepared.

D. Isolation of core particle and western blotting.

Cytoplasmic core particles were prepared as previously described (Kim et al., 2004). Isolated core particles were electrophoresed on a 1% native agarose gel and resolved core particles transferred to PVDF membranes. Immunoblotting was performed using hom-made
polyclonal Rabbit HBc antibody (diluted 1:1000) as described previously (Kim et al., 2004). Horseradish-peroxidase conjugated anti-rabbit secondary antibody (DAKO) and enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ, USA) were employed to visualize HBV core particles. Total lysates were then subjected to SDS-PAGE on 12% gels and the resolved proteins transferred to polyvinylidene fluoride (PVDF) membranes. These membranes were incubated with monoclonal mouse anti-tubulin (diluted 1:1000; Calbiochem, San Diego, CA, USA), mouse anti-PKα (diluted 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or polyclonal rabbit anti-luciferase (diluted 1:500; Santa Cruz, CA, USA) antibodies. Immunoreactive bands were visualized by a horseradish-peroxidase conjugated secondary antibody (DAKO) using ECL. The relative intensities were measured using the Fujifilm Image Gauge V4.0 program (Fuji film Science). Transfection efficiencies were normalized and compared by Western blotting of core particles and/or luciferase.

E. In vivo phosphorylation assay

Transfected HuH7 cells were labeled with 200 µCi [P32] orthophosphoate (New England Nuclear) per 10^7 cells for 8hr, rinsed in ice-cold phosphate buffered saline, and lysed in 500ul lysis buffer (50 mM Tris-Hcl, PH 7.5, 2mM EDTA, 2mM EGTA, 0.5% NP-40, 50mM NaF, 25mM b-glycerophosphate, 2 mM sodium vanadate, and protease inhibitors). Lysates were precleared for 1 h at 4°C with protein A-Sepharose and then immunoprecipitated with rabbit anti-HBe antiserum for 2 h at 4°C and 20ul protein A/G – Sepharose (Calbiocam). Beads washed four times in lysis buffer. Beads were resuspended in
SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer and resolved by 13.5% SDS-PAGE, transferred to a polyvinylidene difluoride Immobilon-P membrane (Millipore), exposed for autoradiography, and probed with an anti-HBc antiserum.

F. Southern blotting.

To analyze HBV DNA synthesis by Southern blotting, HBV DNA extracted from core particles was separated by agarose gel electrophoresis and hybridized to a $^{32}$P-labeled random-primed probe specific for HBV sequence as described previously (Kim et al., 2004). The relative intensities were measured using the Fujifilm Image Gauge V4.0 program (Fujifilm Science).

G. RNase protection analysis (RPA)

For analysis of cytoplasmic and encapsidated pgRNA, RPA was performed as previously described (Kim et al., 2004). The 446 nt of the HBV sequence (nt 1805 to 2187) was synthesized in vitro and the protected sequence was 369 nt long (Kim et al., 2004). The relative levels of pgRNA from isolated core particles or cytoplasmic pgRNA were measured with Fujifilm Image Gauge V4.0 program.

H. Endogenous polymerase assay (EPA)

Isolated core particles were incubated overnight at 37 °C in EPA reaction buffer (50 mM Tris–HCl, 75 mM NH₄Cl, 1 mM EDTA, 25 mM MgCl₂, 0.1% β-mercaptoethanol, 0.5% Nonidet P-40) supplemented with 0.5 mM each of dCTP, dGTP, dTTP, and 10 μCi $\alpha^{32}$P-
dATP (Kim et al., 2004). The resulting $^{32}$P-labeled reaction mixtures were electrophoresed on a 1% native agarose gel and then subjected to dry gel autoradiography (Kim et al., 2004).

I. Immunofluorescence assay (IFA)

HuH7 cells were seeded on cover slips and transfected with HBV wt or phosphorylation mutant and HBV pol ΔPS. Two days after transfection, cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature, followed by washing with PBS. Cell membranes were permeabilized by 0.075% Triton X-100 in PBS for 3 min at room temperature, followed by washing with PBS. After blocking with 3% bovine serum albumin in PBS, cover slips were incubated with diluted antibodies in blocking solution for 2hrs at room temperature. For respective immunofluorescence staining, 1:500 diluted rabbit anti-HBcAg (DAKO, Glostrup, Denmark) for core particles, 1:100 diluted mouse anti-α-tubulin (Calbiochem, San Diego, CA) for microtubule were used. After washing the first antibodies, 1:100 diluted fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG or 1:200 diluted rhodamin-conjugated anti-rabbit IgG, the secondary antibodies, were employed for 1hr at room temperature. The nuclei of cells were stained with 1:50000 diluted 4′,6′-diamidino-2-phenylinolde (DAPI). The immunostained cells were visualized the Zeiss LSM 710 confocal laser-scanning microscope.

