N-Linked Glycosylation at an Appropriate Position in the Pre-S2 Domain Is Critical for Cellular and Humoral Immunity against Middle HBV Surface Antigen

Hao Liu,^{1,2} Shixia Wang,^{3,4} Yiqiong Jia,¹ Jun Li,¹ Zuhu Huang,^{1,3} Shan Lu^{3,4} and Yiping Xing¹

¹Department of Infectious Diseases, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China ²Department of Infectious Diseases, Nanjing Children's Hospital, Affiliated to Nanjing Medical University, Nanjing, China

³China-US Vaccine Research Center, Nanjing Medical University, Nanjing, China

⁴Department of Medicine, University of Massachusetts Medical School, Worcester, MA, USA

Infection with hepatitis B virus (HBV) remains a worldwide health problem, and DNA-based vaccines against HBV have been tested for therapeutic applications. HBV possesses three envelope lipoproteins that are translated from a single reading-frame: large, middle, and small HBV surface antigens. Among these envelope proteins, the middle HBV surface antigen (MHBs) contains a constitutive N-linked glycosylation site at position 4 (Asn4) in the amino-terminal portion (MQWNSTTFHQ) of pre-S2 domain. Asn4 (shown in bold) is essential for secretion of viral particles and conserved among all serotypes of HBV, but its influence on the immunogenicity of MHBs remains unknown. Here, we constructed four MHBs genes carrying mutations, underlined, in the amino-terminal portion of pre-S2 domain. One mutant protein contains Q at position 4 (MQWQSTTFHQ). In addition, each of three mutant MHBs proteins contains a N-linked glycosylation site (N-X-S/T), relocated to position 5 (MQWQNTTFHQ), 6 (MQWQSNTSHQ) or 7 (MQWQSTNFTQ) in pre-S2 domain. The expression and immunogenic properties of mutant DNA vaccines were examined in 293T human renal epithelial cells and in BALB/c mice, respectively. We showed that Asn4 was critical for secretion and immunogenicity of MHBs. Moreover, the MHBs protein that carries a N-linked glycosylation site at position 5 or 7 retained the properties similar to wild-type MHBs. In contrast, the secretion-defective mutant protein carrying Asn at position 6 induced only marginal humoral and cellular immune responses in mice, despite the N-linked glycosylation. In conclusion, N-linked glycosylation at an appropriate position in pre-S2 domain is an essential requirement for DNA vaccine expressing MHBs.

Keywords: DNA vaccine; hepatitis B virus; immunogenicity; middle hepatitis B virus surface antigen; N-linked glycosylation

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Introduction

Hepatitis B virus (HBV) remains an important worldwide health problem; its infection leads to acute or chronic viral hepatitis and is also considered a high risk factor for the development of cirrhosis and hepatocellular carcinoma (HCC) (Trépo et al. 2014). HBV is a small enveloped DNA virus in the hepadnavirus family, and it comprises hepatotropic DNA viruses sharing with HBV most of the genetic structure and replicative characteristics (Ganem and Schneider 2001). The HBV genome consists of a partially double-stranded relaxed circular DNA of about 3,200 nucleotides in length and contains four partially overlapping open-reading frames coding for the viral polymerase, the HBc and HBe antigen, the regulatory protein HBx and the pre-S/S gene encoding the three surface antigens.

HBV has a lipid envelope with three surface antigens: large HBV surface antigen (LHBs = pre-Sl + pre-S2 + S), middle HBV surface antigen (MHBs = pre-S2 + S), and small HBV surface antigen (SHBs = S), which encoded by one open reading-frame that is divided by three in-frame AUG start codons into the following domains: pre-S1, pre-S2 and S (Heermann et al. 1984; Prange 2012). LHBs consists of the pre-S1 domain (108, 118, or 119 amino acids depending on the genotype), the pre-S2 domain (55 amino acids) and the S domain (226 amino acids); MHBs contains the pre-S2 and S domain; and SHBs contains only the S domain (Ni et al. 2010). LHBs and SHBs exist in nongly-

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cosylated and monoglycosylated forms, due to a facultative N-linked glycosylation site at N146 (Asn146) in the S domain. In contrast, MHBs contains an additional N-linked glycosylation site at N4 (Asn4) in the pre-S2 domain and consequently exists in monoglycosylated or diglycosylated form (Ito et al. 2010). Indeed, MHBs is present in human serum as the monoglycosylated and diglycosylated forms (Heermann et al. 1984).

