C-terminus of Hepatitis B virus RNase H Domain is Important for HBV replication

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- ABSTRACT -

C-terminus of Hepatitis B Virus RNase H Domain is Important for HBV replication

Hepatitis B Virus (HBV) DNA polymerase (P) protein consisting of terminal protein (TP), spacer, reverse transcriptase (RT), and RNase H, plays critical roles in viral assembly and replication. RNase H domain is required for HBV DNA replication, however critical motif or amino acid residues in the RNase H domain for the HBV replication has not been extrensively demonstrated yet. In the present study, several chimeras of P protein by substituting Duck hepatitis B virus (DHBV) sequences were constructed. Accordingly, we tested a series of P protein chimeras in which several substitution mutants were disigned to contain various amino acids of DHBV P protein. It is found that amino acid residues from 800 to 826 (^{800}SRPLLRLPFQPTTGRTSLYAVSPSVPS^{826}) in C -terminus of the RNase H domain are required to complete HBV replication. HBV P protein mutants in which single amino acid residue was substituted were examined for the rescue of HBV replication. Among these mutants tested, L806T mutant P protein have a defect in pgRNA encapsidation and viral DNA synthesis, demonstrating that leucine at position 806 is critical for HBV replication.

Key Words: Hepatitis B Virus, HBV P protein, RNase H, HBV replication, pgRNA encapsidation, Chimera

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Ⅰ. INTRODUCTION

Hepadnaviruses are small, enveloped DNA viruses that replicate preferentially in liver cells and are involved in hepatocellular carcinoma (Wands and Blum, 1991). Although new infections are preventable throught vaccination, the treatment of hepatitis B virus (HBV) infection with nucleoside analog is limited by emergence of drug-resistant viruses. Therefore, understanding of the nature of HBV replication and their components is a prerequisite for the solution of the serious global health problem.

HBV, a prototype of hepadnavirus, has a partially duplex relaxed circular DNA genome, which is replicated by reverse transcription of the RNA genome to produce DNA. Pregenomic RNA (pgRNA) serve as an mRNA for core protein (C) and polymerase (P) proteins and as a template for reverse transcription (Ganem and Varmus, 1987).

Following the HBV P protein recognizes the epsilon (Ɛ) sequence, which is the encapsidation signal of pgRNA, HBV P protein is packaged together with pgRNA within HBV core particles, forming P-Ɛ ribonucleoprotein (RNP) complex. In addition, HBV P protein begins the protein-priming reation (initiation of reverse transcription), in which the hydroxyl group of a Tyr in the TP covalently linked with the first deoxyribonucleotide (Weber et al., 1994; Zoulim and Seeger, 1994; Lanford et al., 1997). Subsequently, the 2-3 deoxyribonucleotides are copied from the bulge region of Ɛ to make TGAA or GAA, the nascent minus-strand DNA. However, previous study indicated that the covalent linkage between TP and the first nucleotide is not a prerequisite for initiating of DNA synthesis since short oligomer is synthesized without protein priming (Kim

et al., 2004). Even so this protein priming reaction should be crucial for translocation since priming deficient mutant cannot translocate the nacent DNA (Kim et al., 2004). Inside core particle, the oligomer covalently linked to the P protein translocates from ε to direct repeat (DR) 1 at 3'end of pgRNA, leading to the elongation of minus-strand DNA (Tavis and Ganem, 1995; Tavis et al., 1994). Minus-strand DNA synthesis continues until the 5'end of pgRNA, while RNA template is degradad by the RNase H activity except the short stretch of RNA. This short terminal RNA oligomer is translocated to DR2, from which it serves as a primer for plus-strand DNA synthesis (Ganem and Schneider, 2001). Once plus-strand DNA synthesis has reached the 5'end of minus-strand DNA, a final translocation to the 3'end of minus-strand DNA ocuurs, resulting in partially double-stranded relaxed circular DNA genome.

The multifuctional hepadnavirus P protein plays a key role in HBV DNA synthesis and encapsidation of pgRNA (Bartenschlager et al., 1990; Hirsch et al., 1990), and acts as a primer for minus-strand DNA synthesis (Wang and Seeger, 1992; Weber et al., 1994; Zoulim and Seeger, 1994). HBV P protein has been divided into four charaterized domains, terminal protein (TP), spacer, reverse transcriptase (RT), and RNase H .

The TP utilizes an invariant tyrosine residue to function as a protein primer to initiate reverse transcription (Weber et al., 1994; Zoulim and Seeger, 1994; Lanford et al., 1997). The spacer region connects the TP and RT/RNase H domains. This region is not essential for P protein function, because much of it can be deleted without affecting P protein function (Bartenschlager and Schaller, 1988; Chang et al., 1990). However, it has been shown recently that the conserved cysteine resdues in the spacer domain are ciritical for pgRNA encapsidation since P protein mutants that

have mutation of three conserved cysteine residues in spacer domain failed to support pgRNA encapsidation. RT and RNase H regions of HBV P protein display homology with RT of retrovirus with highly conserved sequence motifs of RT and RNase H (Loeb et al., 1991). RNase H activity is specific for the RNA strand in a RNA:DNA hybrid. Therefore, RNase H domain is required to remove the pgRNA after it has been copied into minus-strand DNA during reverse transcription (Radziwill et al., 1990). The RNase H domain of moloney murine leukemia virus (MMLV) RT can be expressed independently of the P protein domain and retain high levels of RNase H activity (Tanese and Goff, 1988). Several groups have expressed and purified the RNase H domain of human immunodeficiency virus type-1 (HIV-1) reverse transcriptase, but these proteins are inactive or have extremely low levels of RNase H activity (Restle et al., 1992).

Heterologous complementation and then the chimeric proteins with the related viruses could be performed to identify the critical amino acid residues or motif of viral proteins for the replication. The genome of HBV and woodchuck hepatitis virus (WHV) are about 60% identical (Galibert et al., 1982) and the genome of HBV and DHBV are about 40% identical (Mandart et al., 1984). However, heterologous complementation with related hepadnaviruses, such as WHV or DHBV, could not be performed because HBV replication could be complemented easily by WHV C and/or P proteins, and vice versa, but could not be complemented at al by DHBV C and/or P proteins, and vice versa (Okamoto et al., 1990; Ziermann and Ganem, 1996). Therefore, chimeric viruses or chimeric proteins with the HBV and DHBV or the HBV and WHV had never tried.

In previous study, chimeric P proteins were constructed to map the essential parts of the P gene product that support HBV replication (Park,

2005). It had been demonstrated that HBV pgRNA encapsidation and viral DNA replication could not tolerate the change in the P protein (Park, 2005). In accordance with the previous report (Chen et al., 1996), it had been demonstrated that C-terminus of RNase H was also important for HBV replication (Park, 2005). However, significant amino acid sequences in C-terminus of RNase H have not been extrensively demonstrated yet.

In this study, RNase H chimeras were constructed by exchanging the C-terminus of HBV RNase H with the corresponding region of DHBV RNase H to identify the critical amino acids or motif in C-terminus of RNase H for pgRNA encapsidation or HBV DNA replication (Park, 2005). In this regard, Chimeric P proteins were designed to contain various lengths of corresponding DHBV RNase H sequences, while the N-terminus of HBV RNase H was retained (Park, 2005). It was demonstrated that amino acid residues from 800 to 826 (^{800}SRPLLRLPFQPTT GRTSLYAVSPSSVPS^{826}) in C-terminus of the HBV RNase H are required to complete HBV replication (Park, 2005). And it also showed that each of the motif from 800 to 826 in C-terminus of the RNase H, 800 to 802, 803 to 807, 808 to 811, 812 to 818, and 819 to 826, is required for HBV replication. Accordingly, chimeric P proteins that have single amino acid substitution mutation from 803 to 811 were constructed. Among these mutants, mutant with leucine 806 to threonine substitution in RNase H (RH-L806T) had more profound defect in pgRNA encapsidation and viral DNA synthesis than other mutants, indicating that L806 residue in RNase H of HBV P protein is critical for HBV replication.

