

## Bacterial expression, purification, and in vitro N-myristoylation of fusion hepatitis B virus preS1 with the native-type N-terminus

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### Abstract

Very low-level expression of hepatitis B virus (HBV) preS1 with the native-type N-terminus hampered the biochemical and functional studies on its myristoylation. In the present study, the fusion HBV preS1 with the native-type N-terminus and a His6-Tag fused to C-terminus (HBV preS1-HT) was highly expressed in *Escherichia coli*. This was due to an introduced mutation of the rare codon GGA found in the HBV preS1 to the codon preferred by *E. coli*, GGU. The protein was rapidly purified from bacterial lysate by Ni-IDA affinity chromatography. The experimental assays using <sup>3</sup>H-labeled substrate demonstrate that the purified HBV preS1-HT can be effectively N-myristoylated by recombinant human protein N-myristoyltransferase (NMT) in vitro.

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Hepatitis B virus (HBV)<sup>1</sup> causes the public health problems throughout the world because it is easily spread by blood, saliva, and semen. Until now, HBV vaccines [1] and HBV genome [2] have been widely studied. The envelope of HBV consists of large, middle, and small or major surface proteins designated LS, MS, and SS proteins, respectively. MS is coterminal with SS, having an additional 55-amino-acid extension (named preS2) at the N-terminus. LS is coterminal with SS and MS and is characterized by an additional 108- or 119-amino-acid extension (named preS1) at the N-terminus of MS [3]. The HBV preS1 that represents the most peripheral structure of the viral particle and attaches to the liver cell membrane is responsible for some of the biological properties of HBV [4]. It has been found that the myristic acid linked to the N-terminal glycine residue of HBV preS1 [5] is involved in its intracellular retention

[6] and, thus, essential for viral infectivity [7,8]. Although N-myristoylation of many proteins [9,10] has been demonstrated to play an important role for the subcellular targeting or membrane binding, little is known on the role of HBV preS1 N-myristoylation in the process of viral replication, assembly, and infectivity. Given the essential role of N-myristoylated proteins in many physiological and pathological events such as signal transduction, carcinogenesis, and viral replication or assembly, N-myristoylation has been recognized as possible chemotherapeutic targets for anti-viral, anti-fungal, and anti-neoplastic therapy [11]. Accordingly, based on studies of the specific inhibition of the N-myristoylation of HBV preS1, we may develop new anti-HBV chemotherapeutic drugs through interfering N-myristoylation of HBV preS1 to stop viral infection [7,8]. For such studies, a good supply of HBV preS1 is essential. However, so far only very low-level expression of HBV preS1 with the native-type N-terminus, which is necessary for study on the molecular mechanism of its myristoylation, has been reported both in *Escherichia coli* and vaccinia virus systems [12,13] and hampered the biochemical and functional studies on its myristoylation. In this study, by introducing mutation of the rare codon GGA found in the HBV preS1 to the codon

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<sup>1</sup> Abbreviations used: HBV, hepatitis B virus; NMT, myristoyl-CoA: protein N-myristoyltransferase; mC $\alpha$ 4H,  $\alpha$  subunit of mouse cAMP-dependent protein kinase; LB, Luria-Bertani medium; IPTG, isopropyl- $\beta$ -thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride.

GGU preferred by *E. coli*, the HBV preS1 with the native-type N-terminus was highly expressed in soluble form, can then be rapidly purified by Ni-IDA affinity chromatography, and effectively N-myristoylated by recombinant human myristoyl-CoA:protein N-myristoyltransferase (NMT) [14] *in vitro*. It provides a good system for the screening of specific inhibitors for the N-myristoylation reaction of HBV preS1.

