

Modulating gene expression using DNA vaccines with different 3'-UTRs influences antibody titer, seroconversion and cytokine profiles

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Abstract

To determine if modulating the amount of foreign antigen produced by a DNA vaccine can influence the overall intensity and cytokine polarization of the ensuing immune response, three different plasmids, each encoding the hepatitis B (HB) surface antigen, were constructed. In each construct, HBs gene expression was driven by the cytomegalovirus immediate early promoter, but differed in the 3'-untranslated regions (3'-UTR) containing the polyadenylation sequence. These 3'-UTR sequences were derived from either the hepatitis B virus (HBVpA), bovine growth hormone (BGHpA), or rabbit β -globin (β pA). BALB/c mice were immunized intramuscularly with equimolar amounts of each plasmid and blood was collected bi-weekly. Following immunization, total IgG titers correlated with in vitro antigen production levels (from transfected CHO cells), as evidenced by the following response pattern: HBVpA > BGHpA \gg β pA. All groups demonstrated a heavy bias toward a Th1 immune response, as evidenced by high serum IgG2a/IgG1 ratios and the predominance of IFN- γ over IL-4 secretion from cultured splenocytes. In addition, the HBVpA construct resulted in a seroconversion rate of 100%, in comparison to 40–50% in the BGHpA, and 0% in the β pA group. Surprisingly, splenocytes isolated from mice immunized with the β pA construct secreted the highest levels of IFN- γ . Taken together, these findings suggest that altering the level of gene expression not only affects the overall titer and seroconversion rates of vaccinated animals, but also may play a role in modulating cytokine profiles.

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1. Introduction

Over the past decade, genetic (or DNA) vaccines have emerged as a viable alternative to traditional vaccine approaches, such as live-attenuated, subunit, peptide or protein vaccines. DNA vaccines consist of bacterial plasmid DNA that encode antigens, usually under the control of strong viral promoters [1]. The ability of foreign DNA to induce an immune response to the expressed antigen in a mammalian host was shown by several key studies performed in the early 1990s. In one study, intramuscular (i.m.) injection of mice with naked plasmid reporter constructs resulted in immune responses to the encoded antigens [2]. The feasibility of using naked DNA for vaccination purposes was shown in another study in which mice were immunized with a plasmid DNA vaccine encoding the influenza A

nucleoprotein. Immunized mice were protected against influenza A challenge, as evidenced by decreased viral titers in lungs and prolonged survival [3]. These and other publications gave a better understanding of the mechanisms involved in genetic immunizations and further stimulated research to expand and improve the potential of DNA vaccines.

DNA vaccines can be delivered by several different methods, including needle injection, particle bombardment (gene gun), microspheres and liposomes. Following antigen expression, processing and presentation of the antigen occurs in the context of both MHC I and MHC II molecules, thus stimulating cell-mediated and humoral immune responses. The route of immunization can also be used to influence the cytokine profile of the immune response. For instance, i.m. injections of naked plasmid DNA typically yield a Th1-biased immune response, as evidenced by high levels of IL-2, IFN- γ , IgG2a and strong cell-mediated immunity [4–6]. Conversely, gene gun immunization generally induces a Th2 response, as indicated by the Type 2 cytokine profile (IL-4, IL-5, IL-6) and the predominance of IgG1 over

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IgG2a [7–9]. Other factors such as DNA dose, timing, inclusion of cytokine/co-stimulatory molecules and the directed expression of gene product (e.g. secreted, transmembrane, intracellular) can also influence the type and intensity of the ensuing response [10–15].

Many of the approaches discussed above have been evaluated due to a persistent shortcoming that plagues DNA vaccines—the low levels of gene expression, resulting in relatively low levels of immune response and short duration of immunity. The object of the work described here is to evaluate the impact of increasing gene expression on the intensity of the immune response.

Gene expression can be manipulated by altering a plasmid's regulatory elements, such as promoters, enhancers, introns and polyadenylation (polyA) sequences. For example, the expression of the gene encoding cystic fibrosis transmembrane conductance regulator was highest in an epithelial-like mouse cell line when a combination of the cytomegalovirus (CMV) promoter, a hybrid intron and the bovine growth hormone (BGH) polyadenylation signal were used [16]. Similar findings were observed in mouse lung epithelial cells after intranasal delivery of various plasmids expressing a reporter gene. Additionally, mice that were injected i.m. with luciferase constructs using promoters of different “strengths” yielded varying degrees of reporter gene expression and also influenced the subsequent immune response [17]. Norman et al. [18] found that a synthetic polyadenylation site [19] derived from the rabbit β -globin gene augmented expression of a target gene construct by nearly 2-fold over the BGH polyA upon injection of naked DNA to muscle.