Ⅱ. MATERIALS AND METHODS

A. HBV plasmid DNA construction

The HBV wild-type (wt) of subtype adw R9, pPB, was used as the wt HBV construct in which transcription of pgRNA is controlled by cytomegalovirus immediate early (CMV IE) promoter (Kim et al., 2004). To supply pgRNA and C protein *in trans,* P protein deficient mutant, P def, the P gene was frame-shifted and the AUG start codon was modified to ACG in C ORF (Kim et al., 2004). To supply HBV P protein *in trans,* HBV P protein construct in which HBV P ORF was inserted into pcDNA3, was used to express full length HBV P gene (Park, 2005). The DHBV P protein construct in which DHBV P ORF was inserted into pcDNA3, was used to express full length DHBV P gene (Park, 2005). RNase H chimeras were constructed by exchanging the C-terminal RNase H domain of HBV P protein with the corresponding region of DHBV RNase H (Park, 2005). Chimeric RNase H mutants were generated by site-directed mutagenesis using a fusion PCR.

DH800-826 mutant of RNase H had 27 amino acid residues substitutions from residue 800 to 826 of HBV RNase H to corresponding residues of DHBV RNase H (Park, 2005). DH800-811 and DH812-826 mutants of RNase H had N-terminus 12 or C-terminus 15 amino acid residues of DHBV RNase H, respectively (Park, 2005). DH800-802 mutant of RNase H was replaced C-terminus 3 amino acid residues from 800 to 802 of HBV RNase H by corresponding amino acid residues of DHBV RNase H (Park, 2005). DH803-807 mutant of RNase H was replaced

C-terminus 5 amino acid residues from 803 to 807 of HBV RNase H by corresponding amino acid residues of DHBV RNase H (Park, 2005). DH808-811 mutant of RNase H was replaced C-terminus 4 amino acid residues from 808 to 811 of HBV RNase H by corresponding amino acid residues of DHBV RNase H (Park, 2005). DH812-818 mutant of RNase H was replaced C-terminus 7 amino acid residues from 812 to 818 of HBV RNase H by corresponding amino acid residues of DHBV RNase H (Park, 2005). DH819-826 mutant of RNase H was replaced C-terminus 8 amino acid residues from 819 to 826 of HBV RNase H by corresponding amino acid residues of DHBV RNase H (Park, 2005).

To generate a leucine 803 to proline mutant of HBV RNase H, the forward mutagenic promer HBV 220 (5'-TCTCGTCCCCCTCTCCGTCTG CCGTTCCA-3', targeting nt 3318 to 3290) and antisense prime SP1124 (5'-ACAGTGGGAGTGG CACCTTC-3', targeting nt 1105 to 1124) that binds to upstream of SP6 promoter of pcDNA3, were used for one PCR reaction, while a second PCR reaction was performed with sense primer HBV 6 (5'-CCTTTACCCCGTTGCTCGGC-3', targeting nt 2947 to 2967) and antisense mutagenic primer HBV 221 (5'-TGGAACGGCAGACGGA GAGGGGGACGAGA-3', targeting nt 3290 to 3318). The resulting 350 bp and 610 bp of PCR products were conbined by fusion PCR. 940bp of fusion PCR product was digested with SacⅡ and XhoI (New England Biolabs, Inc, USA) and cloned into the corresponding restriction sites of wt HBV P protein, yielding pRH-L803P construct.

For constructing the leucine 804 to aspartate mutant of HBV RNase H, sense mutagenic primer HBV 222 (5'-TCTCGTCCCCTTGATCGTCT GCCGTTCCA-3', targeting nt 3318 to 3290) and antisense primer SP1124 (5'-ACAGTGGGAGTGGCACCTTC-3', targeting nt 1105 to 1124), were

used for one PCR reaction, while a second PCR reaction was performed with sense primer HBV 6 (5'-CCTTTACCCCGTTGCTCGGC-3', targeting nt 2947 to 2967) and antisense mutagenic primer HBV 223 (5'-TGGAACGGCAGACGATCAAGGGGACGAGA-3', targeting nt 3290 to 3318). The resulting 350bp and 610bp of PCR products were conbined by fusion PCR. 940bp of fusion PCR product was digested with SacⅡ and XhoI and cloned into the corresponding restriction sites of wt HBV P protein, yielding pRH-L804D construct.

For constructing the arginine 805 to tryptophan mutant of HBV RNase H, sense mutagenic primer HBV 224 (5'-TCTCGTCCCCTTCTGGCTGCCGTTCCA-3', targeting nt 3318 to 3290) and antisense primer SP1124 (5'-ACAGTGGAGTGGCACCTTC-3', targeting nt 1105 to 1124) were used for one PCR reaction, while a second PCR reaction was performed with sense primer HBV 6 (5'-CCTTTACCCCGTTGCTCGGC-3', targeting nt 2947 to 2967) and antisense mutagenic primer HBV 225 (5'-TGGAACGGCAGCCAGAGAAGGGGACGAGA-3', targeting nt 3290 to 3318). The resulting 350bp and 610bp of PCR products were conbined by fusion PCR. 940bp of fusion PCR product was digested with SacⅡ and XhoI and cloned into the corresponding restriction sites of wt HBV P protein, yielding pRH-R805W construct.

For constructing the leucine 806 to threonine mutant of HBV RNase H, sense mutagenic primer HBV 226 (5'-TCTCGTCCCCTTCTCCGTACGCCGTTCCA-3', targeting nt 3318 to 3290) and antisense primer SP1124 (5'-ACAGTGGGAGTTTGGCACCTTC-3', targeting nt 1105 to 1124) were used for one PCR reaction, while a second PCR reaction was performed with sense primer HBV 6 (5'-CCTTTACCCCGTTGCTCGGC-3', targeting nt 2947 to 2967) and antisense mutagenic primer HBV 227 (5'-T

GGAACGGCGTACGGAGACGGGGACGAGA-3', targeting nt 3290 to 3318). The resulting 350bp and 610bp of PCR products were conbined by fusion PCR. 940bp of fusion PCR product was digested with SacⅡ and XhoI and cloned into the corresponding restriction sites of wt HBV P protein, yielding pRH-L806T construct.

For constructing the proline 807 to alanine mutant of HBV RNase H, sense mutagenic primer HBV 228 (5'-TCCCCTTCTCCGTCTGGC TTTCCAGCCG-3', targeting nt 3323 to 3295) and antisense primer SP1124 (5'-ACAGTGGGAGTGGC ACTTC-3', targeting nt 1105 to 1124) were used for one PCR reaction, while a second PCR reaction was performed with sense primer HBV 6 (5'-CCTTTACCCCGTTGCTCGG C-3', targeting nt 2947 to 2967) and antisense mutagenic primer HBV 229 (5'-CGGCTGGAAAGCCAGACGGAGAAGGGGA-3', targeting nt 3295 to 3323). The resulting 350bp and 610bp of PCR products were conbined by fusion PCR. 940bp of fusion PCR product was digested with SacⅡ and XhoI and cloned into the corresponding restriction sites of wt HBV P protein, yielding pRH-P807A construct.

Single amino acid substitution mutants of the pRH-Q809P and pRH-P810Y have proline substitution mutation of glutamine at position 809, tyrosine substitution mutation of proline at position 810, respectively (unpublished). 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 and transfection

HuH7 Hepatoma cell lines were used for the transfection of HBV P protein and chimeric P protein constructs. HuH7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, invitrogen, USA) and 100 IU of penicillin per milliliter at 37℃ in 5% CO_2 and split every third day. HBV wt and mutant plasmids (8 μg) were transfected into HuH7 cells on a 10-cm plate using polyethylenimine (PEI, 8 μg). One day post-transfection, cells were rinced in PBS and fed with fresh media. .