## Materials and methods

### Expression vectors and reagents

Expression vector pMFHT [15] with the His6-Tag coding sequence was constructed in our laboratory. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Boehringer–Mannheim or GIBCO BRL. Taq DNA polymerase was from Sino-American Biotechnology Company. Agarose was obtained from GIBCO BRL. *Pseudomonas acyl CoA synthetase*, LiCoA, acrylamide, bisacrylamide, IPTG, imidazole, and iminoacetic acid (IDA)–Sepharose 6B were ordered from Sigma. [9,10(*n*)-<sup>3</sup>H]Myristic acid, Amplify™, and Hyperfilm were from Amersham. The recombinant human NMT [14] and  $\alpha$  subunit of mouse cAMP-dependent protein kinase (mC $\alpha$ 4H) [16] were prepared in our laboratory.

### Construction of expression plasmids for fusion HBV preS1-HT

The primer 1 (5'-AGGCCATGGGAGGTTGGTCTTC-3'), primer 2 (5'-ATTCCATGGGTTGGTTGGTCTTCC-3'), primer 3 (5'-ATTCCATGGGTTGGTTGGTCTTCCAAACCACGAGAAG-3') as the forward primers with an *Nco* I site (underlined), and primer 4 (5'-AGGGAATTCGCCTGAGGATGACTGTCT-3') as the reverse primer with an *Eco*R I site (underlined) were designed for amplifying the native and mutant primer containing the synonymous mutations (boxed) of the second codon or both of the second and eighth codons, respectively, and synthesized in Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The HBV preS1 coding region was amplified from plasmid pADR 1 containing human HBV genome DNA [17] by PCR under the reaction condition of 94 °C, 5 min; 94 °C, 30 s, 50 °C, 30 s, 72 °C, and 30 s for 30 cycles; 72 °C, 10 min. The amplified DNA fragments were inserted upstream from the His6-Tag sequence in the expression vector pMFHT under the control of T7 promoter. Three expression plasmids pMF-preS1-HT1 (wild), pMF-preS1-HT2 (mut1), and MF-preS1-HT3 (mut2) were obtained by analysis of restriction digestion and DNA sequencing. All of the DNA manipulation or identification in-

cluding the digestion with restriction enzymes, the agarose gel electrophoresis, the purification of DNA fragments, and the ligation with T4 DNA ligase were performed as described by Sambrook et al. [18].

### Analysis of fusion HBV preS1-HT expressed in *E. coli*

*E. coli* BL21(DE3) cells harboring the different expression plasmids were individually grown to reach about 0.4–0.6 OD<sub>600</sub> in LB medium containing 100  $\mu$ g/ml ampicillin, and then induced to produce the fusion HBV preS1-HT by adding IPTG to a final concentration of 0.5 mM and extending incubation for an additional 3 h. The fusion HBV preS1-HT produced from bacteria was analyzed by SDS-PAGE and Western blotting. First, the lysated samples of bacteria were directly subjected to 15% SDS-PAGE and, subsequently, electroblotted onto nitrocellulose membrane. Then, the electroblotted membrane was preincubated with 5% milk in TBST (150 mM NaCl, 25 mM Tris, and 0.1% Tween 20, pH 7.5) for 1 h, probed with anti-preS1 antibody conjugated with horseradish peroxidase (provided by Zhu-Chuan Zhang at Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences) at 37 °C for another 1 h, and washed with TBST. Finally, the membrane was developed with ECL reagent (Amersham), followed by exposure to X-film.

### Purification of fusion HBV preS1-HT

The fusion HBV preS1-HT was purified by one step using Ni-IDA affinity chromatography [19]. Briefly, the induced *E. coli* BL21(DE3) cells harboring the expression plasmid pMF-preS1-HT2 were harvested by centrifugation for 10 min at 5000g, resuspended in buffer A (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 10% glycerol, 1 mM PMSF, and 40 mM imidazole), and sonicated 30 min on ice for 30 times (with 1 min interval per time). The supernatant fraction was obtained from the sonicated bacterial lysate by centrifugation for 30 min at 10,000g and applied to Ni-IDA agarose column (Sigma) at a constant flow rate of 4 ml per min. Then, the non-specific bound proteins were removed with buffer A (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 10% glycerol, 1 mM PMSF, and 40 mM imidazole) and the fusion HBV preS1-HT was eluted with buffer B (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 10% glycerol, 1 mM PMSF, and 200 mM imidazole). The eluted proteins were analyzed by SDS-PAGE, as described above.