The studies described above demonstrated that manipulation of certain regulatory elements can influence levels of gene expression, but few studies have examined the impact of differing levels of antigen expression on the overall immune response. In one study, Galvin et al. showed that macaque humoral and T-cell proliferative responses to an HIV-1 *gag/env* gene expressed from two plasmids differing only in their promoters correlated with promoter strength [20]. Macaques were immunized with a plasmid expressing the *gag/env* gene under the control of either the CMV immediate early promoter or the AKV murine leukemia long terminal repeat promoter. The results indicated that the CMV-driven vaccine consistently elicited higher immune responses and required fewer injections than the vaccine driven by the AKV promoter. With rare exception, the CMV promoter has been found to induce the highest level of expression and the strongest immune response, and for this reason has been selected to drive expression of most DNA vaccines. Much less attention has been given to the sequences and genetic elements included in the 3' sequences that flank the gene of interest. This region contains not just the polyadenylation sequences required for stabilization of mRNA and transcription termination, but may also contain enhancer sequences that can dramatically alter the level of gene expression.

HBV is a DNA virus with a partially single-stranded 3.2 kb genome encoding several viral proteins, including the core and envelope proteins. Of these proteins, the secreted major “S” (surface) envelope protein is known to be highly immunogenic and can elicit both humoral and cell-mediated immune responses [4,5,21–27]. The “S” gene is followed by two overlapping HBV gene sequences (polymerase and “X” gene) containing two transcriptional enhancers [28]. Deletion of either enhancer element leads to a drastic reduction in viral transcripts [29] and both have been shown to have stimulatory effects when paired with certain HBV promoters [28,30–32]. In vitro studies examining the effects of transcription/translation, in the presence or absence of the enhancer sequences, demonstrated that the HBV surface antigen gene promoter activity was stimulated by 20-fold when the enhancer sequence was included [33]. In the current study, we have investigated how differing levels of antigen expression affect the intensity and cytokine polarization of the immune response. To achieve this, we utilized three different DNA vaccine constructs that differ only in their polyA sequences: hepatitis B virus (HBVpA, including enhancer), bovine growth hormone (BGHpA) and the minimal rabbit β -globin (β pA) resulting in substantial differences in antigen expression.

2. Materials and methods

2.1. Animals

Female BALB/c (H-2^d) mice 6 to 8 weeks old were obtained from Charles River (Charles River Laboratories, Wilmington, MA) and housed in a controlled animal facility. All procedures involving animals were approved by the University of Connecticut's IACUC committee and adhered to National Institutes of Health guidelines.

2.2. Plasmid construction

pRc/CMV-HBs(S) (subsequently referred to as pCMV/HBs-HBVpA) was provided by Aldevron (Aldevron, LLC, Fargo, ND) and served as the recipient plasmid for all subsequent cloning steps (Fig. 1A). pCMV/HBs-HBVpA constitutively expresses and secretes the HBsAg under the control of the CMV promoter and HBV polyadenylation sequence (HBVpA). The first of two constructs was produced by excising the HBs gene and HBVpA sequence from pCMV/HBs-HBVpA via restriction digestion with *KpnI* and *BsmI*. PCR amplification of the HBsAg gene and BGHpA sequence from the pVax plasmid (Invitrogen, Carlsbad, CA) was followed by restriction digestion with *KpnI* and *BsmI*. The resulting fragment was ligated into the recipient plasmid resulting in pCMV/HBs-BGHPA (Fig. 1B). Construction of the second construct was performed similarly, in that the HBsAg gene and rabbit β -globin polyadenylation (β pA) sequences were amplified from pTRE2 (Clontech, Palo

