Posttranscriptional Regulatory Elements Enhance Antigen Expression and DNA Vaccine Efficacy

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In higher eukaryotes, introns are usually required for efficient pre-mRNA processing. However, some viruses have alternative approaches involving posttranscriptional regulatory elements (PREs) to enhance intronless heterologous gene expression through enabling stability and 3′ end formation, and to facilitate the nucleocyto-plasmic export of unspliced mRNAs. In the current study, we compared the human cytomegalovirus (hCMV) immediate/early (IE) intronA, as well as virus-derived PREs—the PRE of Hepatitis B virus (HPRE) and Wood-chuck Hepatitis virus (WPRE) on their ability to enhance antigen gene expression *in vitro* and immune responses induced by DNA vaccination in animal. Among all the constructs, the plasmids carrying the HPRE element showed the highest gene expression level in both *in vivo* and *in vitro* models. During immunization of mice with low doses (10 μg) of HIV-1 DNA vaccine, only −intronA/+HPRE and +intronA/+HPRE vaccine constructs in-duced anti-Gag antibodies, although the −intronA/+WPRE construct also elicited antigen-specific cellular im-mune responses. In addition, pInHGag (+intronA/+HPRE) at a 10 μg dose could induce higher anti-Gag antibody level than that induced by pGag (−intronA/−HPRE) or pInGag (+intronA/−HPRE) at 40 μg dose ($p < 0.05$). Our data are useful for the optimization of heterologous expression and immunogenicity of DNA vaccines.

Introduction

VACCINES HAVE PLAYED a substantial role in controlling epidemic diseases since Jenner's smallpox vaccine in 1796. In 1990 Wolff and his colleagues found that injecting plasmid DNA into mouse muscle could directly induce protein expression within muscle cells (Wolff *et al.*, 1990). Further, they reported gene expression lasting for a year after intramuscular injection of plasmid DNA (Wolff *et al.*, 1992). Subsequently, the efficacies of a number of other plasmid DNA vaccines to induce humoral and cell-mediated immune responses against influenza A virus, human immunodefi-ciency virus (HIV), hepatitis B virus, and malignant tumor have been demonstrated. The discovery of DNA immuni-zation in 1990s fundamentally changed our view of the na-ture of a vaccine: a genetic material that encodes for antigens, rather than the actual antigens themselves, can serve as an effective vaccine component (Lu *et al.*, 2008).

Antigen-encoding naked DNA is relatively safe, stable, and easy to produce, and can mediate sustained antigen expression *in vivo* (Donnelly *et al.*, 1997; Gurunathan *et al.*, 2000). In addition, because DNA vaccines do not elicit anti-bodies against dsDNA itself in the host, they can be ad-ministered repeatedly. However, the development of DNA vaccine candidates has been limited due to their relatively modest immunogenicity. Therefore, great effort has been made to enhance the immune response through optimization of promoter/enhancer elements or the coding sequence ac-cording to the codon-usage bias of the host to achieve higher gene expression level (Xu *et al.*, 2001; Garmory *et al.*, 2003). Other approaches to improve the immunogenicity of DNA vaccine include the application of high-efficiency gene deliv-ery systems such as gene gun or *in vivo* electroporation device (Fynan *et al.*, 1993; Fuller *et al.*, 2006; Luckay *et al.*, 2007; Sharpe *et al.*, 2007; Tsang *et al.*, 2007), the use of cytokine genetic adjuvants such as GM-CSF, IL-2, and IL-12 (Lori *et al.*, 2006), the employment of innate immunomodulatory signals (Li *et al.*, 2007), and the modification of the properties of antigen-presenting cells (Tsen *et al.*, 2007). Because the antigen ex-pression level would influence activation, strength, and the duration of the specific immune response, it is generally be-lieved that optimization of antigen expression is one of the most promising approaches proposed to enhance the immu-nogenicity of DNA vaccine candidates.

Posttranscriptional regulatory elements (PREs) of Hepatitis B virus (HPRE) and Woodchuck Hepatitis virus (WPRE) are both hepadnaviral *cis*-acting RNA elements that can increase the accumulation of cytoplasmic mRNA of an intronless gene

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by promoting mRNA exportation from the nucleus to the cytoplasm, enhancing 3′ end processing and stability. Usually, unspliced mRNAs are exported into the cytoplasm with low efficiency. However, PRE elements function independently of virally encoded proteins, and it is believed that some cellular factors may interact with the PRE element and mediate its posttranscriptional transport (Donello *et al.*, 1998). HPRE can functionally substitute for the HIV type 1 (HIV-1) Rev-Rev-responsive element (RRE) complex in a transient transfection reporter assay (Huang and Liang, 1993; Huang and Yen, 1995). WPRE, as has been demonstrated by others, has been successfully used for efficient expression of intronless genes from plasmid (Garg *et al.*, 2004; Callendret *et al.*, 2007).