### *In vitro* N-myristoylation assay

First, <sup>3</sup>H-labeled myristoyl-CoA was synthesized as described by Towler and Glaser [20] and the reaction mixture containing 20 mM Tris-HCl (pH 7.4), 1 mM

dithiothreitol, 10 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 5 mM ATP, 10 mM LiCoA, 1 μM [9,10-<sup>3</sup>H]myristic acid (5.2 μCi) (Amersham), and 0.3 U/ml *Pseudomonas acyl* CoA synthetase (Sigma) was allowed to incubate at 30 °C for 30 min. Then, the NMT assay was carried out in a final 20 μl volume of NMT buffer containing 30 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.45 mM 2-mercaptoethanol, 10 μM peptide, 1% (v/v) Triton X-100, and the purified recombinant human NMT. The NMT reaction was initiated by the addition of 10 μl synthesized [<sup>3</sup>H]myristoyl-CoA in each reaction, with incubation at 30 °C extended for 30 min and stopped by boiling for 5 min. Finally, the boiled NMT reaction mixture was subjected to 15% SDS-PAGE and the [<sup>3</sup>H]myristoyl peptides were analyzed by autoradiography.

**Results and discussion**

*Constructs for the expression of fusion HBV preS1-HT with the native-type N-terminus*

For the N-myristoylation of HBV preS1, the presence of a native N-terminus containing the N-end-glycine is essential. So far, however, very low-level expression of HBV preS1 in such a state has been reported both in the *E. coli* and the vaccinia virus systems [12,13], which hampered the biochemical and functional studies on its myristoylation. Although, Kim et al. [12] postulated that

a hairpin structure of HBV preS1 mRNA between the sequence 5'-GAGGUU at nucleotide 5–10 and 5'-AACCUC at nucleotide 20–25 affected the expression of HBV preS1 and produced a mutated gene (5'-AAAAAA replaced 5'-GGAGGU at nucleotide 4–9) to achieve high level production of HBV preS1. But the N-terminus of the product was mutated (Lys-Lys replaced Gly-Gly) and could not be used to study its myristoylation. To gain high level production of HBV preS1 with the native-type N-terminus, the 5'-region hairpin structure (wild) of HBV preS1 mRNA is designed to destabilize (mut1) or restabilize (mut2) by synonymous mutation (underlined base) of the second codon (GGA to GGU) or both the second (GGA to GGU) and the eighth (CCU to CCA) codons in stem sequence (boxed) shown in Fig. 1A. Accordingly, three different forward primers were synthesized for PCR. The wild and mutant DNA fragments were amplified from human HBV genome DNA (adr subtype) [17] template. The three different amplified and separated DNA fragments (371 bp, Fig. 1B) were inserted into the His6-Tag expression vector pMFHT to obtain three expression plasmids pMF-preS1-HT1 (wild), pMF-preS1-HT2 (mut1), and pMF-preS1-HT3 (mut2). The three constructs were identified by restriction digestion and DNA sequencing, and used to express the same fusion HBV preS1-HT (153 amino acids), which consists of HBV preS1 (2–119 amino acids) and a 34-amino-acid C-terminal (120–153 amino acids) extension containing His6-Tag (123–128 amino