J. Primer extension assay

Oligonucleotide DNA primers were end labeled with $[\gamma^{32}\text{P}]$ATP. Oligonucleotide 1 (5′-CTCTTGGACTCTCAGCAATGTCAAC-3′) annealed to minus-strand DNA beginning at nt
1661 and was used to measure the level of minus-strand DNA. For analyses of each variant by primer extension, 10 ng of internal standard DNA was added to each viral DNA. Next, the mixture of DNA was heated to 95°C for 5 min, treated with 1 Unit RNase A for 1 h at 37°C, precipitated with ethanol, and resuspended in H₂O. Primer extension analysis was performed on each with Vent Exo (-) polymerase (New England Biolabs, Ipswich, MA). Products of the primer extension reactions were electrophoresed through 5% polyacrylamide gels with 8 M urea. Gels were dried, and autoradiography was performed using a phosphorimager as described for Southern blot analysis.
III. RESULTS

A. Amino acids sequence alignment of HBV core proteins with the related hepadnaviruses

Amino acids sequence alignment of HBV core proteins with the related hepadnaviruses demonstrated that putative protein kinase A (PKA; RRXS/T motif) and serine/arginine protein kinase (SRPK; SPRRR motif) phosphorylation sites are highly conserved except DHBV (Fig. 1). Three serine-phosphorylation sites at positions 157, 164 and 172 (adw subtype) of core protein have been demonstrated to be involved in the core particle trafficking, RNA packaging, and DNA synthesis (Kann et al, 1994; Köck et al, 2003; Gazina et al, 2000; Lan et al, 1999; Melegari et al, 2005). These serine residues can be phosphorylated by kinases such as SRPK and protein kinase C (PKC) (Daub et al, 2002; Kann et al, 1994). Additional three residues, threonine 162, serine 170, and serine 178, in C-terminus of HBV core protein had been suggested as the target of PKA as the putative PKA phosphorylation sites by in vitro study (Okabe et al, 2006) without the implication for HBV replication.
Fig. 1. Sequence alignment of C-terminal domain of HBV and related viruses core protein. C-terminal domain of HBV adwR9 subtype, woolly monkey hepatitis B virus (WMHBV), woodchuck hepatitis B (WHV), ground squirrel hepatitis B virus (GSHV), and Duck hepatitis B virus (DHBV) are shown and aligned. SRPK and PKA phosphorylation sites of HBV are marked with closed and opened arrowheads, respectively. Underlined, boldface residues indicate highly conserved PKA phosphorylation motif sites.
B. In vivo phosphorylation of core protein

In order to examine that threonine 162, serine 170, and/or serine 178, the putative PKA phosphorylation sites, can be phosphorylated, AAAAAA mutant, as the negative control, was constructed, in which all of the six putative phosphorylation sites by SRPK and PKA were abolished to alanine. Then SRPK-AAA (S157A, S164A, S172A) and PKA-AAA (T162A, S170A, and S178A) mutants were constructed by substituting three SRPK or PKA phosphorylation sites to alanines (Fig. 2A). Then threonine 162, serine 170, or serine 178 residues was singly restored from the AAAAAA mutant background, resulting the ATAAAAA, AAASAA, and AAAAAS mutants (Fig. 2A). Western blot analysis was performed to ensure the expression of mutant core proteins in HuH7 cells, demonstrating that all mutant core proteins were expressed in the comparable levels (Fig. 2B, bottom panel). In vivo phosphorylation assay was performed and the negative control AAAAAA mutant was not phosphorylated at all (Fig. 2B, lane 5). Thus, the lack of phosphorylation of AAAAAA mutant protein was not due to its instability or a low level of expression in HuH7 cells. Then we singly restored residue at 162, 170, or 178 site in the AAAAAA mutant background, resulting the ATAAAAA, AAASAA, and AAAAAS mutants (Fig. 2B, upper panel). As the positive control, $^{32}$P-labeled core protein was clearly detectable from the P-deficient mutant transfected cells (Fig. 2B, lane 1). SRPK-AAA has no SRPK phosphorylation sites but maintained PKA phosphorylation sites with the RRXS/T motif. PKA-AAA has no PKA phosphorylation but maintained SRPK phosphorylation sites with the SPRRR motif. Since both SRPK-AAA and PKA-AAA were phosphorylated (Fig. 2B, lanes 6-7), respective RRXS/T and SPRRR can be phosphorylated independently. The ATAAAAA, AAASAA, and
AAAAAS mutant core proteins which have respective threonine 162, serine 170, or serine 178 residues, could be phosphorylated with different efficiencies (Fig. 2B, lanes 2-4). The AAASAA mutant of core protein was phosphorylated at the lowest level among threonine 162, serine 170, or serine 178 from transfected HuH7 cells. This result is the first demonstration that threonine 162, serine 170, or serine 178, the putative PKA phosphorylation residues, could be phosphorylated in vivo, suggesting that these conserved residues might have roles in HBV replication.
Fig. 2. Localization of the major phosphorylation sites in the core protein. (A) Schematic representation of the amino acid sequence of the C-terminal region of WT and serine substitution mutants of core protein. (B) In vivo phosphorylation assay of the core protein. (top) HuH7 cells were transfected with P-deficient, ATAAAA, AAASAA, and AAAAAS mutant by PEI method. Two days after transfection, the cells were used for $^{32}$P-orthophosphate-labeling and radioimmunoprecipitation experiments. Western blot was conducted to measure expression of core protein (bottom). Arrowheads mark the locations of the core protein bands.
C. Core protein expression and core particle formation by phosphorylation site mutant core proteins.