In the current study, we constructed a series of DNA expression vectors carrying either the HPRE or the WPRE, with and without human cytomegalovirus (hCMV) immediate/early (IE) intronA. Wild-type HIV-1 CN54 *Gag* coding sequence, a prevailing strain in many areas of China, was used as a model antigen to compare both the gene expression–enhancing and immunogenicity-improving effects of different PREs with or without hCMV IE intronA on the DNA vaccine constructs. We demonstrated that HPRE significantly improved *Gag* gene expression in 293T cell. The highest level of gene expression was observed when both hCMV intronA and HPRE were present in the same plasmid. Further, this vaccine construct elicited higher cellular and humoral responses with a ¼ dosage in comparison with the *Gag* DNA vaccine construct carrying neither of the PRE elements.

Materials and Methods

Plasmid construction

pVR1012 Is an optimized mammalian expression vector containing intronA sequence of CMV promoter and BGH polyA signal,which was kindly provided by Dr. Gary Nabel from the Vaccine Research Center, NIAID, NIH (Bethesda, MD). A plasmid vector pCMV containing CMV promoter and BGH polyA signal was constructed from pVR1012 through deleting the CMV IE intron. The HPRE and WPRE elements were synthesized by overlapping PCR according the nucleotides 2641–3214 of GenBank accession no. X02763 and nucleotides 1093–1684 of GenBank accession no. J04514, respectively. The HPRE and WPRE PCR fragments were digested with *Xba*I and *Bgl*II and ligated into the *Xba*I-*Bgl*II–digested pVR1012 backbone or pCMV backbone to generate the vectors pInH, pInW, pH, and pW. The *Gag* gene of HIV-1 CN54 (97CN001 GenBank accession no. AF286226), a prevailing strain in many areas of China, was subcloned into the *Sal*I-*Eco*RV sites of the vectors pCMV, pH, pW, pVR1012, pInH, and pInW, producing pGag, pHGag, pWGag, pInGag, pInHGag, and pInWGag, respectively. DNA used for transfection and vaccination was prepared using the EndoFree Plasmid Giga Kit (Qiagen, Hilden, Germany). The final endotoxin level was less than 0.1 EU/µg DNA (0.1 endotoxin units/µg plasmid DNA). The supercoiled plasmid was no less than 80% as detected by agarose gel electrophoresis.

Cell culture and plasmid transfection

293T Cells (ATCC, CRL-11268) were cultured in DMEM containing 10% fetal bovine serum and plated in six-well tissue culture plates at a density of 5×10^5 cells/well the day before transfection. pCMV, pGag, pHGag, pWGag, pInGag, pInH-Gag, and pInWGag were transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a ratio of 5 µg lipofectamine 2000 to 2 µg plasmid DNA. The cell was harvested 48 h after transfection. *Gag* mRNA and protein expression were measured by real-time RT-PCR and Western blot, respectively.

Real-time RT-PCR

Total RNA from transfected cells was extracted using RNeasy Kit (Qiagen), amplified in duplicates using One Step SYBR PrimeScript™ RT-PCR Kit (Takara, Dalian, China), and detected using Applied Biosystems 7500 real-time PCR System (ABI, Foster city, CA). *Gag* RNA was amplified by RT-PCR using the primers GagF (5′-AGACAAGATAGAGGAAGAA CAAAAC-3′) and GagR (5′-ATGTCTCCTACTGGAACAG GTGGGT-3′). Serving as an internal standard, β-actin RNA was amplified with primers actinF (5′-CCAGCCATGTACG TTGCTATC-3′) and actinR (5′-CAGGTCCAGACGCAGGA TGGC-3′) for each sample.

Western blot assay for Gag expression

Forty-eight hours after transfection, cell lysates were denatured and subjected to denaturing SDS-PAGE and then blotted onto PVDF membrane (Millipore, Bedford, MA). Blocking was done with 5% defatted milk powder/PBS containing 0.05% Tween (PBST) for 2 h. HIV human serum and rabbit anti-β-actin polyconal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used as the detecting antibodies at 1:500 dilution incubated for 1 h. Subsequently, the membranes were washed with PBST and then incubated with HRP-labeled anti-human IgG (1:2000) and HRP-labeled anti-rabbit IgG (1:2000), respectively. After final wash, chemiluminescence reagent was applied to the membranes. Then, the Anti-Gag Western blot membranes were scanned and quantified using the Gel/Chem doc program Quantityone (Bio-Rad, Milan, Italy). Protein expression levels were obtained from chem images using the Quantity One software (v.4.5.1; Bio-Rad).