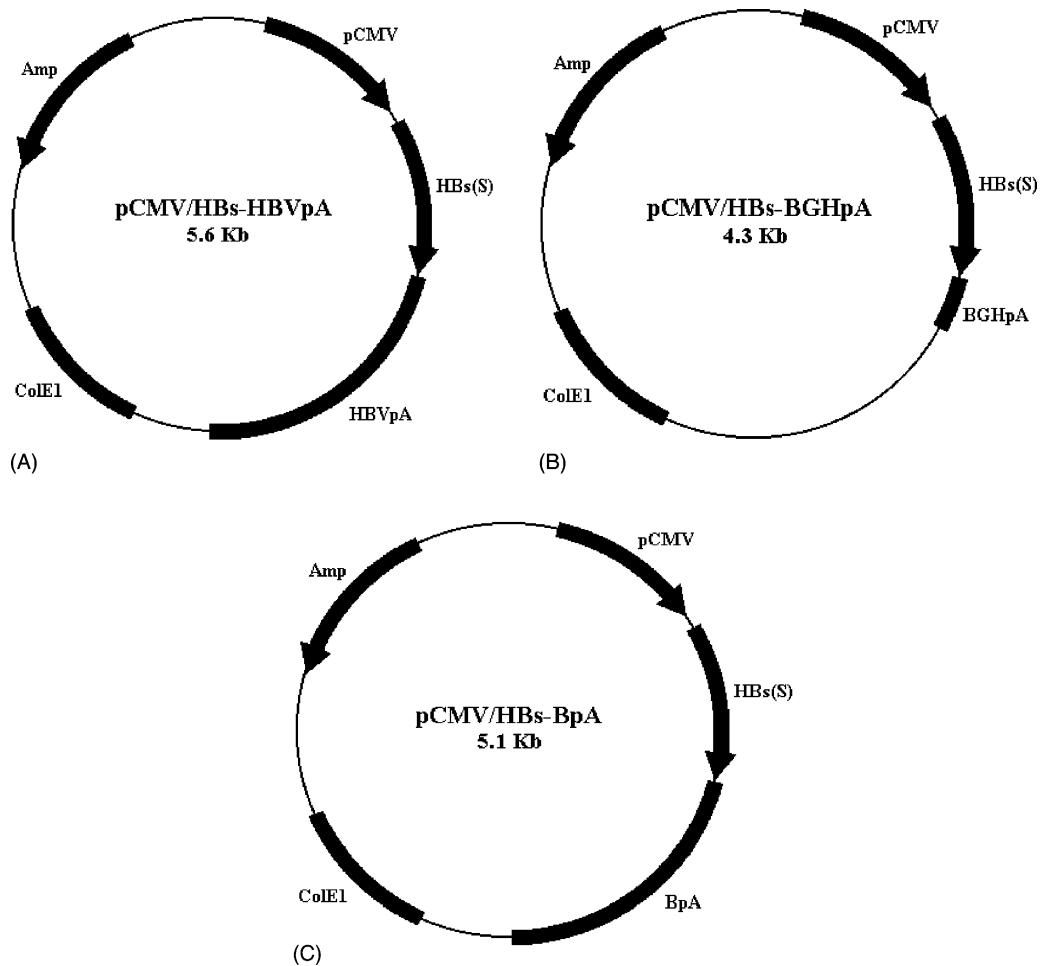


Fig. 1. Plasmid maps of pCMV/HBs-HBVpA (A), pCMV/HBs-BGHpA (B), and pCMV/HBs- β pA (C) used for DNA immunization.

Alto, CA) and digested using the same restriction enzymes. The resulting fragment was cloned into the recipient vector, producing the pCMV/HBs- β pA plasmid (Fig. 1C). DNA sequencing of the cloned regions of pCMV/HBs-BGHpA and pCMV/HBs- β pA was used to confirm base pair identity with published sequences.

Plasmids were propagated in TOP10 cells (Invitrogen, Carlsbad, CA) and purified using Qiagen Mega columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. Endotoxin removal was performed using Triton X-114. Briefly, 0.1 vol. 3 M NaOAc was added to plasmid DNA and the samples were brought up to 1.2 ml with distilled water, then placed in an ice water bath for 5 min. Triton X-114 (0.03 vol.) was then added, and each sample was briefly vortexed, then placed into a 50°C water bath for 5 min. Samples were then centrifuged briefly at 12,000 \times g in a microcentrifuge, followed by removal of the upper aqueous phase containing the DNA. These samples were brought up to 1.2 ml with distilled water using 0.1 vol. of Triton X-114. The DNA was then precipitated with 2 vol. of 100% ethanol at room temperature. All plasmids were determined to have <20 endotoxin units/mg DNA (confirmed by Alde-

vron, Fargo, ND). Plasmid DNA was resuspended in 0.9% sterile saline for vaccinations.

2.3. DNA immunization

Three groups of mice ($N = 10$) were immunized i.m. with an equimolar amount of plasmid based on the injection of 100 μ g of pCMV/HBs-HBVpA. Thus, only 77 μ g pCMV/HBs-BGHpA and 91 μ g pCMV/HBs- β pA were required for immunizing their respective groups. All mice were immunized while under isoflurane anesthesia. DNA was diluted in 0.9% sterile saline and 75 μ l was injected into each tibialis anterior muscle using a 30-gauge needle (Becton Dickinson, Franklin Lakes, NJ).

2.4. In vitro transfections

The level of HBsAg mRNA expression and the amount of antigen secreted were determined using Chinese Hamster Ovary (CHO) cells transfected with each of the three plasmids described above. Cells were maintained in a 37°C humidified 10% CO₂ incubator with Minimal Essential

Media (Hyclone, Logan, UT), 10% fetal bovine serum (Clontech, Palo Alto, CA), supplemented with non-essential amino acids (Gibco, Gathersburg, MD). Briefly, CHO cells were seeded at a density of 7×10^5 ml in 35 mm culture dishes (Fisher Scientific, Pittsburgh, PA) for 24 h, followed by a 3 h transfection with equimolar amounts of each plasmid using Gene Porter liposome reagent (Gene Therapy Systems, San Diego, CA). Cells were subsequently washed with complete medium followed by a 48 h incubation. CHO cell RNA was extracted (as outlined later) and culture supernatants were recovered and concentrated approximately 5-fold using Centricon-10 filter units (Amicon, Bedford, MA). The level of secreted surface antigen was measured using a quantitative ELISA (see following sections).

2.5. Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was used to determine the relative amount of HBsAg mRNA expression from transfected CHO cells. Total RNA was extracted from CHO cells 48 h post-transfection using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was treated with RNase-free DNase (Ambion, Austin, TX) followed by reverse transcription of approximately 0.2 μ g of total RNA using the Retroscript RT-PCR kit (Ambion, Austin, TX). To determine the optimum number of PCR cycles required for a near linear relationship between the amount of RNA and amplified DNA band intensity, a variable cycle number PCR was performed (data not shown). Using internal primers specific for HBsAg and the house-keeping gene β -actin, multiplex PCR was performed using a pre-determined number of cycles. cDNA products were separated on a non-denaturing 8.0% polyacrylamide gel, and the level of HBsAg mRNA produced was determined based on the intensity of ethidium bromide staining relative to β -actin staining using Quantity One software (Bio-Rad, Hercules, CA).