Since putative PKA phosphorylation sites are conserved, these residues might affect the HBV replication. To investigate this, more core protein phosphorylation site mutants were constructed by substituting threonine or serine to alanine or glutamic acid in the P-deficient mutant background to ensure that P protein did not affect by PCR mediated site-directed mutagenesis (Fig. 3). PKA-AAA mimicked the nonphosphorylated core protein (Fig. 2) and PKA-EEE (T162E, S170E, and S178E) has the glutamic acid substitution and mimicked the phosphorylated core protein (Fig. 3A). SRPK-EEE mutant was also constructed by substituting three SRPK phosphorylation sites to glutamic acids (Fig. 3A). Finally, EEEEEE mutant which mimicked phosphorylated core protein at all six phosphorylation sites was constructed (Fig. 3A).

To examine the expression and core particle assembly by mutant core proteins, HuH7 cells were transfected with P-deficient, SRPK-AAA, SRPK-EEE, PKA-AAA, PKA-EEE, AAAAAA, and EEEEEE. By Western blot analysis the expression of the respective core protein mutants were investigated after 3 days after transfection from HuH7 cells. HBV wt and P-deficient were used as positive controls. All core protein mutants expressed the comparable levels with wt core protein (Fig. 3B, top panel). The renilla luciferase expression plasmid phRL-CMV was co-transfected into HuH7 cells as a transfection control (Fig. 3B, bottom panel). Native agarose gel electrophoresis followed by Western blotting with polyclonal anti-HBc antibody revealed that core particles formed by core protein mutants produced slightly different migration patterns (Fig. 3B, second panel, lanes 2–8),
suggesting that phosphorylation site mutants might affect core particle formation or stability to some extent, even though the amino-terminal assembly domain remained intact in these variants. When levels of core particle formation were compared with core protein expression by normalization to the phRL-CMV transfection control, all variants exhibited no significant differences (Fig. 3C).
Fig. 3. Mutations within the phosphorylation site of HBV core protein have little to no impact on core particle formation. (A) Schematic diagram of HBV *wt*, P-deficient, HBV pol ΔPS, YMHA pol ΔPS and HBe phosphorylation mutants. The positions of point mutations are indicated as opened arrowheads. Four ORFs of HBV are shown at the top as open boxes. The CMV promoter is denoted by an open arrow. P proteins from HBV pol
ΔPS providing wt P protein with splice acceptor site mutation or YMHA polΔPS providing RT reaction deficient P protein can encapsidate pgRNA in core particles with core proteins from phosphorylation mutant. C-terminus of core protein and putative phosphorylation site by PKA or SRPK are presented. Phosphorylation sites were mutated to Glu to mimic the phosphorylated mutant or to Ala to make the unphosphorylated mutant. (B) Identification of core protein and core particles by phosphorylation mutants. To examine expression of core protein variants, lysates from HuH7 cells transfected with HBVwt and phosphorylation mutants were electrophoresed on 13.5% SDS-PAGE gels and protein levels visualized by Western blotting using polyclonal rabbit anti-HBc antibody (top panel). P-deficient mutant used as positive control. Transfection experiments were repeated more than three times. To detect core particles formed by core protein variants from native agarose gels, isolated core particles were transferred to PVDF membranes and incubated with polyclonal rabbit anti-HBc antibody. HRP-conjugated secondary antibody and enhanced chemiluminescence were used to visualize core protein, α-tubulin, and core particles. (C) Relative levels of core protein expression and core particle assembly by phosphorylation mutants. Relative levels of core proteins and core particles were measured with the Fujifilm Image Gauge V4.0 program.
D. PgRNA encapsidation, DNA synthesis, and endogenous polymerase activity by the nonphosphorylated and phosphorylated core protein mutants.