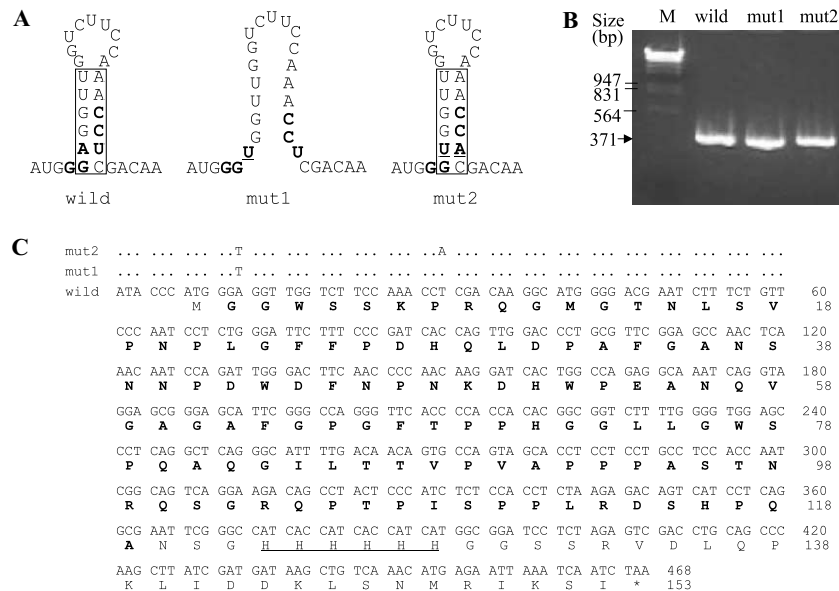


Fig. 1. Construction of expression plasmids for the fusion HBV preS1-HT with native-type N-terminus. (A) Mutation strategy and schematic structure of HBV preS1 mRNA 5'-region. The 5'-region hairpin structure (wild) of HBV preS1 mRNA is designed to destabilize (mut1) or restabilize (mut2) by synonymous mutation (underlined base) of the second codon (GGA to GGU) or both the second (GGA to GGU) and the eighth (CCU to CCA) codons in stem sequence (boxed). (B) Separation of the amplified wild and mutant DNA fragments by agarose gel electrophoresis. M, molecular weight marker. (C) DNA sequences and their encoding amino acids of HBV preS1-HT. The wild (dotted) and mutated bases in 5'-sequences of mut1 and mut2 are shown. The amino acids of HBV preS1 (2–119 amino acids) are bolded. The 34-amino-acid (120–153 amino acids) C-terminal extension containing His6-Tag (123–128 amino acids, underlined) is encoded by vector sequence (unbolded).

acids, underlined) encoded by vector sequence (Fig. 1C). We have not removed the 25-amino-acid C-terminal extension (129–153 amino acids), since it has little affected the expression and purification of many recombinant proteins in our experiments by using this vector. The further experiments also indicate that the 25-amino-acid C-terminal extension do not affect the expression, purification, and N-myristoylation of HBV preS1-HT.

#### Increasing expression of fusion HBV preS1-HT by synonymous mutation

Three constructed expression plasmids pMF-preS1-HT1 (wild), pMF-preS1-HT2 (mut1), and pMF-preS1-HT3 (mut2) were individually transformed and the fusion HBV preS1-HT was induced under the same condition. SDS-PAGE (Fig. 2A) and Western blot (Fig. 2B) results indicate that the fusion HBV preS1-HT (lanes 2, 4, and 6) can be induced from *E. coli* BL21(DE3) cells harboring the above three constructed expression plasmids by comparison with their uninduced samples (lanes 1, 3, and 5). But the amount of induced fusion HBV preS1-HT (about 17 kDa) from both mut1 (lane 4) and mut2 (lane 6)