DNA immunization

Six- to eight-week-old female Balb/C mice were purchased from the Institute of Laboratory Animal Science, the Chinese Academy of Medical Sciences & Peking Union Medical College. Animals were used in compliance with institutional animal health and care regulations, and all procedures used in the experiments with animals were approved by the local Institutional Animal Care and Use Committee. Groups of eight Balb/C mice each were injected intramuscularly with 100 µL plasmid DNA (50 µL in each tibialis anterior muscle) in PBS. Mice were injected with 40 µg or 10 µg pGag, pHGag, pWGag, pInGag, pInHGag, and pInWGag or 40 µg pCMV (as negative control) plasmid DNA at weeks 0, 3, and 6.

ELISPOT assay

The ELISPOT assay described by BD™ ELISPOT Mouse IFN-γ ELISPOT Set (BD, San Diego, CA) protocol was modified to detect HIV-1 *Gag*-specific T-cell responses. Three weeks after the last immunization, mouse splenocytes were harvested. Ninety-six-well plates were coated at 4°C overnight with 10 µg/mL of anti-mouse IFN-γ in sterile PBS. The

plates were washed four times with 200 μL/well PBST and blocked with RPMI 1640 containing 10% FBS at room temperature for 2 h. Mouse splenocytes were isolated, and RBCs were lysed according to the protocol described previously (Li et al., 2007). Splenocytes were then seeded into wells with 50 μL/well (5×10^5 cells/well) in addition to 50 μL Gag overlapping peptides (acid sequence: AAMQILKDTINEEAA) and incubated for 30 h at 37°C in 5% CO_2. After incubation, the ELISPOT plates were developed according to the kit instruction. Finally, plates were air-dried, and the spot-forming cells (SFC) were quantified by a Bioreader-4000 automated ELISPOT reader (BioSys, Karben, Germany) and normalized for 10^6 splenocytes.

Enzyme-linked immunosorbent assay

Ninety-six-well flat-bottom plates (Costar, Corning, NY) were coated with purified recombinant p55 protein (a recombinant protein of HIV-1 CN54 strain was expressed in E. coli and purified to 95% purity) at a concentration of 0.01 μg/mL in coating buffer (0.012 M Na_2CO_3 and 0.038 M $NaHCO_3$, pH 9.6) at 4°C overnight. Plates were washed five times with PBST, and blocked with 3% BSA in PBST at 37°C for 1 h. Mouse sera were serially twofold diluted in block solution (starting at 1:100), and 100 μL was added to each well. After incubation at 37°C for 1 h, the plates were washed five times with PBST and then incubated with 1:5000 diluted HRP-labeled goat anti-mouse IgG antibody (Santa Cruz Biotechnology) at 37°C for 1 h. After the final wash, 100 μL of fresh TMB substrate (Sigma, St. Louis, MO) was added per well, and plates were incubated for 5 min. The reaction was stopped by adding 25 μL of 2M H_2SO_4, when the optical density of the plate measured at 450 nm by Multiscan enzyme-linked immunosorbent assay (ELISA) plate reader (Thermo Life Sciences, Hampshire, United Kingdom).

Statistical analysis

Values were expressed as means ± standard deviations (SD). Analysis of differences in means between groups was conducted by one-way analysis of variance (ANOVA); $p < 0.05$ was considered significant.

Results

Construction of HIV-1 Gag expression vectors and Gag expression in transfected cells

As depicted in Figure 1, a total of six Gag DNA vaccine plasmids were constructed with various combinations of CMV IE promoter/IntronA, PREs, and Gag gene of HIV-1 CN54. The RNA export elements of the HBV and Woodchuck Hepatitis virus, know as HPRE and WPRE (Donello et al., 1998; Heise et al., 2006), were inserted to upstream of the BGH poly A signal, respectively.

The Gag expression levels of these plasmids in transiently transfected 293T cells differed significantly as shown in Figure 2. Approximately 48 h after transfection, the cells were harvested, and total RNA was isolated. The amount of Gag transcripts in 293T cells transfected by the above DNA vaccine plasmids were determined by real-time RT-PCR. As internal standard, the amount of β-actin RNA was determined for each sample (Fig. 2).