To investigate the effect the phosphorylation of core protein on pgRNA encapsidation, RT-deficient P protein expressing plasmid, pYMHA-pol-ΔPS, was co-transfected with the pP-deficient, pSRPK-AAA, pSRPK-EEE, pPKA-AAA, pPKA-EEE, pAAAAAA, or pEEEEEE mutant plasmids to HuH7 cells. Assembly of replication-competent HBV core particles requires interaction of pgRNA with the P and core proteins. In this system, core proteins and pgRNA were supplied in trans from P-deficient mutant, and RT-deficient P protein for pgRNA encapsidation were provided from pYMHA-pol-ΔPS for transcomplementation (Fig. 4A). pgRNA from pYMHA-pol-ΔPS and P-deficient co-transfected cells was used as a positive control. The total cellular RNA and the RNA extracted from core particles were analyzed by RNase protection assay (RPA). pgRNA from total cellular RNA was all expressed at comparable level from the P-deficient mutant plasmid under the cytomegalovirus (CMV) immediate early promoter (Fig. 4B, middle panel). However, encapsidated pgRNA extracted from core particles by mutant core proteins was drastically decreased in the alanine substitution mutants but not in the glutamic acid substitution mutants (Fig. 4B, top panel). Like the core protein mutants at Ser157, Ser164, and Ser172 residues which have been demonstrated to contribute pgRNA encapsidation, pgRNA encapsidation was decreased significantly by dephosphorylated core protein mutants to alanine at Thr162, Ser170, and Ser178 residues, while decreased marginally by phosphomimetic core protein mutants to glutamic acid (Fig. 4B, top panel). This result was quantified with densitometric analysis (Fig. 4C).
Fig. 4. PgRNA encapsidation in core particles with core protein phosphorylation mutants. (A) RNase protection assay (RPA) to detect encapsidated pgRNA. *In vitro* transcribed radiolabeled antisense RNA probe (446 nt) was hybridized overnight at 50°C with pgRNA from isolated core particles. Following RNase digestion, the protected pgRNA (369 nt), nt 1819-2187 of the HBV sequence, was run on a 5% polyacrylamide–8 M urea gel and visualized by autoradiography. Core particle formation (bottom panel) was determined as described for Fig. 3B. (B) Relative levels of encapsidated pgRNA were measured with the Fujifilm Image Gauge V4.0 program and compared after normalization to total cellular RNA level. The data represent the mean ± SD from three independent experiments. *p < 0.05, **p < 0.005 (n=3).
Next, HBV DNA replication was also analyzed from HuH7 cells co-transfected with pHBV-pol-ΔPS and phosphorylation core protein mutants plasmid; pP-deficient, pSRPK-AAA, pSRPK-EEE, pPKA-AAA, pPKA-EEE, pAAAAAA, or pEEEEEE. HBV core particles were isolated and Southern blot analysis was conducted to measure HBV DNA extracted from mutant core particles. Single- (SS) and double-stranded linear (DL) and partially double-stranded relaxed circular (RC) HBV DNA molecules isolated from intracellular core particles were detected from HBV wt or P-deficient and pHBV-pol-ΔPS transfected cells (Fig. 5A, lanes 1 and 2). HBV DNA level was very low from HuH7 cells co-transfected with pHBV-pol-ΔPS and the alanine substitution mutants. These defects may be due to the drastically decreased pgRNA encapsidations (Fig. 4B, lanes 2, 4, and 6 vs Fig. 5A, lanes 3, 5, and 7). From pHBV-pol-ΔPS and glutamic acid substitution mutants co-transfected HuH7 cells, HBV DNA level was decreased but not as much as alanine substitution mutants (Fig. 5A, lanes 4, 6, and 8). However, DNA was migrated faster than SS DNA (Fig. 5A, lanes 4, 6, and 8), which is consistent with the previous reports showing spliced DNA from putative glutamic acid substitution mutants of SRPK phosphorylation site (Köck et al, 2004; Gazina et al, 2000). This result was quantified with densitometric analysis (Fig. 5B). To further analyze small-sized DNA in detail (Fig 5C, top panel), polymerase chain reaction (PCR) was performed from core particles isolated from co-transfected HuH7 cells. Sequence analysis revealed that the PCR-amplified small DNA was spliced from nucleotides (nt) 2455–491, one of the most spliced sites, deleting 1257 nt (Fig 5C, bottom panel).