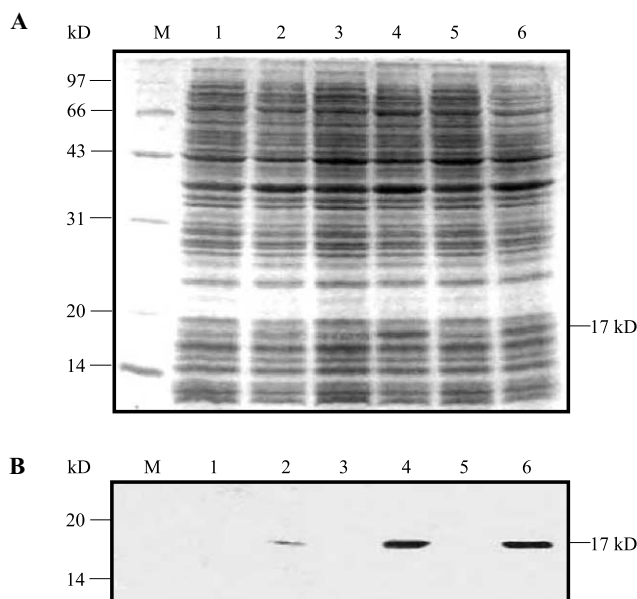


Fig. 2. SDS-PAGE and Western blot analysis of fusion HBV preS1-HTs expressed in *E. coli* BL21(DE3). (A) SDS-PAGE analysis. The *E. coli* BL21(DE3) cells harboring pMF-preS1-HT1 (wild), pMF-preS1-HT2 (mut1), and MF-preS1-HT3 (mut2) were, respectively, grown for 2 h as controls (lanes 1, 3, and 5) and then induced by IPTG for another 3 h (lanes 2, 4, and 6). The bacterial lysates were directly subjected to 15% SDS-PAGE with molecular weight marker sample (lane M). (B) Western blot analysis. After electrophoresis, the above samples with the same order were subsequently electroblotted onto a nitrocellulose membrane. The electroblotted membrane was incubated with anti-preS1 antibody conjugated with HRP. The immunoreactive proteins were visualized by using the ECL detection system.

was increased to the same level and much higher than that of wild (lane 2), suggesting that the expression of fusion HBV preS1-HT was highly promoted by synonymous mutation of the second rare codon GGA to major codon GGU with high usage frequency in *E. coli*. These results also suggest that the major factor affecting the expression of HBV preS1 gene in *E. coli* is the unfavored second codon rather than the hairpin structure at HBV preS1 mRNA 5'-region.

#### Purification of fusion HBV preS1-HT by Ni-IDA affinity chromatography

The *E. coli* BL21(DE3) cells harboring the expression plasmid pMF-preS1-HT2 (mut1) were induced to the high production of fusion HBV preS1-HT by IPTG. The induced bacterial supernatant was directly used to purify the fusion HBV preS1-HT by one step using Ni-IDA chromatography. The SDS-PAGE result indicates that the expressed fusion HBV preS1-HT (lane 2 in Fig. 3) is mainly in a soluble form (lane 3 in Fig. 3) and can be purified by Ni-IDA agarose column (lane 5 in Fig. 3). Fusion HBV preS1-HT protein (5.5 mg) has been purified from 1 L of induced bacteria by one-step affinity chromatography (Table 1). As for the N-terminal methionine, it is usually removed from the recombinant products having penultimate residues of alanine, glycine, and threonine expressed in *E. coli* [21,22]. Our further result of the effective N-myristoylation also indicates that the recombinant HBV preS1-HT contains the N-end of