2.6. Enzyme linked immunosorbent assay (ELISA)

In vitro secreted HBsAg was measured using a quantitative capture ELISA. Briefly, Immulon 4 HBX (Costar, Cambridge, MA) 96-well microtiter plates were coated with a monoclonal anti-HBs antibody (Aldevron, Fargo, ND) at 10 μ g/ml diluted in coating buffer (0.05 M sodium bicarbonate, pH 9.6). Plates were allowed to incubate for 1 h at room temperature. Following coating, plates were blocked (50 mM Tris, 0.15 M NaCl, 1% BSA, pH 8.0) for 1/2 h at room temperature. Concentrated CHO cell supernatants were applied in duplicate and incubated for 1 h at room temperature, after which a polyclonal goat-anti-HBs antibody (Cliniqa Corp., Fallbrook, CA) was applied. Rabbit-anti-goat IgG conjugated to alkaline phosphatase (Cappel, Aurora, OH) was added for 1 h at room temperature followed by *p*-nitrophenylphosphate substrate (Sigma, St. Louis, MO). Plates were read at 405 nm using a Bio-Tek EL-311 plate

reader (Bio-Tek Instruments, Winooski, VT). The relative amount of HBs antibody in each sample was interpolated from a standard curve using purified HBs antigen (Cliniqa Corp., Fallbrook, CA). All wash steps were performed using wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0).

Total IgG, IgG1 and IgG2a serum anti-HBs antibody responses were measured using mouse IgG quantitative kits (Bethyl Laboratories, Montgomery, TX). Briefly, 96-well Immulon 4 HBX plates (Costar, Cambridge, MA) were coated with anti-mouse IgG, IgG1 or IgG2a (for standard curve) in addition to a polyclonal goat-anti-HBs antibody (Cliniqa Corp., Fallbrook, CA) in coating buffer (0.05 M sodium bicarbonate, pH 9.6) for 1 h at room temperature. Plates were then blocked with blocking buffer (50 mM Tris, 0.15 M NaCl, 1% BSA, pH 8.0) for 1/2 h. Purified reference mouse serum was diluted in sample diluent (50 mM Tris, 0.15 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) at varying concentrations to generate the standard curve (10–200 ng/ml), which was included on each plate. Purified HBs antigen (Cliniqa, Fallbrook, CA), diluted in sample diluent (10 μ g/ml) was then applied and incubated for 1 h at room temperature. Following incubation, serial dilutions of mouse serum samples were applied and incubated for 1 h such that at least one value would fall within the range of the standard curve. After washing, goat-anti-mouse horseradish peroxidase labeled secondary antibody was applied and incubated for 1 h. Antibody detection was completed with the addition of tetramethylbenzidine substrate (KPL, Gaithersburg, MD) followed by a 50-min incubation. Product formation was terminated with the addition of 2 N H₂SO₄ stop solution. The absorbance of each well was measured at 450 nm using a Bio-Tek EL-311 plate reader (Bio-Tek Instruments, Winooski, VT). All wash steps were performed using wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0).

2.7. Antibody avinity determination

Antibody avinity measurements were performed using a 50% inhibition competitive ELISA. Briefly, Immulon 4 HBX microtiter plates (Costar, Cambridge, MA) were coated with purified HBs antigen (Cliniqa, Fallbrook, CA) at 10 μ g/ml in coating buffer (0.05 M sodium bicarbonate, pH 9.6) overnight at room temperature. Plates were subsequently blocked (50 mM Tris, 0.15 M NaCl, 1% BSA, pH 8.0) for 2.5 h and incubated at room temperature. During incubation, mouse serum samples (varying dilutions, previously determined) were pre-incubated with increasing amounts of purified soluble HBs (0.1–33 μ g/ml, calculated MW $\sim 2.5 \times 10^6$ Da) in sample conjugate diluent (50 mM Tris, 0.15 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) for 2 h at 37 °C. Following blocking, pre-incubated HBs/mouse sera were added and incubated for 2 h at 37 °C. Biotinylated goat-anti-mouse IgG (Cappel, Aurora, OH) antibody was added for 1 h at room temperature, followed by a wash step,

then streptavidin/alkaline phosphatase conjugate (Gibco, Gaithersburg, MD) was applied for 1 h at room temperature. The optical density of each sample was measured at $\lambda = 405$ nm following the addition of *p*-nitrophenylphosphate substrate (Sigma, St. Louis, MO). All wash steps were performed using wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). The 50% inhibition was interpolated by plotting the percent inhibition against increasing amounts of soluble HBs using a log/linear quadratic equation. The concentration of soluble HBs required for 50% inhibition was used as an index of average avidity.