Prophylactic immunization against HBV infections can be achieved with vaccines composed solely of HBV envelope proteins. For vaccination purposes, recombinant vaccinia viruses that express MHBs or LHBs may have advantages over those that express only SHBs. Experiments have also demonstrated that the amino acids in the pre-S2 region of MHBs are highly immunogenic (Neurath et al. 1984; Cheng and Moss 1987). Therefore, DNA vaccines, which are efficacious but inexpensive, may be a promising means for the control of HBV infections. Moreover, DNAmediated immunization has been shown to break immune tolerance in an HBV transgenic mouse model, indicating that DNA vaccines may also be beneficial for immunotherapy of chronic HBV infections (Chen et al. 2011).

The immunogenicity of MHBs is affected by various factors. Although glycosylation has been reported to influence the immunogenicity of MHBs (Lu et al. 2001), its effect remains unclear. The N-terminal pre-S2 domain of mammalian hepadnavirus MHBs possesses both N-linked and O-linked glycosylation sites (Yu et al. 2014). The O-linked glycosylation motif is not conserved across all HBV genotypes of HBV. However, the N-linked glycosylation site is highly conserved across all HBV serotypes, which might reflect its importance (Schmitt et al. 2004). MHBs possesses two N-linked glycosylation sites: Asn4 in the pre-S2 domain and Asn146 in the S domain. A previous analysis showed that deglycosylation at N-linked glycosylation site (Asn146) in the S domain resulted in a significant decrease in HBs-specific cell-mediated immune responses (Xing et al. 2008), and there is evidence that N-linked glycosylation (Asn4) in the pre-S2 domain plays an essential role in the secretion of MHBs and formation of HBV particles (Block et al. 1994).

In the current study, we investigated the biological and immunological properties of DNA vaccines encoding mutant MHBs. With this aim, we generated four mutant constructs with a defective or relocated pre-S2-specific N-linked glycosylation site (N-X-S/T), where X represents any amino acid. Wild type MHBs-encoding DNA vaccines and mutated MHBs-encoding DNA vaccines were produced for in vitro expression and in vivo immunogenic studies. We show that N-linked glycosylation at position 4 in pre-S2 domain of MHBs is critical for the secretion of recombinant MHBs protein and the immunogenicity of DNA vaccine expressing MHBs antigen.

Materials and Methods

Construction and identification of DNA vaccines

To construct MHBs mutant-encoding DNA vaccines from the HBV strain (adr serotype, C genotype) (GenBank: AF052576.1), an existing DNA vaccine, pSW3891/MHBs/adr, was used. The mutated amino acid residues in the pre-S2 domain of MHBs are underlined in bold (Table 1). Four pairs of complementary primers were designed (Table 2). Site-directed mutagenesis was conducted according to the manufacturer's instructions (Stratagene, La Jolla, CA). Four mutated MHBs-encoding DNA vaccines were produced; the N-linked glycosylation site (Asn residue at position 4) was either replaced with Gln (MHBs-dN4) or relocated to position 5 (MHBs-N4-5), position 6 (MHBs-N4-6), or position 7 (MHBs-N4-7). The resulting vaccine plasmid was verified by digestion with restriction enzyme and gene sequencing (Shanghai Invitrogen Biotechnology Co., China). Wildtype MHBs and mutated MHBs-encoding DNA vaccines were produced and purified using a Qiagen Mega prep kit (Hilden, Germany) for in vitro and in vivo studies.