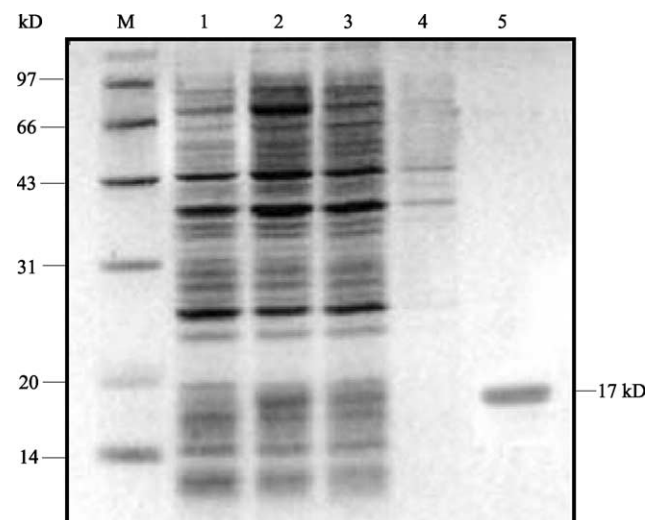


Fig. 3. SDS-PAGE analysis of fusion HBV preS1-HT's purification. The bacterial lysates were prepared from the uninduced (lane 1) and induced (lane 2) *E. coli* BL21(DE3) cells harboring pMF-preS1-HT2 (mut1) by sonication. Then the supernatant (lane 3) and pellet (lane 4) were obtained from the above induced bacterial lysate by centrifugation. Finally, the HBV preS1-HT (lane 5) was purified from supernatant by Ni-IDA affinity chromatography. All of the above samples were directly subjected to 15% SDS-PAGE with molecular weight marker sample (lane M).

Table 1  
Ni-IDA affinity purification of HBV preS1-HT

Step	Volume (ml)	Total protein (mg)	Estimated purity (%)
Culture medium	1000	ND	ND
Soluble lysate	75	220.5	3.2
Ni-IDA	10	5.5	>95

Note. ND, not determined.

glycine residues. Thus, the successful purification by Ni-IDA affinity chromatography suggests that the expressed fusion HBV preS1-HT with the native-type N-terminus containing the N-end-glycine can be obtained for the further study on its N-myristoylation.

#### *In vitro* N-myristoylation of fusion HBV preS1-HT

The purified fusion HBV preS1-HT and  $\alpha$  subunit of mouse cAMP-dependent protein kinase (mC $\alpha$ 4H) [16] as control were used in N-myristoylation assay *in vitro*. The results illustrate that the purified HBV preS1-HT can be efficiently N-myristoylated by NMT (Fig. 4A) as same as the control protein substrate mC $\alpha$ 4H containing the N-end-glycine (Fig. 4B), which is essential for N-myristoylation of proteins [23]. It also indicates that the methionine of purified HBV preS1-HT is removed. Therefore, the HBV preS1 with the native-type N-terminus has been highly expressed, rapidly purified, and

effectively N-myristoylated, providing a good system for screening specific inhibitors on N-myristoylation of HBV preS1 and the biochemical studies on the role of N-myristoylation of HBV preS1, which is essential for viral infectivity.

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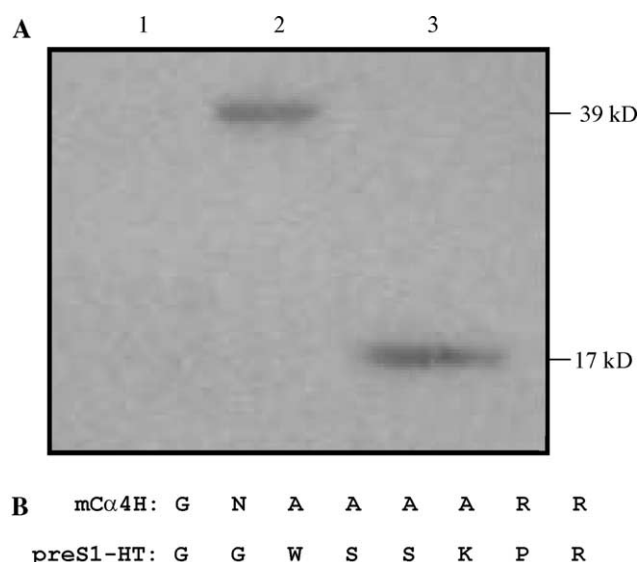


Fig. 4. *In vitro* N-myristoylation assay. (A) Autoradiography of the  $^3\text{H}$ -labeled myristoyl peptides. *In vitro* N-myristoylation assay was carried out by using the purified HBV preS1-HT and mC $\alpha$ 4H as NMT substrates. [ $^3\text{H}$ ]myristoyl peptides were separated by 15% SDS-PAGE and then analyzed by autoradiography, which indicated the negative control without NMT substrate (lane 1), positive control with mC $\alpha$ 4H (lane 2), and the expressed HBV preS1-HT (lane 3). (B) N-terminal amino acids of HBV preS1-HT and mC $\alpha$ 4H as NMT substrates.

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