2.8. IFN- γ and IL-4 measurement

To measure T-cell activation and assess cytokine polarization in immunized mice, IFN- γ and IL-4 ELISA kits (eBioscience, San Diego, CA) were used. Mice were sacrificed 8 weeks following immunization and the spleens were harvested aseptically and placed into 60 mm culture dishes (Becton Dickinson, Lincoln Park, NJ) containing 5 ml of complete splenocyte medium (RPMI 1640, 10% FBS, 50 μ M 2-ME, 100 U/ml penicillin, 100 μ g/ml streptomycin sulfate). Splenocytes were collected by injection/aspiration of the spleen using two 22-gauge syringes (Becton Dickinson, Franklin Lakes, NJ). Medium containing the splenocytes was removed and centrifuged at $200 \times g$ for 10 min. The supernatants were removed and 5 ml of RBC lysis buffer (0.15 M NH_4Cl , 10 mM KHCO_3 , 0.1 mM NA_2EDTA) was added and allowed to incubate for 10 min. at room temperature. After further centrifugation, lysis buffer was removed and splenocytes were resuspended in complete medium and counted using a hemocytometer. Splenocytes were added to 24-well cell culture plates (Corning, Corning, NY) at a concentration of 3×10^6 cells/ml. Purified HBs antigen (Cliniqa, Fallbrook, CA) or bovine serum albumin was added at 2 μ g/ml followed by a 72 h incubation. Following incubation, supernatants were removed and applied directly to the IFN- γ or IL-4 coated capture ELISA plates. Assays were carried out following the manufacturer's protocol.

2.9. Statistical analysis

Differences between groups were determined by Student's *t*-test (two groups) or one-way ANOVA (three groups) with $P < 0.05$ being considered statistically significant (SAS Institute Inc., Cary, NC).

3. Results

3.1. Construction of HBsAg expression plasmids and mRNA expression following transient transfection

To produce differing levels of antigen expression, three common polyA sequences were used to flank the gene of interest at the 3'-end. All three plasmids constitutively ex-

pressed and secreted the hepatitis B major surface antigen (HBsAg, subtype *ayw*) under the control of the human CMV immediate early promoter. We anticipated that the pCMV/HBs-HBVpA would result in the highest level of antigen secretion due to the transcriptional enhancer sequence discussed above, however it was uncertain how this level would compare with the others. The pCMV/HBs-BGHpA and pCMV/HBs- β pA plasmids were derived from pCMV/HBs-HBVpA and for ease of discussion will be referred to as BGHpA, β pA and HBVpA, respectively. Following cloning, the nucleotide sequence was confirmed by dideoxy sequencing to ensure that no PCR or insertion errors had occurred (data not shown). To verify in vitro production of HBsAg mRNA from each construct, the relative level of mRNA was examined following transient transfection of CHO cells with equimolar amounts of each plasmid. Following extraction and purification, the RNA was used as template in a relative RT-PCR experiment in which two sets of primers were used, one specific for the HBsAg, the other for the housekeeping gene β -actin. By comparing the ratio of the resulting signal, it was clear that transfection with the HBVpA resulted in significantly higher levels of HBsAg specific mRNA than did the β pA construct ($P < 0.05$, Fig. 2). However, the differences were indistinguishable from either the BGHpA or β pA construct ($P > 0.05$).

3.2. Levels of HBs secretion following transient transfection

To determine if the levels of secreted HBsAg correlated with the mRNA expression levels, an equivalent number

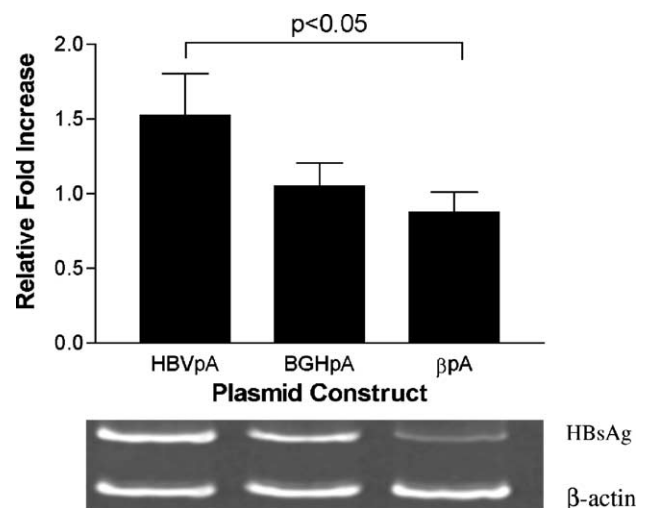


Fig. 2. Relative levels of HBs mRNA expression. CHO cells were transfected with equimolar amounts of each plasmid (Fig. 1) and incubated for 48 h. Following incubation, RNA was extracted and subjected to RT-PCR. The graph shows the mean values obtained from four independent experiments (\pm S.E.M.). The PAGE gel (lower panel) shows the raw data representative of a single experiment. Non-transfected CHO cells served as the control and resulted in no amplified product (data not shown).

Table 1
IgG isotype profiles following i.m. immunization

Construct	IgG1 ^a	IgG2a ^a	IgG2a/IgG1
HBVpA	612 ± 302*	2540 ± 735 [†]	4.1
BGHpA	174 ± 93*	670 ± 225 ^{b,†}	3.9
βpA	<78	<195	NA

^a Data ($N = 8$ per group) measured in ng/ml (mean ± S.E.M.).