C. Isolation of core particles

Three days after transfection, HuH7 cells were treated with lysis buffer (10 mM Tris-Cl [pH8.0], 50 mM NaCl, 5 m M EDTA, 1% Noniodet P-40). Then nuclei were removed by centrifugation. The clarified lysate was adjusted with 10 mM $MgCl_2$ and 8 mM $CaCl_2$ solution, and incubated for 1hr at 37℃ with 20 U DNase I (Roche, USA) and 60 U micrococcal nuclease (Fermentas, Canada). Cytoplasmic core particles were precipitated with 6.5% polyethylene glycol.

D. RNase protection assay (RPA)

Encapsidated pgRNA was analyzed by RNase protection assay (RPA). Core particles were isolated as described above. pgRNA from core particles was extracted following proteinase K (100 μg/ml) and DNase I (20 U) treatment (Kim et al., 2004). To analyze cytoplasmic pgRNA, total

RNA was extracted with RNAzol B (Tel-test INC, USA). In brief, cells were lysed with 1 ml of RNAzol B, then 200 ul chloroform was added to lysate. The cell lysates were incubated at room temperature for 5 min and then centrifugated at 12,500 rpm for 15 min at 4℃. The aqueous phase that transferred to fresh tube was precipitated by addition of equal volume of isopropanol. Mixing and centrifuge at 12,000 rpm for 15 min at 4℃. Total RNA pellet was washed with 1 ml of 70 % ethanol, dissolved in nuclease-free water, and then subjected to RPA analysis. To prepare riboprobe for RPA, part of the HBV sequence (nt 1805-2187) was cloned into pGEM3Zf(+) vector, generating pRPAFD (Kim et al., 2004). From this construct, 446 nt of radiolabeled anti-sense probe was synthesized in vitro using SP6 RNA polymerase with α-^{32}P-UTP (specific activity, 800 Ci/mmol). The RPA procedure was performed using the manufacturer's protocol (RPA II, Ambion, USA). The protected pgRNA is 369 nt long.

E. Core particle Western blotting

Core particles were isolated as described above. Isolated core particles were dissolved 15 ul of Tris-Acetate EDTA buffer and electrophoresed on a 1% native agarose gel and blotted onto a polyvinylidene fluoride (PVDF) membranes. Immunoblotting was performed using an anti-HBc antibody (DAKO, Denmark). Horseradish peroxidase-conjugated anti-rabbit secondary antibody and enhanced chemical luminescence (ECL) were employed to visualize HBV core particles.

F. Southern blotting

To analyze HBV DNA synthesis by Southern blotting, HBV DNA was extracted from core particles, separated by agarose gel electrophoresis, and hybridized to a ^{32}P-labeled random-primed probe specific for the HBV sequence (Kim et al., 2004).

G. SDS-PAGE and Western blotting

Three days post-transfection, cells were treated with lysis buffer (50 mM Tris-Cl [pH8.0], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail (Calbiochem, USA). The lysates were mixed with sample buffer (100mM Tris-Cl [pH6.8], 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM β-mercaptoethanol). After boiling, the protein samples were resolved through 8% sodium dodecyl sulfate-polyacrylamide gels and then transferred to a polyvildene fluoride (PVDF) membrane (Millipore, USA) for 1 hour 30 min at 100 V. After blocking, the membrane was incubated for 16 hours at 4℃ with 1:200 of anti-HBV P protein-spacer antibosy (8D5) sc-81591 (Santa Cruz Biotechnology, USA) and then washed with phosphate buffered saline (PBS) containging 0.1% Tween-20 (Sigma, USA) for 10 min three times. After washing, the membrane was reacted with horseradish peroxidase (HRP)-Mouse anti-mouse IgG (DACO, Denmark) for 1 hour at room temperature. After washing the membrane, they were exposed to enhanced chemical luminescence (ECL) solution and then X-ray film. The α-Tubuin was detected by using a mouse anti-tubulin antibody (1:2000, Oncogene, USA).

Ⅲ. RESULT

A. HBV P constructs containing DHBV P residues in RNase H domain.

The HBV wild-type (wt) construct of subtype adw R9 (Blum et al., 1991) in which transcription of pgRNA was controlled by cytomegalovirus immediate early (CMV IE) promoter (Kim et al., 2004) was used for this study. P deficient mutant could express pgRNA and core, surface, and X protein except P protein (Kim et al., 2004). This P deficient mutant was used to co-transfect with various RNase H mutants of HBV P protein. For the construction of chimeric P proteins of HBV and DHBV (Park, 2005), amino acid sequence of P protein was aligned (Fig. 1). Then each of the RNase H mutants is named according the exchanged amino acid residues of DHBV P protein. A previous study showed that DH1C and DH2C, had C-terminal 35 amino acid of DHBV RNase H region, but joining site with HBV RNase H were different, could not support HBV pgRNA encapsidation and viral DNA synthesis (Park, 2005). The Del C mutant, which is deleted construct from 827 to 845 amino acid residues in HBV RNase H, failed to support HBV replication (Radziwill et al, 1990) and used a negative control in this study.