In vitro expression of MHBs

The expression of MHBs-encoding DNA vaccine constructs was examined in 293T human renal epithelial cells, as previously reported (Pear et al. 1993). Transfection was performed when cells had reached approximately 50% confluence on 60-mm dishes, by calcium phosphate co-precipitation using 10 μ g of plasmid DNA per dish. The supernatants and cell lysates were harvested at 72 hours after transfection.

Western blot analysis

Proteins from the supernatants and cell lysates were separated on SDS-polyacrylamide gels and transferred to membranes. Blocking was performed with 0.1% I-Block (Tropix, Bedford, MA). MHBsspecific mouse sera produced in a previous study (Xing et al. 2008) were used as the detecting antibody at 1:500 dilution with incubation for 45 min. Subsequently, membranes were washed with blocking buffer and then reacted with alkaline phosphatase (AP)-conjugated goat anti-rabbit (Tropix) at 1:5000 dilution. After the final wash, Western-Light substrate was applied to the membranes for 5 min. Once dried, Kodak films were exposed to the membrane and developed using an XOmat processor.

Immunization of mice

Female BALB/c mice, aged of 6-8 weeks, were purchased from the Shanghai Animal Center, at the Chinese Academy of Science.

Table 1. The amino-terminal amino acid sequence of pre-S2 domain of MHBs and its mutated sequences.

Constructs	First 10 amino acids
Wild type MHBs	MQWNSTTFHQ
MHBs-dN4	MQWQSTTFHQ
MHBs-N4-5	MQW <u>QN</u> TTFHQ
MHBs-N4-6	MQWQS <u>N</u> T <u>S</u> HQ
MHBs-N4-7	MQWQST <u>N</u> F <u>T</u> Q

A N-linked glycosylation site (Asn residue) is shown in bold. Each substituted amino acid is underlined.

Table 2. Sequences of pairs of complementary primers.

Name	Primer	Sequences
MHBs-dN4:	MHBs-dN4-F:	ATGCAGTGGCAGTCCACAACATTCCACC
	MHBs-dN4-B:	GGTGGAATGTTGTGGACTGCCACTGCAT
MHBs-N4-5:	MHBs-N4-5-F:	ATGCAGTGGCAGAACACAACATTCCACCAA
	MHBs-N4-5-B:	TTGGTGGAATGTTGTGTTCTGCCACTGCAT
MHBs-N4-6:	MHBs-N4-6-F:	ATGCAGTGGCAGTCCAACACATCCCACCAAGCTCTG
	MHBs-N4-6-B:	CAGAGCTTGGTGGGATGTGTTGGACTGCCACTGCAT
MHBs-N4-7:	MHBs-N4-7-F:	ATGCAGTGGCAGTCCACAAACTTCACACAAGCTCTC
	MHBs-N4-7-B:	GCAGAGCTTGTGTGAAGTTTGTGGACTGCCACTGCT

The mice were randomly divided into 6 groups (6 mice per group). The mice were housed at the Department of Animal Medicine, the Nanjing Medical University, in accordance with approved protocols. Animals were sedated with ketamine (0.1 mg/g of body weight) prior to immunization. Electroporation was used for DNA vaccine delivery, as previously described (Wang et al. 2008), using an electroporator (SCIENTZ-2C) manufactured by Scientz Co., Ltd. (Ningbo, China). Briefly, following intramuscular (IM) injection of MHBsencoding DNA vaccines or the vector control (100 μ g per mouse), the injection sites were electroporated using the following parameters: 50 V, 30 ms, and 30 Hz. The DNA plasmid was delivered to two different sites in the quadriceps muscle for each immunization. Four immunizations were given at weeks 0, 2, 4, and 6. Serum samples were collected prior to the first immunization and two weeks after each immunization to measure HBs-specific antibody responses. Spleens were removed and single-cell suspensions of splenocytes were prepared, as previously described (Xing et al. 2008). This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of The First Affiliated Hospital of Nanjing Medical University.