To demonstrate viral polymerase activity in core particle by phosphorylation
mutant core proteins, an endogenous polymerase assay (EPA) was performed. This assay is based on the fact that core particle-associated viral polymerase can incorporate nucleotides into the viral genome in vitro. Consistent with Southern blotting (Fig. 5A), EPA result show the DNA synthesis defect by alanine substitution mutants and small DNA synthesis by glutamic acid substitution mutants (Fig. 5A and D). Core particles from phosphorylated mutant core proteins are competent for nucleotide incorporation albeit mostly small DNA from spliced RNA (Fig. 5D). Since RPA with 5’-end probe detect both the full-length pgRNA and spliced RNA, RPA clearly show that RNA encapsidation is severely impaired in alanine substitution mutants, suggesting that defective replication might be due to the defective encapsidation.
Fig. 5. DNA synthesis and Endogenous polymerase reaction in core particles with core protein phosphorylation mutants. (A) To examine HBV DNA synthesis in core particles, HBV pol ΔPS and the HBc phosphorylation mutants, SRPK-EEE, SRPK-AAA, PKA-AAA, PKA-EEE, AAAAAA or EEEEEE were co-transfected into HuH7 cells. HBV DNA was extracted from isolated core particles and Southern blot analysis performed. Briefly, HBV DNA was separated, transferred to nylon membranes, hybridized with a random-primed $^{32}$P-labeled HBV specific probe, and subjected to autoradiography. Transfection experiments were repeated more than three times. Single-, double-, and partially double-stranded relaxed circular forms of HBV DNA are marked as SS, DS, and RC, respectively. Core particle formation (bottom panel) was determined as described for Fig. 3B. (B) Relative levels of HBV DNA from isolated core particles were measured with the Fujifilm Image Gauge V4.0 program. (C) Polymerase chain reaction (PCR) was performed to further examine the small-sized DNA (Fig. 5A, arrowhead). Small DNA was amplified from glutamic substitution variants co-transfected cells (Fig. 2C, lanes 1–3 lanes), further indicating that small DNA was from spliced RNA. Thirty five cycle PCR reaction with DNA extracted from core particles show that full-length DNA was also amplified from glutamic substitution variants co-transfected cells even though full-length DNA was very faint band in southern blot (Fig. 5A, 4, 6, and 8 lanes). Sequence analysis revealed that PCR amplified small DNA was spliced from nucleotides (nt) 2455–491 which was one of the mostly spliced sites, deleting 1257 nt (Fig. 2C, bottom panel). (D) The replication competence of core particles isolated from HuH7 cell lysates after transient expression of phosphorylation mutant was tested by performing an endogenous polymerase assay. Labeled nucleic acids were isolated from core
particles and separated on a 1% agarose gel and, after drying of the gel, detected by autoradiography.
E. Distribution of nonphosphorylated and phosphorylated core protein mutants.

Next we investigated the intracellular trafficking by HBV core protein phosphorylation mutants. To investigate the possible relationship between phosphorylation and nuclear localization of the core protein, the subcellular localizations of wt and phosphorylation mutant core proteins from transfected HuH7 cells were assessed by immunofluorescence assay (IFA) and confocal immunofluorescence laserscan microscopy. Localization of the wt core protein in HuH7 cells was heterogeneous, with roughly 70% of the transfected cells displaying predominantly cytoplasmic staining, 30% of the transfected cells displaying both nuclear and cytoplasmic staining of the core protein (Fig. 6). This subcellular localization of core protein was significantly altered if cells were transfected with phosphorylation mutants. The representative wt and mutant core protein-positive cells are shown and, unlike previous report, phosphorylation mutants were predominantly localized in the nucleus (Fig. 6), possibly due to the impaired nuclear export or accelerated nuclear import.
Fig. 6. Intracellular distribution of phosphorylation mutant core protein.

Immunofluorescence assay of fixed cells co-transfected with P-deficient, PKA-AAA, PKA-EEE, SRPK-AAA, or SRPK-EEE; (A) the core protein of P-deficient is localized preferentially in the cytoplasm. (B-E) Fluorescence micrograph of HuH7 cells transfected with phosphorylation mutants; The core protein of phosphorylation mutants shows nucleocytoplasmic distribution pattern. Each picture was taken at day 2 post-transfection.
F. Individual roles of putative PKA phosphorylation sites of the core protein in pgRNA encapsidation and DNA synthesis.

To investigate the individual roles of Thr162, Ser170, and Ser178 of the core protein for HBV replication, additional mutants were constructed with different combinations to conduct trans-complementation assays (Fig. 7A). RPA was performed from HuH7 cells co-transfected with phosphorylation mutant core proteins and pYMHA-pol-ΔPS to examine the RNA encapsidation. Similar to the Fig. 4A, pgRNA expressions were comparable (Fig. 7B, second panel), however pgRNA encapsidation by T162A/S170A and T162A/S178A mutants were decreased significantly (Fig. 7B, top panel), although core particle assembly and stability did not seem to be responsible as shown by native agarose gel Western blotting (Fig. 7B, bottom panel).
Fig. 7. Role of individual phosphorylation sites in HBV pgRNA encapsidation. (A) Schematic representation of the amino acid sequence of the C-terminal region of WT and serine substitution mutants of core protein. Phosphorylation site at Thr162, Ser170, and Ser178, within the core protein was substituted by alanine or glutamic acid, respectively. (B) RNase rotection assay (RPA) to detect encapsidated pgRNA. Relative levels of encapsidated pgRNA were measured with the Fujifilm Image Gauge V4.0 program. Transfection
experiments were repeated more than three times.