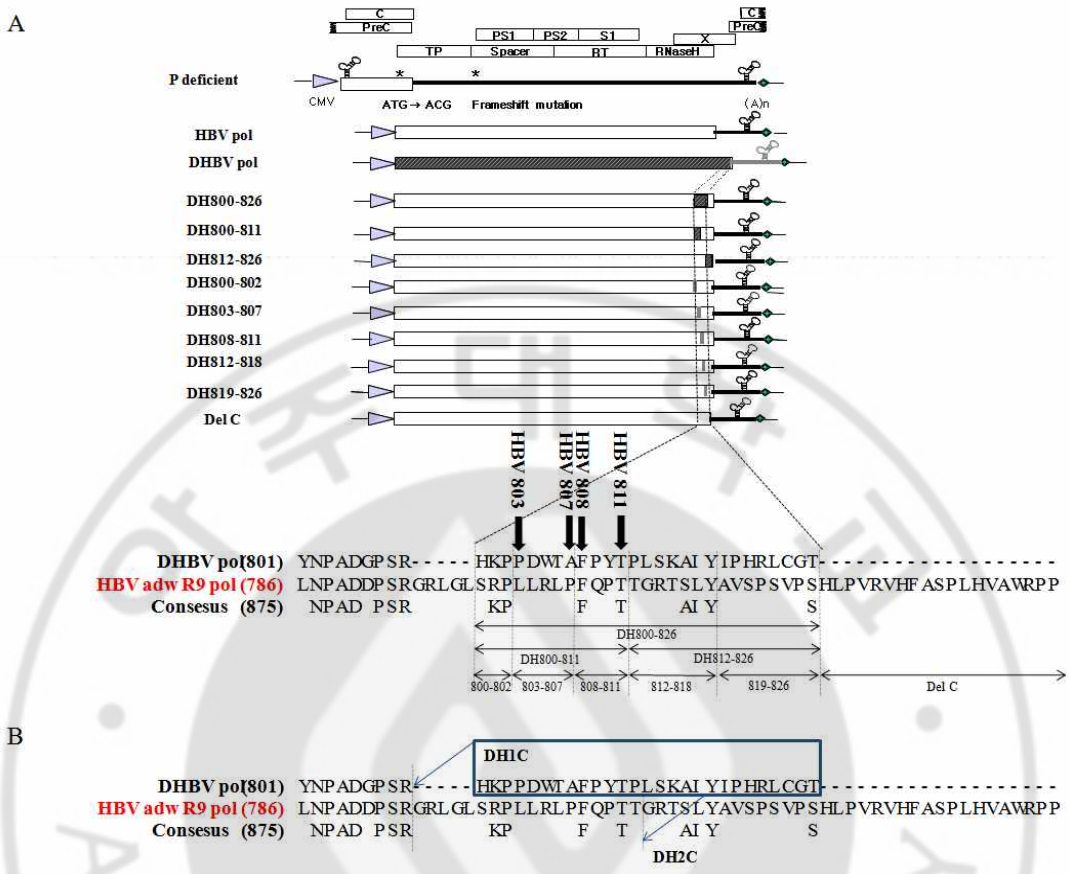


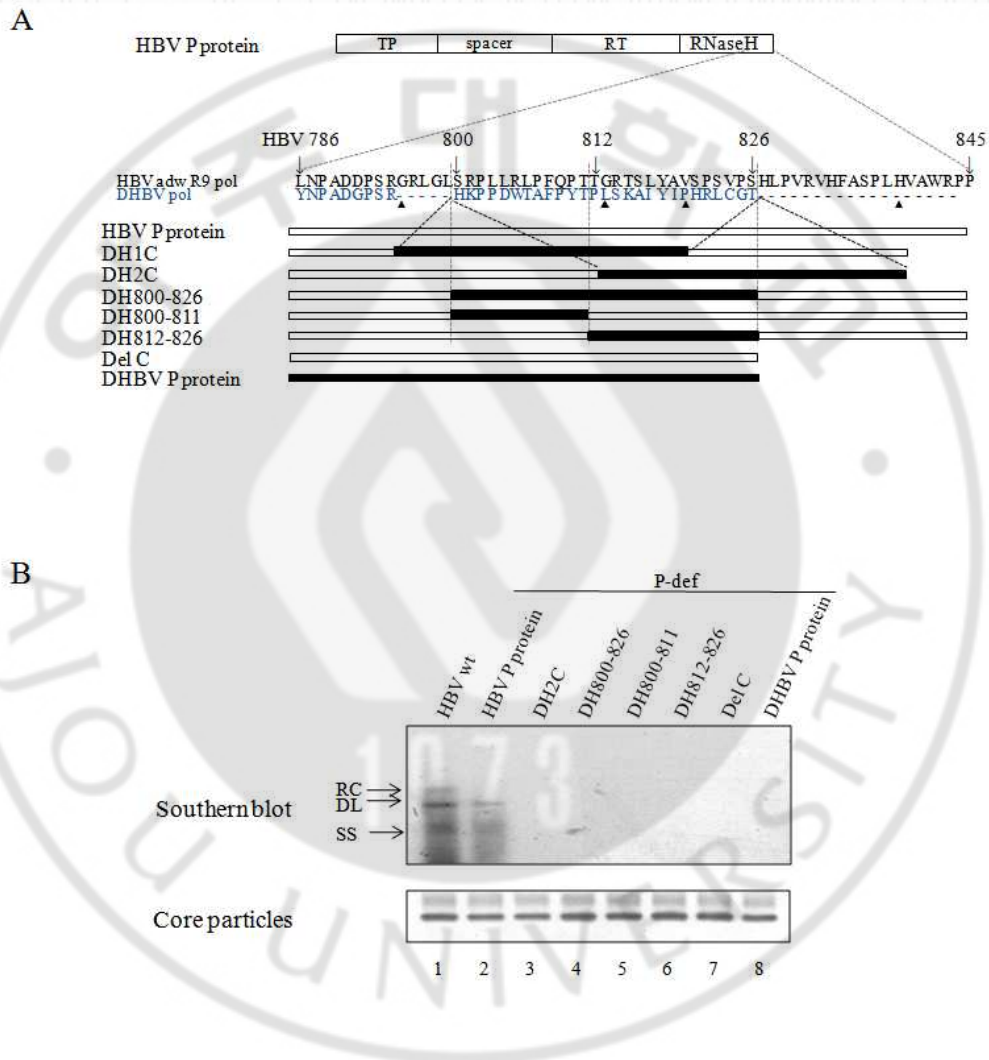
Fig. 1. Schematic diagram of chimeric RNase H constructs with substituted DHBV P protein sequence. (A) Open boxes and hatched boxes indicate HBV and DHBV sequences, respectively. The ORFs of C, P, S, and X genes are presented as open boxes. Each domain (TP, spacer, RT, and RNase H) of the P protein is indicated. The cytomagalovirus immediate early (CMV) promoter is represented by a filled arrowhead. For P deficient mutant, mutated sites are marked with asterisks. The C-terminus of RNase H is subdivided into several motifs, DH800-826, DH800-811, DH812-826, DH800-802, DH803-807, DH808-811, DH812-818, and DH819-826, as denoted by double-headed arrows. (B) DH1C and DH2C mutant of RNase H contain 3'-end sequences of DHBV RNase H.

B. Amino acid residues from 800 to 826 (^{800}SRPLLRLPF QPTTGRTSLYAVSPSVVPS^{826}) in C-terminus of the RNase H are critical for pgRNA encapsidation and HBV DNA replication.

In the previous study, it was demonstrated that amino acid residues from 795 to 845 of HBV RNase H were required for HBV replication since DH1C and DH2C RNase H chimeras (Fig. 2A) failed to support pgRNA encapsidation and viral DNA synthesis (Park, 2005). The DH800-826 mutant of HBV RNase H with corresponding region of DHBV RNase H (Fig. 2A) was tested to determine whether amino acid residues from 800 to 826 is required for HBV replication. In addition, two mutants of HBV RNase H, DH800-811, DH812-826, which have subdivided DH800-826 into two motifs (Fig. 2A), were tested to examine which motif is important for HBV replication. To investigate HBV DNA genome replication, cytoplasmic core particles that were isolated from HuH7 cells were co-transfected with HBV P protein or chimeric RNase H mutants and P def mutant. Replicating HBV DNA was observed in core particles with single-stranded (SS), double-stranded (DL), and partially double-stranded relaxed circular HBV DNA (RC). HBV wt construct was used as a positive control (Fig. 2B, lane 1). The HBV P protein construct and P deficient mutant co-transfected cells had ability to rescue the viral DNA synthesis and encapsidate pgRNA (Fig. 2B, lane 2 and 2C, lane 3). The DHBV P protein construct and P deficient mutant co -transfected cells were defective in HBV replication (Fig. 2B, lane 8 and 2C, lane 9) since HBV replication could not be complemented by DHBV P protein, and vice versa (Ziermann and Ganem, 1996). DH2C, DH800-826, DH800-811, and DH812-826 mutants of RNase H also failed to support viral genome replication (Fig. 2B, upper

panel, lanes, 3, 4, 5, 6), suggesting that amino acid residues from 800 to 826 are indispensable for HBV DNA synthesis. To detect core particles, the isolated core particles were electrophoresed on the native agarose gel and then Western blot analysis was conducted using the anti-HBc antibody (Kim et al., 2004). Core particle assembly should be indentical since core proteins participate in core particle assembly are solely provided from P def mutant. As expected, all of the core particles were displayed similar on native gel (Fig. 2B and 2C). RPA analysis was performed to detect cytoplasmic and encapsidated pgRNA using riboprobe specific for the detection of pgRNA (Fig. 3C). Similar levels of cytoplasmic pgRNA were observed in HBV P protein construct or RNase H mutants and P def co-transfected HuH7 cells (Fig. 3C, top panel). Following extraction of HBV pgRNA from isolated core particles, RPA analysis showed that encapsidated pgRNA levels of the DH2C, DH800-826, DH800-811, and DH812-826 RNase H mutants significantly reduced (Fig. 3C, middle panel, lanes, 4, 5, 6, 7) compare to that of the HBV P protein construct (Fig. 3C, lower panel, lane, 3). Core particle Western blot analysis ensures that identical amounts of core particles had been loaded (Fig. 3C, bottom panel). To examine whether mutants of RNase H affected the expression of HBV P protein, Western blot analysis was performed (Fig. 4D). The P protein expression levels derived from HBV wt, HBV P protein, and RNase H mutants were comparable (Fig. 4D, upper pane, lanes, 1, 2, 3, 4, 5, 6, 7), but there is no P protein expression derived from DHBV P protein since DHBV P protein could not be detected by anti-HBV P protein-spacer antibody (Fig. 4D, upper panel, lane 8). Overall, HBV P protein could not tolerate 27 amino acid residues substitutions from residues 800 to 826 of HBV RNase H to corresponding resiudues of DHBV RNase H, suggesting