^b The group average was determined based on 7/8 mice, as it was determined by Grubb's test that an outlier (15,630 ng/ml) existed within this group, which was omitted from the data analysis.

* $P > 0.05$.

[†] $P < 0.04$.

of CHO cells were transiently transfected with equimolar amounts of each plasmid. Following incubation, supernatants were concentrated and used in a quantitative ELISA. In keeping with previous observations [34], the level of secreted antigen from HBVpA was significantly higher than both BGHpA ($P < 0.05$) and βpA ($P < 0.001$) indicating that the enhancer sequence of the HBVpA contributed to higher levels of HBs expression.

3.3. Total IgG anti-HBs antibody responses following i.m. immunization

BALB/c mice were immunized with equimolar amounts of each plasmid (week 0) and sera samples were collected bi-weekly. Antibody responses for both the HBVpA and BGHpA groups were evident by week 2 and increased markedly through week 8. The mean titers of HBVpA immunized mice were consistently higher at weeks 4–8 than those of animals immunized with BGHpA or βpA (Fig. 4, $P < 0.05$). The lack of any detectable antibody response from βpA immunized mice is consistent with the low levels of secreted HBs seen in vitro.

In addition to producing higher antibody titers, the HBVpA construct increased the overall percentage of seroconverting mice. For example, 100% of mice immunized with HBVpA had detectable levels of antibody by week 4, in comparison to only 50% of mice immunized with BGHpA and 0% of mice immunized with the βpA construct. This pattern was also observed at weeks 6 and 8.

3.4. IgG subclass profiles following i.m. immunization

To examine whether a Th1 or Th2 response prevailed, sera samples (week 8) were assayed for HBsAg-specific IgG1 and IgG2a. Mice immunized with either HBVpA or BGHpA produced a heavily Th1 polarized immune response, as evidenced by high IgG2a/IgG1 ratios (Table 1). Although there was no statistical difference in IgG1 titers between the groups, mice immunized with the HBVpA construct produced significantly higher levels of IgG2a than those immunized with BGHpA ($P < 0.05$).

Table 2
Antibody avinities of week 8 anti-sera

Construct	Animal no.	K_A (M)
HBVpA	1	2.0×10^{-8}
	2	1.7×10^{-8}
	3	4.6×10^{-9}
	\bar{X}	1.7×10^{-9}
BGHpA	1	1.9×10^{-8}
	2	6.5×10^{-9}
	3	6.5×10^{-9}
	\bar{X}	4.4×10^{-9}

3.5. Antibody avinities following i.m. immunization

To determine if differing levels of antigen expression affected antibody avinity, avinity constants were calculated for mice injected i.m. with each construct. There were no significant ($P > 0.05$) differences in avinity (measured at week 8) between either the HBVpA and BGHpA groups (Table 2), suggesting that variable levels of antigen expression did not influence the overall avinity of the antibody population. Additionally, no correlation was observed between the antibody avinities and total IgG titers when the two groups were compared (data not shown).

3.6. IFN-γ and IL-4 responses to HBsAg

To further elucidate the effect of antigen expression levels on the Th1/Th2 bias, IFN-γ and IL-4 secretion was measured in splenic tissue culture supernatants following in vitro stimulation with purified HBsAg (Fig. 5). Surprisingly, mice immunized with the βpA construct, in which no antibody response was observed, produced the highest levels of IFN-γ, suggesting that the response was strictly Th1-mediated. IFN-γ levels were not statistically different between mice immunized with either the HBVpA or BGHpA construct. None of the three groups produced measurable quantities of IL-4 (data not shown).

4. Discussion

Improving the efficiency of DNA vaccines has become an important issue since these vaccines often produce relatively low levels of immunity when compared to traditional approaches to vaccination. This deficiency has become particularly apparent in the context of raising humoral immune responses in non-rodent species. Several groups have attempted to improve gene expression levels by manipulating promoter elements or polyadenylation sequences. Lee et al. showed that intramuscular injection of the chloramphenicol acetyltransferase (CAT) reporter gene resulted in optimal levels of expression when under the control of the CMV promoter, when compared to either the RSV or SV40 promoter [35]. Differences in luciferase expression have also

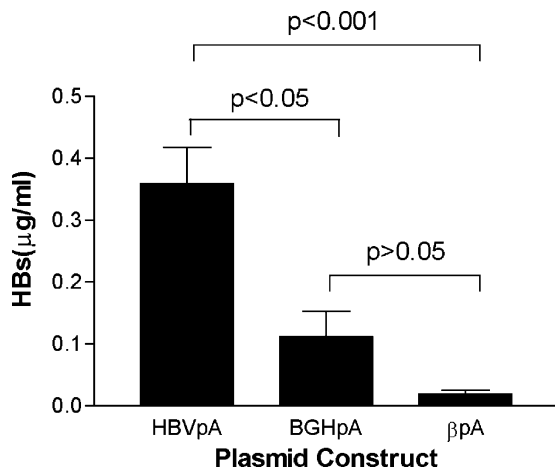


Fig. 3. Levels of secreted HBs antigen. CHO cells were transfected with equimolar amounts of each plasmid and incubated for 48 h. Following incubation, CHO cell supernatants were concentrated to equal volumes and assayed by quantitative ELISA. The graph shows the mean values obtained from four independent experiments (\pm S.E.M.). Non-transfected CHO cells served as the control and no *secreted antigen* could be detected from these samples (data not shown).

been observed in studies in which different polyA sequences were utilized. Hartikka et al. optimized *in vivo* luciferase expression (utilizing CMV promoter) by employing several different polyA termination signals [36]. Additionally, *in vivo* gene expression levels have been controlled through the use of inducible promoter systems [37–41], but none of these studies examined the effect on the immune response.