Detection of anti-MHBs antibody responses in mouse immune sera

Anti-MHBs antibodies in immunized animal sera were evaluated by enzyme-linked immunosorbent assay (ELISA), as previously described (Shen et al. 2010; Ge et al. 2012). Both temporal and peaklevel MHBs-specific antibodies were determined. Flat-bottom 96-well plates were coated with 100 μ l (0.1 μ g) of HBsAg isolated from plasma collected from HBV-infected patients (US Biological, Swampscott, MA, USA). Animal sera were incubated for 1 h at room temperature and washed 5 times with phosphate buffered saline (PBS) containing 0.1% Triton X-100. The plates were then blocked with $200 \,\mu$ l/well of blocking buffer (5% non-fat dry milk, 4% whey, 0.5% Tween-20 in PBS at pH 7.2) for 1 h. After five washes, $100 \,\mu$ l of serially diluted mouse serum was added to duplicate wells and incubated for 1 h. Following another round of washing, the plates were incubated for 1 h at 37°C with 100 µl of biotinylated anti-mouse IgG (US Biological) diluted at 1:1000 in whey dilution buffer (4% whey, 0.5% Tween-20 in PBS). Then, 100 μ l of horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA) diluted at 1:2000 in whey buffer was added to each well and incubated for 1 h. After the final wash, the plates were developed with 3,3',5,5'tetramethylbenzidine (TMB) solution at 100 μ l per well (Sigma, St. Louis, MO, USA) for 3.5 min. The reaction was stopped by adding 25 μ l of 2 M H₂SO₄, and the plates were read at an OD of 450 nm. The end titration titer was determined to be the highest serum dilution with an OD reading twice that of the negative control serum.

Analysis of MHBs-specific T cell responses by enzyme-linked immunospot (ELISPOT) assay

Gamma interferon (IFN-y) ELISPOT assays were performed to detect MHBs peptide-specific T cell responses in mouse splenocytes, as described previously (Xing et al. 2008; Shen et al. 2010; Ge et al 2012; Chuai et al. 2013). A mouse IFN-y kit (U-CyTech Biosciences, the Netherlands) was used to detect MHBs-specific IFN-y responses, according to the manufacturers' directions. Briefly, ELISPOT plates were coated with 5 μ g/ml of purified rat anti-mouse IFN- γ in PBS and incubated at 4°C overnight. After the plates were washed three times with PBS, each plate was blocked by the addition of 200 μ l of the blocking buffer to each well for 2 h at 37°C. The HBs-relevant peptide used was a CD8⁺ cell-restricted HBs peptide (IPQSLDSWWTSL) and the non-relevant peptide was the HIV Env V3 (IGPGRAFYT) peptide, which served as a negative control. Peptides (final concentration 5 μ g/ml) were added to the wells with 100 μ l of freshly isolated splenocytes (200,000 cells/well in R10 medium) in duplicate. The plates were incubated at 37°C with 5% CO₂ for 24 h. The plates were then washed, incubated with 100 μ l of biotinylated rat antimouse IFN- γ (1 μ g/ml in dilution buffer from the kit), and incubated at 37°C for 1 h. After additional washes, 100 µl horseradish peroxidase (HRP)-conjugated streptavidin complex (BD Biosciences) was added to each well in dilution buffer at 37°C for 1 h. The plates were washed, and spots representing individual IFN- γ producing cells were detected after a 35-min color reaction using the AEC coloring system. Spot-forming cells (SFCs) were counted and the results were expressed as the number of SFCs per million input cells. The number of peptide-specific IFN-y-secreting T cells was calculated by subtracting the background (no-peptide) control value from the established SFCs count.

Statistical analysis

All statistical analyses were performed using SPSS13.0 software. Measured data are represented by $\overline{X} \pm s$. Student's *t*-test was adopted to perform comparisons between two groups, and multiple groups were compared with one-way analysis of variance (ANOVA). P < 0.05 was considered statistically significant.