that C-terminus of the RNase H are critical for pgRNA encapsidation and HBV DNA replication.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spacer
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RNaseH
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DH2C
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Del C
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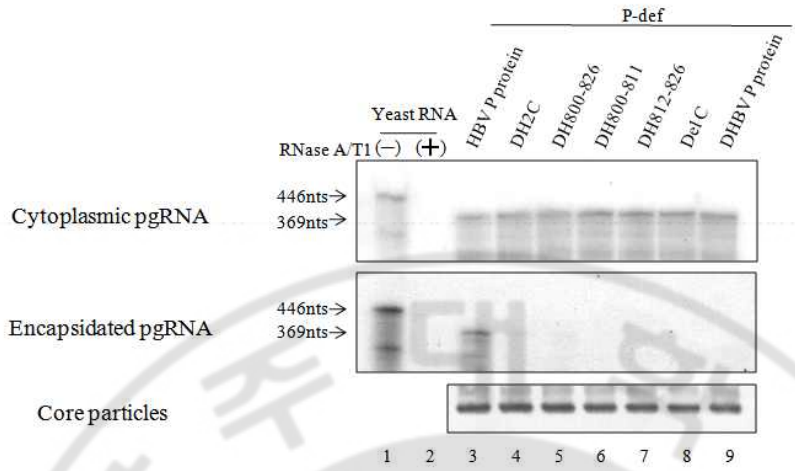
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DH800-811
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446nts
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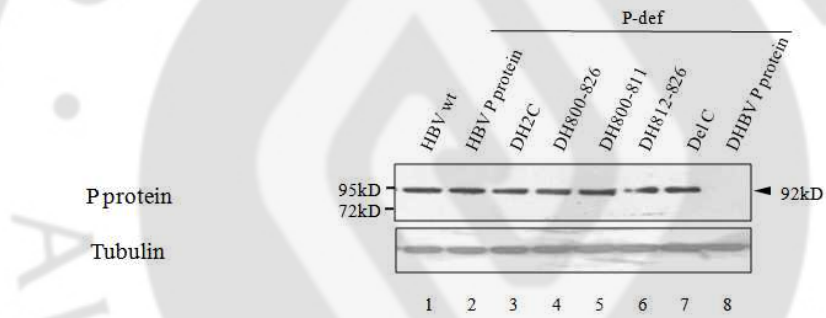
Core particles
1 2 3 4 5 6 7 8 9

D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P protein
95kD
72kD
92kD
Tubulin
1 2 3 4 5 6 7 8

Fig. 2. Amino acid residues from 800 to 826 in C-terminus of the RNase H are critical for pgRNA encapsidation and HBV DNA replication. (A) Schematic diagram of chimeric RNase H mutants with substituted DHBV P protein sequence. Open boxes and hatched boxes indicate HBV and DHBV sequences, respectively. (B) Southern (upperpanel) and Western blot analyses (lower panel) were performed to detect HBV replication. HBV DNA extracted from core isolated core particles and then separated, transferred to nylon membranes, hybridized with a random-primed ^{32}P-labeled HBV specific probe. Single-, double-stranded and partially double-stranded relaxed circular forms of HBV DNA are marked as SS, DS, and RC, respectively. Western blot analysis of core particles were performed using an anti-HBc antibody to normalize the transfection efficiency. (C) RNase protection assay (RPA) to detect HBV cytoplasmic pgRNA (top panel) and encapsidated pgRNA (middle panel). In vitro transcribed radiolabeled antisense RNA probe (446 nt) was hybridized overnight at 50 ℃ with pgRNA from isolated core particles or 10 μg total RNA. Protected pgRNA (369 nt) following RNase digestion was run on a 5% polyacrylamide-8 M urea gel and visualized by autoradiography. Yeast RNA, which served as a negative control, was analyzed with (+) and without (-) RNase treatment. (D) Western blot analysis of HBV P protein was performed using a anti-HBV P protein-spacer antibody (Santa Cruz Biotechnology, USA) to demonstrate equal P protein expression levels (upper panel). Tubulin served as a loading control (lower panel).

C. The small motif substituted HBV RNase H domain mutants at C-terminus have ability to support HBV DNA synthesis.

Based on the result that amino acid residues from 800 to 826 in C-terminus of RNase H are HBV replication (Fig. 2), we made a series of small motif substitution mutants, DH800-802, DH803-807, DH808-811, DH812-818, and DH819-826 (Fig. 3A, Park, 2005). To determine which RNase H mutants are able to rescue the HBV DNA genome replication, HuH7 cells were co-transfected with RNase H mutants and P def mutant. Following extraction of HBV DNA from isolated core particles, Southern blot was performed (Fig. 3B, upper panel). Consistent with the result of Fig. 2B, The DH2C, DH800-826, DH800-811, DH812-826, and Del C mutants of RNase H and P def mutant co-trasfected cells completely failed to supprort viral DNA synthesis (Fig. 3B, upper panel, lanes, 3, 4, 5, 6, 12). The levels of viral DNA synthesis from DH800-802, DH803-807, DH808-811, DH812-818, and DH819-826 RNase H muntants and P def mutant co-transfected cells were modestly reduced (Fig. 3B, upper panel, lanes, 7, 8, 9, 10, 11) compared to that of P protein and P def mutant co-transfected cells (Fig. 3B, upper panel, lane, 2) although each of the reduction levels is different. Core particle Western blot analysis showed that all of the core particles were displayed similar on native gel (Fig. 3B, lower panel). Overall, these results suggested that each of the small motif of RNase H at C-terminus is required for complete HBV DNA replication.