The current study was designed to determine the influence of 3'-polyadenylation sequences on gene expression and to assess its effect on immune response. *In vitro* anal-

ysis demonstrated that the HBVpA construct containing a well-characterized transcriptional enhancer sequence resulted in higher levels of both HBs specific mRNA (Fig. 2) and secreted antigen (Fig. 3). Interestingly, although the levels of mRNA expression from the β pA construct were comparable to the BGHpA construct, the amount of HBsAg secreted *in vitro* was extremely low. This difference in the level of expressed mRNA and secreted antigen may reflect the lack of β pA mRNA stability in supporting high levels of HBsAg expression.

We hypothesized that higher *in vitro* HBsAg expression levels generated by the HBVpA construct would correlate with higher *in vivo* antibody titers, relative to the two other constructs. The results from experiments designed to test this question clearly indicate that mice immunized with the HBVpA construct produced higher levels of total IgG and an increased proportion of seroconverting animals (Fig. 4). These findings are in agreement with those published by Lee et al., which showed that the intensity and seroconversion rate directly correlated with promoter strength [35]. Although the level of mRNA and HBsAg were not measured following immunization, it can be inferred from *in vitro* experiments that an increase in HBsAg expression, driven in part by the enhancer, resulted in the observed increases in antibody titers. In support of these findings, previous studies have shown a direct correlation between the amount of expressed antigen *in situ* and antibody titer. In one study, mice that were immunized *i.m.* with increasing doses of a secreted alkaline phosphatase (SEAP) expressing construct yielded higher SEAP levels and higher anti-SEAP antibody titers relative to lower DNA doses [42]. Similar studies have also reported a correlation between DNA dose and antibody titer [43–45]. However, studies that manipulate the dose of

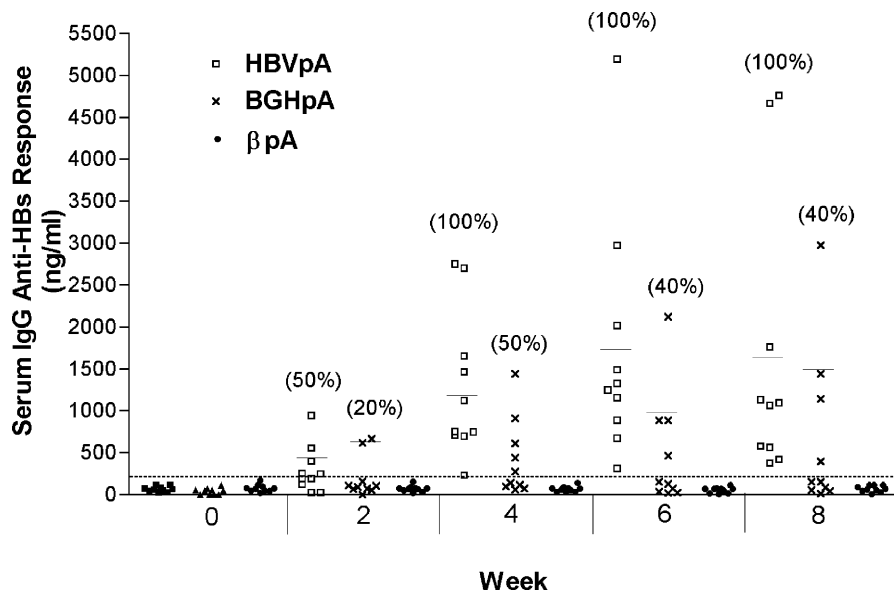


Fig. 4. Total IgG anti-HBs immune response. BALB/c mice ($N = 10$) were immunized *i.m.* on week 0 with equimolar amounts of each plasmid. Following immunization, sera was collected bi-weekly and assayed by quantitative ELISA (\pm S.E.M.). The limit of detection was 200 ng/ml (dashed line), while the small solid lines represent the group means. Numbers in parenthesis represent the percentage of mice that seroconverted.

DNA administered may simply be increasing the number of cells transfected, and are not directly comparable to the current study. Increasing the amount of antigen produced *per cell* may qualitatively influence the type of immune response that ensues. Our data supports this supposition in that cytokine profiles and antibody subclass ratios were altered, not just overall antibody titers.