Results

In vitro expression of mutated MHBs-encoding DNA vaccines

The mutated MHBs-encoding DNA vaccines were constructed as described in the Materials and Methods, and each of them was transiently expressed in 293T cells. The expression of the MHBs-encoding DNA vaccine was confirmed by western blot analysis using the polyclonal mouse immune sera, elicited by an MHBs-encoding DNA vaccine (Xing et al. 2008). The two glycosylation sites of MHBs are schematically shown in Fig. 1A. For the wild-type MHBs-encoding DNA vaccine, MHBs species were identified in both cell lysates and supernatants. In cell lysates, the MHBs appeared predominantly in the 33-kDa monoglycosylated form (gp33) that represents monoglycosylation, and to a lesser extent, in the 36-kDa diglycosylated form (gp36) (Fig. 1B, lane 6). A 30-kDa nonglycosylated form (p30) was faintly visible when total cellular extracts were analyzed. Multiple glycosylated forms of MHBs were efficiently released from the cells. The molecular mass of the MHBs in the supernatants appeared to be larger than that seen in the cell lysates (Fig. 1B, lane 5), suggesting that secreted MHBs may undergo additional glycosylation modifications.

The role of Asn4 was confirmed using the mutant protein carrying the Gln residue at position 4 (MQWQSTTFHQ), encoded by MHBs-dN4. This mutant protein appeared in a nonglycosylated p30 form and in a monoglycosylated gp33 form in the cell lysates (Fig. 1B, lane 4), but the diglycosylated gp36 form of MHBs was not observed. In addition, no MHBs were detected in the supernatant of 293T cells expressing MHBs-dN4 (Fig. 1B, lane 3). Thus, the MHBs-dN4 mutation impairs the glycosylation and the secretion of MHBs; namely, Asn4 is responsible for the N-linked glycosylation in the pre-S2 domain.

We then analyzed the expression of mutated MHBs proteins that contained the N-linked glycosylation site relocated from Asn4 to nearby positions in the pre-S2 domain. DNA vaccines encoding MHBs-N4-5, MHBs-N4-6, and MHBs-N4-7 contained the Asn residue relocated to position 5 (MQWQNTTFHQ), 6 (MQWQSNTSHQ) and 7 (MQWQSTNFTQ), respectively. Similar bands were observed in the lysates of cells transfected with each of these three MHBs-encoding DNA vaccines, which included all three forms of MHBs (nonglycosylated, monoglycosylated, and diglycosylated) (Fig. 1B, lanes 8, 10, and 12). Thus, each relocated Asn residue was correctly glycosylated in 293T cells. However, the same pattern of MHBs secretion as seen for the wild-type MHBs-encoding DNA vaccine was observed only in the supernatants of cells expressing MHBs-N4-5 or MHBs-N4-7 construct (Fig. 1B, lanes 7 and 11), but not in supernatants of cells expressing MHBs-N4-6 (Fig. 1B, lane 9). Thus, the presence of a N-linked glycosylation site in the pre-S2 domain is not sufficient for secretion of MHBs. The impaired secretion of the MHBs-N4-6 mutant protein may be related to the lack of the Phe residue at position 8 (see Table 1).

Antibody responses of BALB/c mice immunized with mutated MHBs-encoding DNA vaccines

BALB/c mice were immunized using electroporation with the wild-type MHBs construct or each of the four mutant MHBs constructs in which the pre-S2-specific N-linked glycosylation site had been replaced with Gln and relocated. Mouse serum samples were assayed for the pres-



Fig. 1. The structure and expression of MHBs protein.

⁽A) Schematic organization of the domain structure of MHBs. Note that the N-linked glycosylation site (NG) is located in pre-S2 domain (Asn4) and S domain (Asn146). (B) In vitro expression of MHBs DNA vaccines in transiently transfected 293T cells. Western blot analysis of MHBs protein expression by DNA vaccines expressing MHBs, MHBs-dN4, MHBs-N4-5, MHBs-N4-6, MHBs-N4-7, or empty vector pSW3891 in supernatant (S) or lysates (L) of transfected 293T cells. Levels of β -actin were used as control for protein loading. Rabbit serum specific for MHBs was used as the detecting antibody.