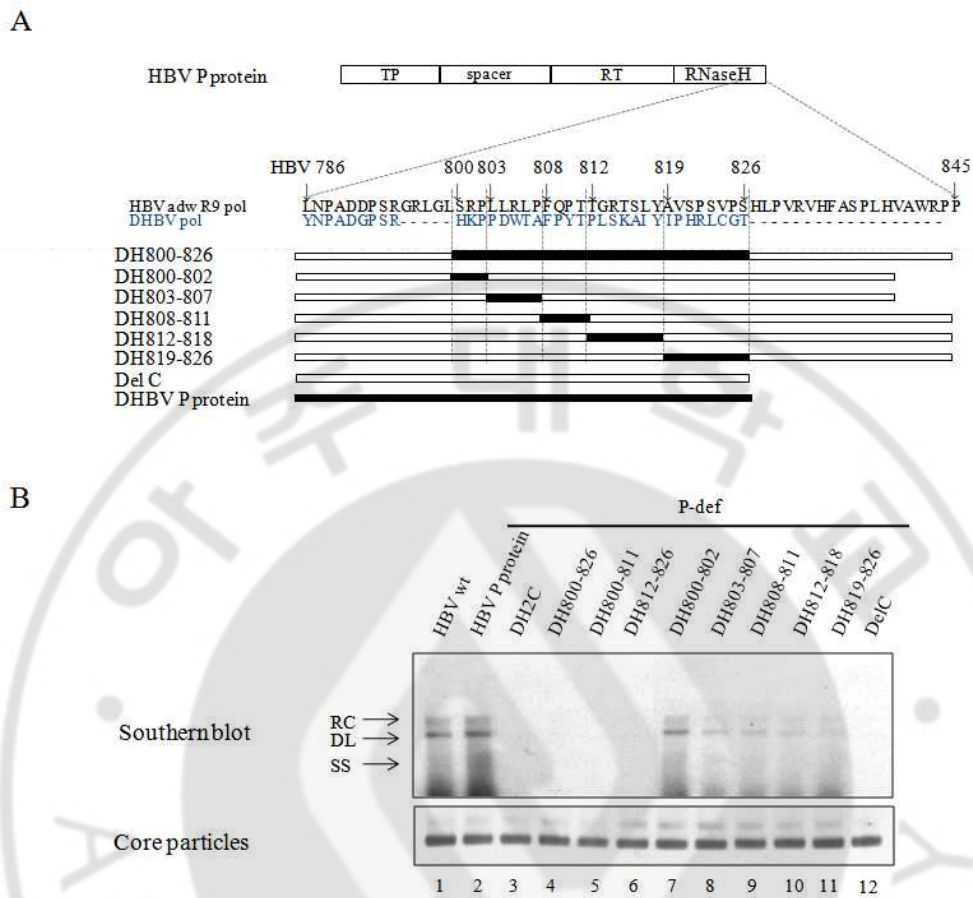

Fig. 3. HBV DNA synthesis by small motif substituted HBV RNase H domain mutants at C-terminus (A) Schematic diagram of chimeric RNase H mutants with substituted DHBV P protein sequence. Open boxes and hatched boxes indicate HBV and DHBV sequences, respectively. (B) Southern blot analysis to detect HBV DNA replication (upper panel). Single-, double-stranded and partially double-stranded relaxed circular forms of HBV DNA are marked as SS, DS, and RC, respectively. Western blot analysis of core particles were performed to normalize the transfection efficiency (lower panel).

D. The small motif substituted HBV RNase H domain mutants at C-terminus have ability to support HBV pgRNA encapsidation.

Following the cotransfection with P protein expression plasmid and P def mutant or with RNase H mutants, RPA was performed to detect cytoplasmic and encapsidated pgRNA (Fig. 4A). Similar levels of cytoplasmic pgRNA were observed in HBV P protein construct or RNase H mutants and P mutant co-transfected HuH7 cells. (Fig. 4A, top panel). The pgRNA from isolated core particles were analyzed with RPA analysis. Consistent with the result of Fig. 2C, HuH7 cells were co-transfected with DH2C and P def mutant or with DH800-826, DH800-811, DH812-826 RNase H mutants led to significantly reduce pgRNA encapsidation levels (Fig. 4B, middle, lanes, 4, 5, 6, 7,). The levels of pgRNA encapsidation from DH800-802, DH803-807, DH808-811, DH812-818, and DH819-826 RNase H mutants and P def mutant co-transfected cells were modestly reduced (Fig. 4B, middle, lanes, 8, 9, 10, 11, 12) compared to that of P protein and P def mutant co-transfected cells (Fig. 4B, middle panel, lane, 3). Native agarose gel electrophoresis followed by Western blotting with an anti-HBc antibody revealed that core particles were formed at comparable levels in HBV P protein construct or RNase H mutants and P def mutant co-transfected cells (Fig. 4B, bottom panel). Western blot analysis confirmed that the expression levels of the P protein were comparable between P protein construct and RNase H mutants. (Fig. 4B, upper panel). Together with the results depicted in Fig. 3, each of the small motif in RNase H at C-terminus, 800 to 802, 803 to 807, 808 to 811, 812 to 818, and 819 to 826, were required for complete HBV replication. Accordingly, single amino acid substitution mutants were constructed to define specific amino

acid residues critical for HBV replication (Fig. 5A).

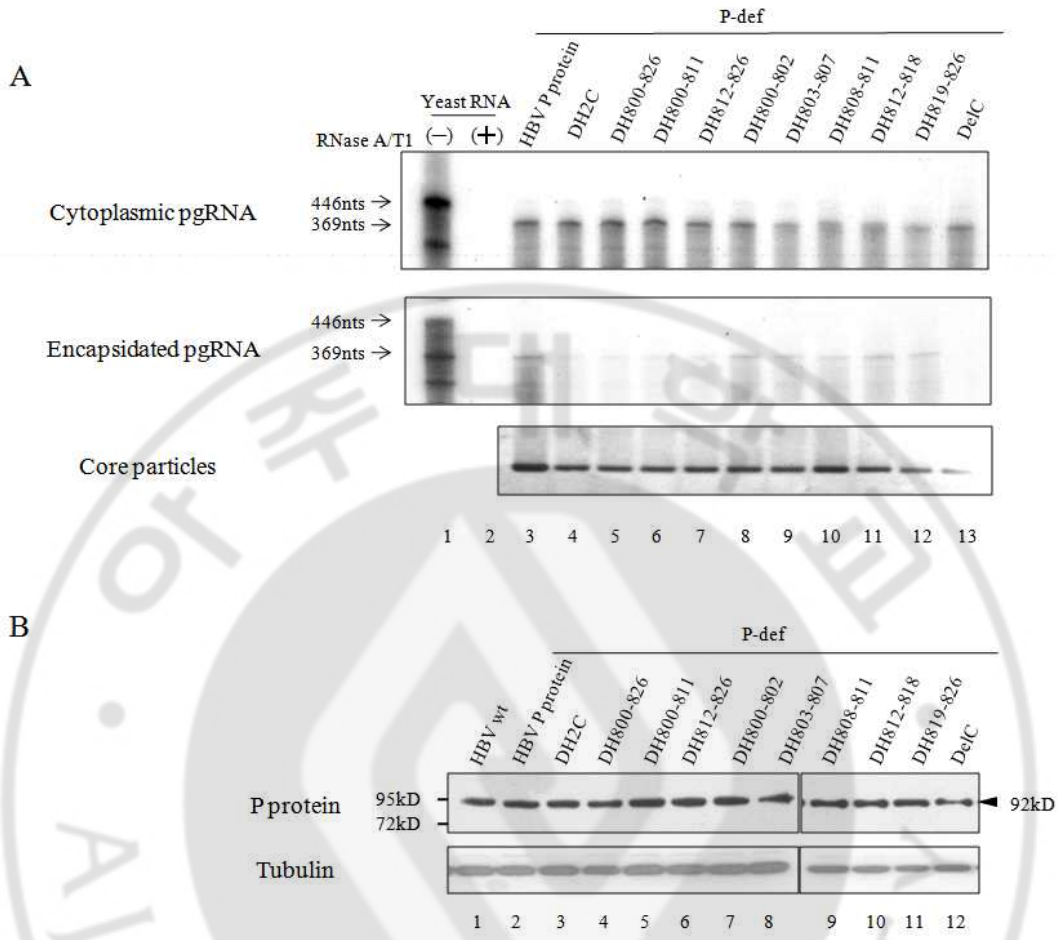

Fig. 4. HBV pgRNA encapsidation by small motif substituted HBV RNase H domain mutants at C-terminus (A) RNase protection assay (RPA) was performed to detect cytoplasmic pgRNA (top panel) and encapsidated pgRNA (middle panel). Western blot analysis was performed to ensure that identical amounts of core particles were loaded onto each gel (bottom panel). (B) Western blot analysis of HBV P protein was performed as described in the legend to Fig. 2.