The lack of any detectable antibody response from β pA immunized mice is consistent with the very low levels of secreted HBsAg measured *in vitro*. One possible explanation for this observation is that the limited amount of antigen expressed was insufficient to activate naïve B cells [42]. In support of this notion, the high levels of IFN- γ measured in splenic supernatants (discussed later), which is known to act as a switch factor for IgM to IgG2a [46] would, in all likelihood, have provided the appropriate stimulus for isotype switching provided that sufficient antigen was present. The absence of B cell activation may also provide an explanation for why only 40–50% of those mice immunized with BGHpA responded over the 8-week study, whereas 100% of all mice immunized with HBVpA had seroconverted.

It is well known that *i.m.* injection of DNA vaccines elicits predominately a Th1 biased humoral immune response as evidenced by a predominance of IgG2a production against both the HBsAg and other antigens [4,6,43,47]. In contrast, DNA vaccines delivered via biolistic inoculation (gene gun) generally evoke a Th2 response, resulting in a predominance of IgG1 production over IgG2a [5]. In the current study, immunization with either the HBVpA or BGHpA construct induced primarily a Th1 biased immune response as indicated by high levels of IgG2a (Table 1). Consistent with the total IgG responses, the levels of IgG2a were highest in mice immunized with HBVpA, however, the overall IgG2a/IgG1 ratios were not significantly different between groups, suggesting that differing levels of antigen expression affects the overall antibody titer more dramatically than it does the isotype distribution. Thus, it appears that the Th1/Th2 cytokine bias is more heavily influenced by the route of immunization than it is by level of antigen expression.

Although it has been shown that high titers of antigen specific antibody correlates with HBV protection [13], optimizing antibody avinities is also an important consideration in improving the immune response to DNA vaccines. For certain viral antigens, a minimum antibody avinity of $\sim 2 \times 10^7 M^{-1}$ was shown to be effective at protecting mice from subsequent virus challenge [48]. In our study, differences in the level of antigen expression did not appear to influence the avinities of either HBVpA or BGHpA immunized mice. It is unclear if a DNA vaccine construct that generates very low levels of antigen would elicit antibodies of differing avinities. The β pA construct was developed for this purpose, but unfortunately did not elicit a sufficient amount of antibody for avinity analysis.

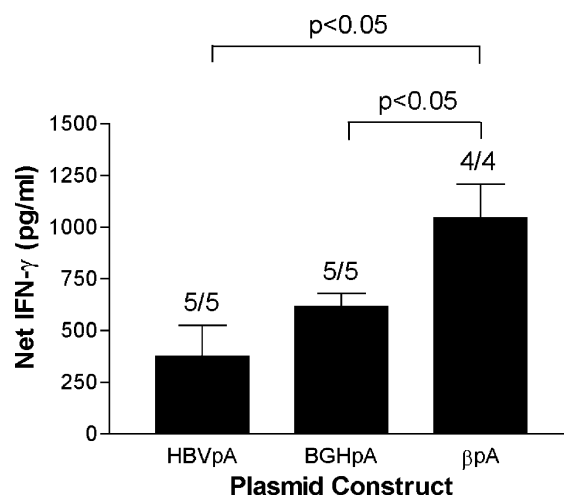


Fig. 5. Splenic IFN- γ responses following DNA immunization. Mice were immunized with equimolar amounts of plasmid on week 0 and sacrificed at week 8. Splenocytes were purified, followed by HBs stimulation in culture for 72 h. Tissue culture supernatants were then harvested and assayed by ELISA (see Section 2). Results were calculated by subtracting background of unstimulated control cultures from the net values of antigen-pulsed cultures. The numbers above each error bar represent the proportion of mice responding in each group (mean \pm S.E.M.).

To further examine the polarization of the immune response, IFN- γ and IL-4 levels were measured as an indication of a Th1 or Th2 immune response, respectively. Although IL-4 could not be detected in splenic supernatants from mice in any of the groups (as would be predicted based upon the IgG2a/IgG1 ratios), very high levels of IFN- γ were observed in all three groups of mice (Fig. 5). Based upon previous studies showing that increases in DNA vaccine-induced antigen production yielded higher IFN- γ levels [45], we hypothesized that the HBVpA construct would induce the highest level of IFN- γ , compared to the other constructs. Surprisingly, mice immunized with the β pA construct yielded the highest levels of IFN- γ . The source of this cytokine was not determined, but could have been contributed by Th1 cells, CD8⁺ CTLs or natural killer cells. One possible explanation for this observation may reside in the reciprocal cytokine cross-regulation that occurs between the Th1 and Th2 cell populations. In the current study, both Th1 and Th2 cell populations were activated, but to dramatically differing degrees. The release of Th2 cytokines, as evidenced by modest levels of IgG1, may in part be responsible for a partial down regulation of Th1 cells, leading to decreased levels of IFN- γ in mice immunized with the HBVpA or BGHpA constructs. Thus, mice receiving the β pA construct may have produced more IFN- γ by virtue of the absence of Th2 cytokines.

In conclusion, this study demonstrates that modulating the level of antigen expression not only affects the overall intensity of the immune response (antibody titer and seroconversion rates), but also plays a role in modulating cytokine profiles. The ability to tailor these variables may prove useful for combating infectious agents by stimulating the most

efficacious arm of the immune system (cell-mediated versus humoral).

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