Temporal HBs-specific antibody responses of MHBs in the immunized mice. Temporal HBs-specific antibody responses induced by MHBs, MHBs-dN4, MHBs-N4-5, MHBs-N4-6, MHBs-N4-7, or empty vector pSW3891 in BALB/c mice. Serum IgG responses were measured by ELISA at 1:1000 serum dilution against HBsAg. Temporal antibody responses were measured using (A) OD value or (B) serum antibody titer with pooled serum samples from each of the animal groups. (C) Peak antibody titers were determined by using individual animal sera collected at 2 weeks after the 4th DNA immunization. Bars show the average IgG titers of each group ± standard deviation and those that differ significantly from the MHBs group are marked (*P < 0.05). Six mice were included in each group.

ence of MHBs-specific antibodies with ELISA. Levels of antibody responses increased after each of the four immunizations, reaching peak levels after the fourth DNA immunization (Fig. 2A and B). The kinetics of the anti-MHBs antibodies in sera from the immunized mice was monitored using both optical density (OD) values with a fixed serum dilution (1:1,000) (Fig. 2A) and end-titration titers (Fig. 2B). The wild-type MHBs-encoding DNA vaccine and two mutants with a changed N-linked glycosylation site (MHBs-N4-5 and MHBs-N4-7) produced overall higher antibody responses throughout the entire immunization period. Two of the mutated MHBs-encoding DNA vaccines, in which the Asn4 N-linked glycosylation site had been removed (MHBs-dN4) or relocated to position 6 (MHBs-N4-6), elicited much lower antibody responses. Peak level HBs-specific antibody titers were further determined at week 8 (2 weeks after the final immunization). A similar pattern was observed, with the highest antibody titers achieved by the wild-type MHBs-encoding DNA vaccine (up to 1:121,500) and slightly lower titers achieved by the MHBs-N4-5 and MHBs-N4-7 DNA vaccines. In contrast, when the secretion of MHBs was blocked due to lack of the Asn4-glycosylation site or by relocation of the glycosylation site to position 6, peak level anti-HBs antibodies were significantly reduced (Fig. 2C).

IFN- γ *T cell-mediated immune responses induced by MHBsencoding DNA vaccines*

Using ELISPOT analysis, high-level antigen-specific IFN- γ responses (at the level of several hundred spots per million splenocytes) were detected in mice immunized with various MHBs DNA vaccines. The wild-type MHBs-encoding DNA vaccine elicited a higher IFN- γ ELISPOT response (138.6/million cells) than the mutant MHBs-encoding DNA vaccines. Similar to the antibody responses, DNA vaccines expressing the MHBs-N4-5 or MHBs-N4-7 antigens elicited MHBs-specific IFN- γ responses similar to those of the wild-type MHBs-encoding DNA vaccine, but DNA vaccines expressing the MHBs-dN4 or MHBs-N4-6 antigens elicited much lower IFN- γ ELISPOT responses than did the wild-type MHBs-encoding DNA vaccine (P < 0.05) (Fig. 3).

Discussion

DNA-based vaccines against HBV are increasingly being tested for therapeutic vaccination applications. They are able to activate both defective humoral and Th1 cellular immune responses in HBV carriers, and have been proposed as a particularly pertinent approach for therapy for chronic hepatitis B therapy (Michel et al. 2011; Cova 2012). This method of vaccination has the advantage of inducing



Fig. 3. IFN-γ T cell-mediated immune responses of MHBs in the immunized mice. ELISPOT analysis of IFN-γ secretion in mouse splenocytes immunized with MHBs, MHBs-dN4, MHBs-N4-5, MHBs-N4-6, MHBs-N4-7, or empty vector pSW3891 at 2 weeks after the 4th DNA immunization. (A) Representative ELISPOT results with mouse splenocytes stimulated with either Con A, mock peptide, or HBs peptide. The number of the pots reflects the degree of the antigen-antibody reaction. (B) Group averages of HBs peptide-specific IFN-γ responses (6 mice/group). Those that differ significantly from the MHBs group are marked (*P < 0.05).</p>