E. A leucine residue at position 806 in RNase H of HBV P protein is important for HBV DNA synthesis.

To further define the critical amino acid residues within the small motif of RNase H at C-terminus, nine amino acid residues from 803 to 811 in RNase H of P protein were individually replaced by corresponding amino acid residues of DHBV RNase H (Fig. 5A). All of the single amino acid substitution mutants, except for the two amino acid residues (F808, T811) that are conserved between the HBV and DHBV RNase H, were constructed and designated RH-L803P, RH-L804D, RH-R805W, RH-L806T, RH-P807A, RH-Q809P, and RH-P810Y (Fig. 5A). These seven single amino acid substitution mutants were evaluated by Southern blot analysis (Fig. 5B, upper panel). HuH7 cells were co-transfected with P protein construct or RNase H single amino acid point mutants and P def mutant. Southern blot analysis indicated that while DH803-807, DH808-811, and RH-L806T mutants of RNase H were unable to rescue the complete HBV DNA synthesis (Fig. 5B, upper pane, lanes, 3, 7, 9), mutants of RNase H harboring mutation of other residues (L803, L804, R805, P807, F808, T811) were able to support complete the viral DNA synthesis (Fig. 5B, upper panel, lanes, 4, 5, 6, 8, 10, 11) similarly to HBV P protein construct and P def mutant co-transfected cells (Fig. 5B, upper panel, lane, 2). Core particle Western blot analysis confirmed that the core particle fomation levels derived from all of constructs were comparable (Fig. 5B. lower panel), suggesting that the L806 residue from 803 to 807 are critical for HBV DNA synthesis.

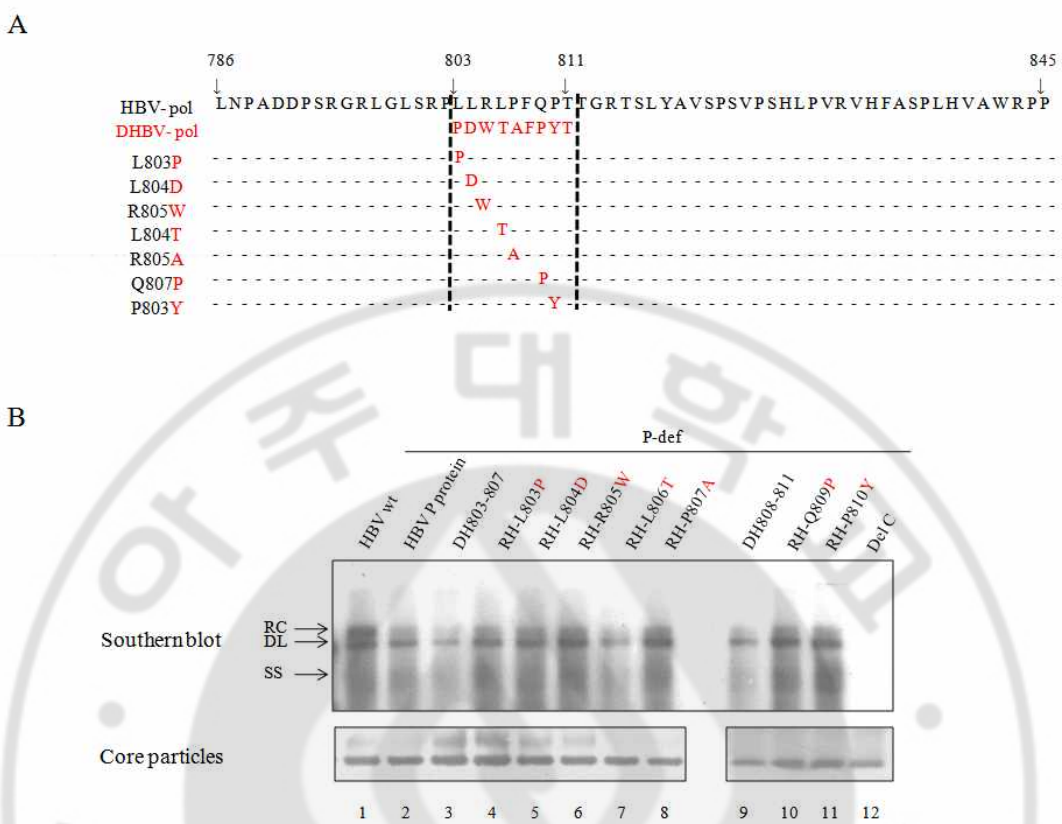

Fig. 5. HBV DNA synthesis by mutant HBV P proteins with single point mutation at C-terminus of RNase H. (A) Schematic diagram of single point mutants. These HBV RNase H mutants were constructed by site-directed mutagenesis. Seven amino acid residues from 803 to 811 in RNase H replaced by corresponding amino acid residues in DHBV RNase H, generating RH-L803P, RH-L804D, RH-R805W, RH-L806T, RH-R807A, RH-Q809P, and RH-P810Y. (B) Southern blot analysis to determine the levels of viral DNA synthesis (upper panel). Core particle Western blot analysis was performed to ensure the transfection efficiency (lower panel).

F. A leucine residue at position 806 in RNase H of HBV P protein is important for pgRNA encapsidation.

Next, single amino acid substitution mutants of HBV RNase H were each inspected for their ability to support pgRNA encapsidation. Following the core particles isolated from HuH7 cells that were co-transfected with P protein expression plasmid and P def mutant or with RNase H mutants, RPA analysis was conducted to detect cytoplasmic pgRNA and to determine the ability of RNase H mutants to encapsidate pgRNA. There were no significant differences in cytoplasmic pgRNA expression levels between P protein or RNase H mutants and P def mutant co-transfected cells (Fig. 6A, top panel). However, Encapsidated pgRNA levels were considerably varied between P protein or RNase H mutants and P def mutant co-transfected cells (Fig. 6A, middle panel). The levels of pgRNA encapsidation from DH803-807 or RH-L806T RNase H and P def mutant co-transfected cells were significantly reduced (Fig. 6A, middle panel, lanes 4, 8) compared to that of P protein or other RNase H mutants and P def mutant co-transfected cells (Fig. 6A, middle panel, lane 3). Encapsidated pgRNA levels from HuH7 cells that were co-transfected with RH-Q809P or RH-P810Y and P def mutant were identical compared to that of P protein and P def mutant co-transfected cells (Fig. 6A, middle panel, lanes, 11, 12). Western blot analysis performed in parallel indicated that the expression levels of the P protein were slightly comparable (Fig. 6B, upper panel). Together with results depicted in Fig. 5, it was indicated that leucine residue at position 806 in RNase H of P protein is critically important for HBV replication.