Next, we analyzed DNA synthesis by Southern blotting (Fig. 8A). The individual phosphorylation mutant core proteins and pHBV-pol-ΔPS were co-transfected into HuH7 cells. Except T162A mutant, RC DNA was detected from single amino acid substituted mutants co-transfected cells. HBV DNA synthesis was decreased significantly from T162A mutant co-transfected cells. HBV DNA synthesis was decreased more in the presence of T162A mutation such as double mutants T162A/S170A and T162A/S178A. These results indicate that Thr162 of core protein is important for DNA synthesis. To confirm the inhibitory effect of T162A mutation for HBV replication, HuH7 cells were triple-transfected with the pHBV-pol-ΔPS, P-deficient (1, 3, 5, or 7µg of DNA), and T162A (7, 5, 3, or 1µg of DNA) mutants. P-deficient mutant provide wt core protein. Equal amounts of DNAs were transfected and all of the constructs had the same CMV promoter, utilizing transcription factors with equal efficiency. With increasing amounts of T162A mutant, HBV DNA synthesis was inhibited significantly (Fig. 8B, right panel) while pgRNA encapsidation was not altered drastically (Fig. 8B, left panel). The core particles that contain T162A mutant core protein may be defective in HBV DNA synthesis. To further investigate for HBV DNA replication, primer extension assay was performed to measure the relative amount of HBV DNA that had completed minus-DNA initiation, plus-DNA initiation, and then circularization of DNA. Internal standard DNA (I.S.) was used to directly compare measurements obtained by primer extension. Each of HBV replication stages, encapsidation, minus-DNA initiation, and replicative intermediate DNAs were quantitated and compared according to the equation (Fig. 9, Table 1, Loeb, 2010).
Fig. 8. Role of individual phosphorylation sites in DNA replication. (A) Southern blot DNA hybridization analysis of core-associated HBV DNA. DNA was extracted from cytoplasmic core particles and analyzed by Southern blot. Whole replicative intermediates were measured with the Fujifilm Image Gauge V4.0 program. (B) Analysis of HBV DNA in cells transfected with increasing doses of T162A. HBV DNA extracted from isolated core particles was measured by Southern blot.
Fig. 9. Role of individual phosphorylation sites in (-) DNA elongation. (A) The position of oligonucleotide primer is shown. Radiolabeled oligonucleotide HBV1661+ was used to detect both the internal-standard (I.S.) and the minus-strand DNA that has undergone the template switch to DRI. A sequencing ladder is shown as a reference. I.S. was used to normalize and compare the level of (-) DNA detected. (B) Formula used to calculate (-) DNA elongation.
Table 1. PgRNA encapsidation, minus-DNA initiation, and replicative intermediate DNAs by phosphorylation mutants

<table>
<thead>
<tr>
<th>Phosphorylation mutants</th>
<th>pgRNA encapsidation (% of WT)</th>
<th>(+)DNA synthesis (% of WT)</th>
<th>Whole E1 density (% of WT)</th>
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<tr>
<td>WT</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>T162A</td>
<td>94.1</td>
<td>83.5</td>
<td>27.6</td>
</tr>
<tr>
<td>T162E</td>
<td>107.1</td>
<td>124.2</td>
<td>96.5</td>
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<tr>
<td>S170A</td>
<td>103.5</td>
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<td>89.8</td>
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<td>S170E</td>
<td>109.9</td>
<td>129.3</td>
<td>111.4</td>
</tr>
<tr>
<td>S178A</td>
<td>98.0</td>
<td>101.1</td>
<td>95.5</td>
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<tr>
<td>S178E</td>
<td>113.5</td>
<td>125.7</td>
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<td>9.6</td>
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<tr>
<td>162/170E</td>
<td>95.2</td>
<td>133.9</td>
<td>47.5</td>
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<tr>
<td>162/178A</td>
<td>67.6</td>
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<td>22.9</td>
</tr>
<tr>
<td>162/178E</td>
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<td>38.9</td>
</tr>
<tr>
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<td>101.5</td>
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<tr>
<td>170/178E</td>
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<td>PKA EEE</td>
<td>74.2</td>
<td>84.7</td>
<td>39.7</td>
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</table>
G. Over expression and knockdown of PKAα on HBV replication