both humoral and cellular immune responses, including cytotoxic and Th1 responses (Donnelly et al. 2005). The pre-S domain of HBV genes carries various B and T cell epitopes, which are highly immunogenic in mice and humans (Shapira et al. 2001). Therefore, MHBs, which also carries the pre-S2 sequences, has been considered to offer an improvement over the HBV vaccine currently in use. The N-linked glycosylation site (Asn4) within the pre-S2 domain is conserved among all serotypes of HBV, which reflects an important role for glycosylation at this position (Schmitt et al. 2004). In the present study, we analyzed the properties of modified MHBs with respect to assembly, secretion, antigenicity, and immunogenicity, using transient expression in 293T cells and immunization of mice.

When four mutated MHBs DNA vaccines were tested, their in vitro expression and in vivo immunogenicity consistently demonstrated the importance of the N-linked glycosvlation site at Asn4 in the pre-S2 domain of the MHBs antigen. First, this N-glycan was shown to be necessary for the secretion of MHBs and elicitation of high-level antibody and T cell immune responses by MHBs. When this site was completely removed, secretion and immunogenicity were very low. Second, the location of the N-glycan within the pre-S2 domain was found to be somewhat flexible; MHBs secretion and high-level immunogenicity were strongly maintained when the N-glycan site was moved to positions 5 and 7, but not position 6. The original amino acid residues at positions 5, 6, 7, 8, and 9 are Ser, Thr, Thr, Phe, and His, respectively. Thus, replacing the Phe residue at position 8 with Ser produced a previously untested MHBs-encoding DNA vaccine (MHBs-N4-6); however, it remains unclear how this change affects the secretion of MHBs.

Findings from the current study are concordant with our previous report showing that N-glycans play a role in the immunogenicity of HBV DNA vaccines (Xing et al. 2008). However, the impact of the N-glycan at position 4 of the pre-S2 differs from that of an N-glycan mutation at position 146 in the main structure of the S protein. When N-glycan was removed from Asn146 in the S protein, T cell immune responses, but not antibody responses, were affected (Xing et al. 2008). It is likely that the presence of the N-glycan in the middle of the S protein may affect intracellular processing of newly synthesized protein antigens, which can affect T cell immune responses, whereas the N-glycan in the pre-S2 domain may mainly affect the secretion of immunogens and thus antibody responses (Liu et al. 2007). Although intracellular expression of MHBs was still observed in our study, N-glycan mutation may also affect T cell immune responses, due to altered intracellular processing of newly synthesized protein antigens.

The results presented here show that the glycan at position 4 is important for the secretion of MHBs, consistent with recent reports (Lambert and Prange 2007; Ito et al. 2010). Our work further extends this finding and indicates that any gene-based vaccines or therapies based on MHBs should consider the impact of the N-linked glycosylation site at position 4 in the pre-S2 domain. HBV surface proteins possess a complicated design and structure, with three different forms (SHBs, MHBs, and LHBs), each of which showed a distinct pattern of immunogenicity when tested as DNA vaccines, as we reported previously (Shen et al. 2010; Ge et al. 2012). Our data also provide new information showing that this N-linked glycosylation site can remain functional after relocation to other positions within the pre-S2 domain. This information may be useful for studying the biological and virological functions of the pre-S2 domain of HBV.

Following the discovery of DNA vaccine technology approximately 20 years ago, significant progress has been made in optimizing their function (Wu et al. 2011; Gao et al. 2013; Obeng-Adjei et al. 2013; Saade et al. 2013; Endmann et al. 2014; Yoon et al. 2015). Post-translational modification of newly synthesized antigens has proven important in determining the level and type of immune response. Data presented in the current report provide information on the role of N-linked glycosylation at the amino-terminal region of a DNA vaccine insert not previously reported in the literature. This unique finding on N-linked glycosylation may help to improve elicitation of the desired immune responses by many types of DNA vaccines.

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Conflict of Interest

The authors declare no conflict of interest.

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