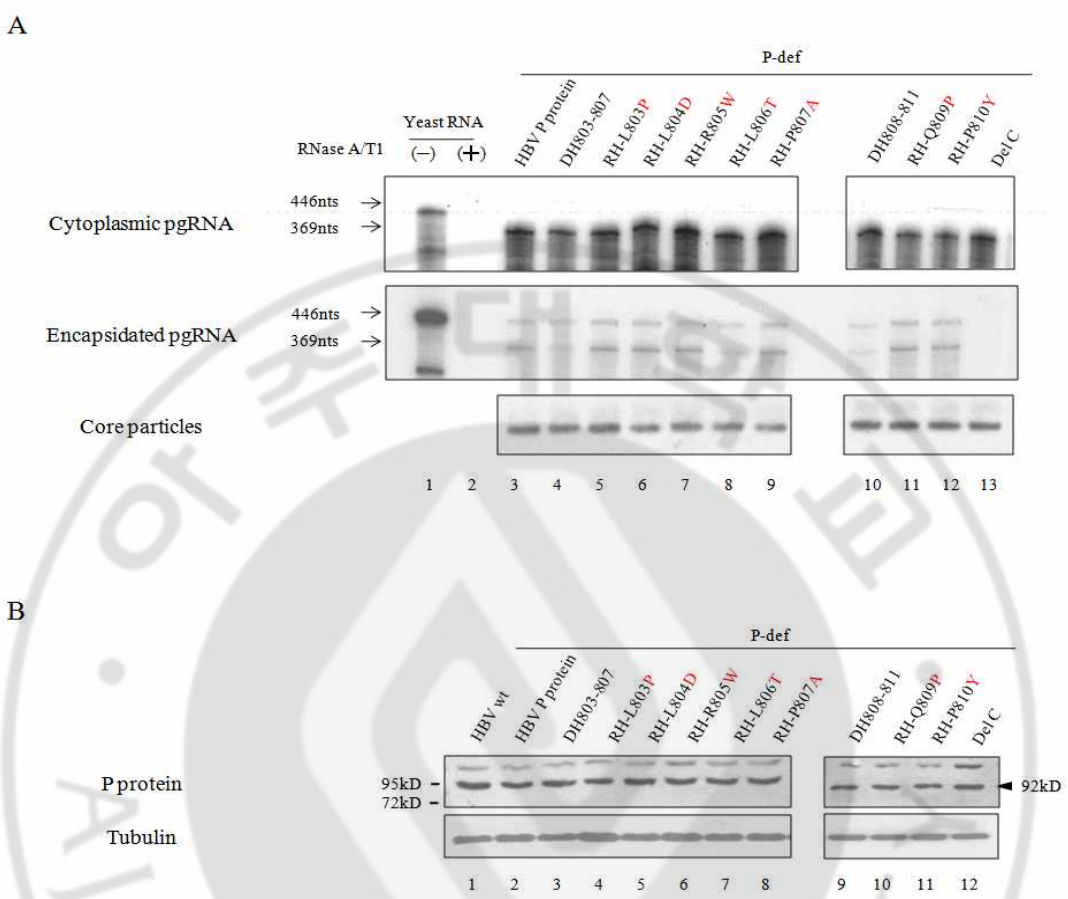

Fig. 6. HBV pgRNA encapsidation by mutant HBV P proteins with single point mutation at C-terminus of RNase H. (A) RPA was performed to detect cytoplasmic pgRNA (top panel) and encapsidated pgRNA (middle panel). The upper panel shows cytoplasmic pgRNA from isolated core particles while the lower panel depicts encapsidated pgRNA. Core particle Western analysis was performed to ensure that identical amounts of core particles were loaded onto each gel (bottom panel). (B) For Western blot analysis of P protein, equvalent amounts of total cell lysate were analyzed using anti-HBV P protein-spacer (upper panel). Tubulin served as a loading control (lower panel).

Ⅳ. DISCUSSION

In this study, amino acid residues from 800 to 826 in RNase H of HBV P protein are critical since encapsidation of pgRNA and DNA synthesis were defective (Figs. 2). Next, single amino acid substitution mutants in C-terminus of RNase H were made and tested to define critical amino acid residues for HBV replication. It was showed that leucine residue at position 806 in RNase H is important for HBV replication since RNase H mutant harboring mutation at position 806 led to reduce pgRNA encapsidation and viral DNA synthesis (Fig. 5 and 6). These results might arise a question of which mechnisms trigger defect in HBV replication.

It is not yet clear from this study why some single amino acid substitution mutations led to reduce HBV replication while others did not. This understanding may be reached only when the crystal structure of RNase H is determined. However, It should be noted that insufficient data have been so far reported for the RNase H activity of entire P protein, whose expression in heterologous cell systems or purification from insected individuals remains very elusive. It had been reported that the carboxylate-rich and negatively charged active site (DDE motif, Asp-Asp-Glu) are crucial for RNase H activity that depends on the presence of divalent cations, such as Mg^{2+} or Mn^{2+}, to bind substrate (Nowotny et al., 2005). Accordingly, Metal ions must be correctly located in active site (702D, 731E, 750D) of RNase H for enzyme activity. These results also identify the findings Potenza et al. (2007), who determined that substrate-binding ablility of isolated RNase H domain may in part be attributed to the catalytic residues H715, R744, and K745 in C-terminus of RNase H. It can be postulated that modification of active site in RNase H

may in part be caused by mutation in C-terminus of RNase H, so that it leads to prevent metal ions from binding active site although there is no DDE motif or carboxylate-rich site in our R Nase H mutant.

It had been reported that heat shock protein (Hsp), such as Hsp70, Hsp90, and p23, play a role in binding between DHBV P protein and the epsilon stem-loop region (Hu et al., 2004). Therefore, it was believed that the family member of the Hsp involved in the initiation of DHBV genome replication. Additional chaperone Hsp 60 has, in part indirectly, been implicated in affecting P protein activity (Park and Jung, 2002). It was shown that TP (amino acids 1-199) and the RNase H (amino acids 680-842) domains of HBV P protein associated with Hsp60 independently, suggesting that C-terminus of RNase H domain is a binding site between Hsp60 and P protein to stablize P protein activity (Park and Jung, 2002). Therefore, it led us to speculate that mutations in C-terminus of RNase H domain may have disrupted RNase H conformation that is requred to Hsp60 binding for RNase H.

In addition to HBV RNase H has a nuclease activity that degrades the RNA strand of RNA-DNA hybrid, RNase H may be involved in pgRNA encapsidation and viral DNA synthesis. I could not examine whether RNase H domain participate in efficient viral DNA synthesis, including initiation of minus-strand DNA synthesis, elongation of minus-strand DNA, and initiation of plus-strand DNA synthesis. These possibilities will be tested in the future.

Overall, it can be suggested that HBV P protein could not tolerate the small changes in the HBV RNase H for complete HBV replication. Accordingly, the HBV RNase H is an attractive molecular target for development of new anti-hepatitis B drugs.

V. CONCLUSION

To identify the critical amino acids in HBV RNase H domain for HBV replication, the approaches using chimeric viruses have employed in this study. To accomplish this goal, several HBV RNase H mutants were constructed from the amino acid sequences comparison from DHBV RNase H. This study has demonstrated that the C-terminus of RNase H domain are critically involved in pgRNA encapsidation and viral DNA synthesis. In addition to the RNase H that specific for the RNA strand in a RNA:DNA heteroduplex, HBV RNase H may be involved in pgRNA encapsidation and viral DNA synthesis.

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- 국문요약 -

B형 간염 바이러스 DNA중합효소 RNase H domain의 C-말단이 바이러스 증식에 미치는 영향

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목적: B형 간염 바이러스 DNA 중합효소의 RNase H domain은 구조적 측면이나 분자생물학적 측면에서 연구된바가 거의 없다. 이 RNase H domain은 pgRNA encapsidation 에도 필수적임이 알려져 있으나 RNA binding에 작용하는 region은 아직 규명되지 않았다. 따라서 HBV 게놈복제 기능에 작용하는 RNase H domain의 아미노산을 규명하고 RNase H domain의 아미노산잔기가 HBV 게놈복제에 작용하는 단계를 알아보고자 하였다.

재료 및 방법: 다양한 RNase H mutant들을 제작하여서 세포 내로 transfection하였다. RPA의 결과를 토대로 각각의 RNase H chimera들의 pgRNA encapsidation 여부와 효율을 측정하고 EPA를 통하여 DNA 중합효소의 활성을 조사하였다. 그리고 나서 Southern blot을 통해서 RNase H chimera들의 DNA의 복제의 효율을 비교 분석하여 HBV DNA replication에 중요한 아미노산을 조사하였다.

결과: 본 연구를 통해 HBV RNase H 의 C 말단이 pgRNA encapsidation 과 DNA replication에 중요하다는 것을 밝혀 내었다. 그리고 B형 간염 바이러스

DNA 중합효소의 806번째 아미노산이 HBV replication에 중요하다는 것을 밝혔다.

핵심되는 말: B형 간염 바이러스, B형 간염 바이러스 DNA 중합효소, RNase H