To test whether PKA can modulate HBV replication, replication competent HBV wt plasmid was co-transfected with catalytic subunit of PKAα an expressing vector. Realtime RT-PCR reveal that HBV RNA expressions were not significantly affected (Fig. 10A). Overexpression of PKAα in HBV wt transfected HuH7 cells decreased core protein and core particle formation significantly 48 hours after transfection (Fig.10B). HBV DNA synthesis was also markedly decreased 48 hours after transfection (Fig. 10C) which may be due to the reduction of core protein. To further examine the functions of PKA on HBV replication, HBV wt and PKA siRNA (siPKA) were co-transfected in HuH7 cells and core protein and HBV replicative intermediates were analyzed (Fig. 10D, second and third panels). Knockdown of PKA by siPKA decreased core particles (middle) and DNA replication significantly (top). This result suggests that PKA might contribute to HBV replication in regulating core protein expression and/or core particle formation.
Fig. 10. Effect of PKAα over-expression and knockdown on HBV replication. Co-transfection of HBV wt and the PKAα over-expressing vector leads to decreased core expression (A, right panel) and HBV DNA synthesis (B) in HuH7 cells. Total RNA was isolated 48 hrs after transfection, and HBV RNA was detected by realtime RT-PCR (A, left panel). HBV DNA and core protein expression were analyzed by southern and western blot,
respectively. Transfection of HuH7 (C) and PEB8(D) cells with siRNA represses HBV replication. HuH7 cells were co-transfected with siPKA together with the HBV wt and analyzed for HBV replication. PEB8 cells were transfected with siRNA and analyzed for HBV replication. Oligonucleotides with scrambled sequences were used as negative controls (si-nontarget). HBV DNA and core particle formation were decreased in HuH7 cells 48 h after co-transfection with siRNA together with HBV wt. siPKA also represses HBV DNA synthesis in PEB8 cells.
IV. DISCUSSION

Comparison of the amino acid sequences of the HBV, woolly monkey hepatitis B virus (WMHBV), woodchuck hepatitis B (WHV), ground squirrel hepatitis B virus (GSHV), and duck hepatitis B virus (DHBV) revealed that phosphorylation sites of C-terminal highly conserved except DHBV. The core protein of HBV is associated with protein kinase activity, capable of phosphorylating the arginine-rich, C-terminal domain of core (Roossinck, 1987; Schlicht, 1989). In this study, HBc de/phosphorylation mutants were generated and HBV replication was investigated.

Core protein phosphorylation

Previous studies reported that phosphorylation of core protein takes place exclusively at serine residues for HBV, DHBV and GSHV (Feitelson, 1982; Liao and Ou, 1995; Kann, 1999). It has been shown that several intracellular protein kinases, such as PKC, cyclin-dependent kinase cdc, 46 kDa serine protein kinase, and two SRPK1 and 2, can phosphorylate core protein in vitro. These kinases may be responsible for the preferential phosphorylation of core protein during pgRNA encapsidation and DNA-replication in HBV-infected cells (Liao and Ou, 1995; Kann, 1999; Perlman, 2005; Basagoudanavar, 2007). In the present study demonstrated that threonine 162, serine 170, and serine 178 residues, preferentially threonine 162 and serine 178, in RRXS/T motif of core protein are phosphorylated (Fig. 2B), showing that, in addition to SRPK phosphorylation sites, PKA phosphorylation sites are also utilized by protein kinases.
Intracellular localization of phosphorylated and non-phosphorylated core protein mutant.

Since many DNA viruses depend on the host cell machinery in the nucleus for genome replication, transcription, and mRNA processing, these DNA viruses have to target their genome and accessory proteins to the nucleus. In this study, we have examined nuclear localization of HBV core particle by phosphorylation mutant core proteins. Many reports have described that phosphorylations either up- or down-regulate nuclear transport of proteins, such as lamins (Leukel, 1995), SV-40 T antigen (Rihs, 1989), and PKC (Boulikas, 1995). The nuclear export of influenza viral RNPs also requires phosphorylation (Whittaker, 1995). As for HBV core proteins, phosphorylation of core protein may play a role in regulating nucleocytoplasmic transport. (Liao and Ou, 1995; Kann, 1999; Perlman, 2005; Basagoudanavar, 2007). Both phosphorylated and non-phosphorylated core protein mutant were all seemed to be imported to the nucleus, almost right after their expressions. This may be due to impaired nuclear export or accelerated nuclear import of core protein. However, this may explain why these mutant can not encapsidate HBV RNA inefficiently. This discrepancy might need to be examined further with different HBV strains and other mutant core proteins.

Phosphorylation of the HBV core protein is important for DNA replication.

Although the phosphorylated mutant core proteins could package the pgRNA relatively well, these mutants were not able to fully support viral DNA replication like wt (Fig. 4, 5 and 8). These PKA phosphorylation mutants are consistent with the previous SRPK mutant experiments. Therefore the previous speculation might apply to the this result
saying that, while the core protein phosphorylation may be important for pgRNA packaging, its dephosphorylation may be important for viral DNA replication. HBV DNA replications by individual core protein mutation on Thr162, Ser170, or Ser178 were examined and T162A mutant shows the significantly decreased replicative intermediate DNA without the reduction of encapsidation, indicating that Thr 162 residue may be important for HBV DNA synthesis (Fig. 7 and 8). Le Pogam et al. (Le Pogam, 2005) suggested that the C-terminal of core protein may function by providing the appropriate number of positive charges in the interior of core particles. These charges neutralize the negative charges associated with encapsidated nucleic acids such that a system containing the core particle with encapsidated nucleic acid is more thermodynamically stable than a system containing the core particle with no nucleic acid (Le Pogam, 2005). C-terminal of core protein may provide a thermodynamic drive for DNA synthesis by phosphorylation of C-protein. Since alanine substitutions of core protein has not putative phosphorylation site, less charges in the C-terminal of core protein could cause the pgRNA to either not be encapsidated or only be partially encapsidated. However, glutamic acid substitutions did not cause significant changes in encapsidation of pgRNA and DNA synthesis. Previous study demonstrated that serine157 is most important for pgRNA encapsidation (Lan et al, 1991) and DNA synthesis (Basagoudanavar, 2007). Since the threonine162 is located 5 amino acids downstream of serine157 and serves as the phosphorylation acceptor site determined by in vivo phosphorylation assay, it is conceivable that highly conserved threonine 162 among mammalian hepadnaviruses (Yeh et al., 1991) plays an important role in HBV replication.

Functions of PKA or related kinases in HBV replicating cells may require more
detailed investigations to elucidate the negative or synergistic effects on HBV replication through phosphorylation or dephosphorylation of core proteins.
V. CONCLUSION

To investigate contribution of PKA-mediated phosphorylation on HBV replication, phosphorylation core protein mutants by substituting the phosphorylation site of HBV core protein with alanine or glutamic acid were constructed and HBV replication were examined. The results shown in this study indicated that the phosphorylation sites in the RRXS/T motif were important for the DNA replication. Especially, T162A and T162A containing double mutant decreased DNA synthesis significantly. In vivo phosphorylation assay also showed that T162 and S178 in the RRXS/T motif of core protein are labeled predominantly with $\text{P}^{32}$-orthophosphate. Together with results from in vivo phosphorylation assay and southern blot by phosphorylation mutants, it suggests that threonine 162 of HBV core protein may be one of phosphorylation site and modulate HBV replication.
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PART II

B형 간염바리러스 Core 단백질의 C-말단에 위치한 인산화
부위가 genome복제에 미치는 영향

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(지도교수: 김 정 민)

B형 간염 바리러스 core 단백질의 C-말단 도메인에 위치한 세린157, 세린164, 세린172는 세린/알지닌 특이적 인산화효소 혹은 단백질 인산화효소 C에 의해서 인산화되는 잔기로 알려져 있으며 이것이 B형 간염 바리러스의 복제에 기여한다고 알려져있다. 이와 더불어 RRXS/T motif에 위치한 트레오닌162, 세린170, 세린178는 cAMP-dependent 단백질 인산화효소 A에 의해서 인산화되는 잠정적인 잔기로 알려져 있다. In vivo 인산화 실험을 통해서 트레오닌162, 세린170, 세린178가 인산화 되는 것을 확인하였다. 인산화효소 A에 의해서 인산화되는 부위가 B형 간염 바리러스의 복제에 미치는 영향을 연구하기위해 인산화-core단백질과 비인산화-core단백질의 유사한 형태를 만들기 위해 글루타민산과 아라닌으로의 돌연변이주를 제작하였다. T162A–
HBc 돌연변이주에서 DNA 합성률이 크게 감소하였다. 또한 T162A와 더불어 다른조합으로 돌연변이를 제작하여 실험한 결과, T162에 A의 돌연변이가 존재할 때 DNA 합성률이 현저하게 떨어지는 것으로 나타났다. 모든 실험을 종합하여 볼 때, Core 단백질의 트레오닌162, 세린170, 세린178가 인산화될 수 있으며 이 부위는 B형 간염 바이러스의 복제에 중요하다는 것을 제시하였다.

핵심어: B형 간염 바이러스, HBV core 단백질의 인산화, 단백질 인산화효소 A