As shown by real-time RT-PCR (Fig. 2), the Gag RNA levels in 293T cells transfected by DNA plasmid with CMV-IE
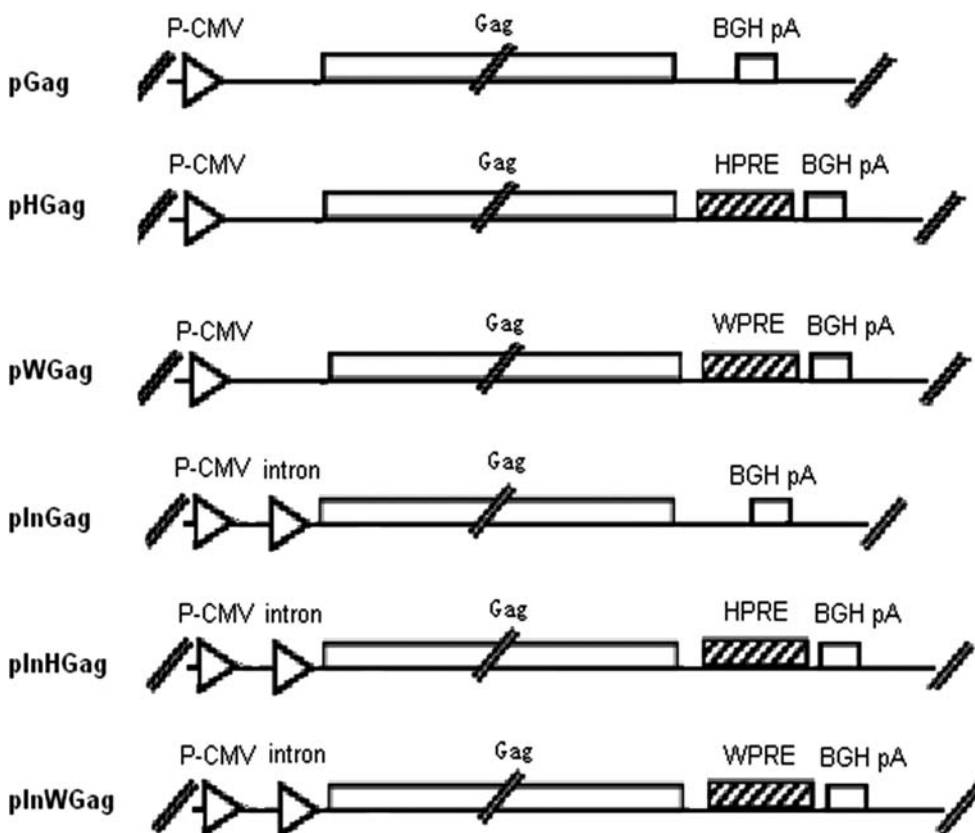

FIG. 1. Schematic maps of Gag expression plasmids. P-CMV, human cytomegalovirus (hCMV) immediate/early (IE) promoter; intron, hCMV IE intronA; HPRE, hepatitis B virus posttranscriptional element; WPRE, Woodchuck hepatitis virus posttranscriptional element; BGH pA, bovine growth hormone polyadenylation signal.
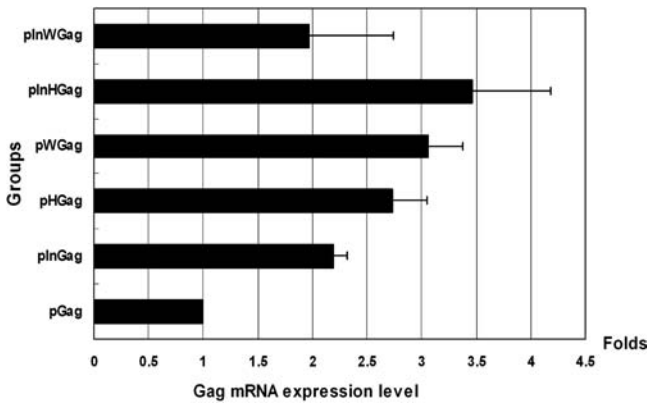
FIG. 2. Relative *Gag* RNA levels. *Gag* mRNA was analyzed by real-time RT-PCR using *Gag*-specific primers. Serving as internal standard, β-actin RNA level was determined by real-time RT-PCR using β-actin–specific primers. The *Gag* RNA level in pGag-transfected 293T cells was set to one, and the RNA levels in other plasmid-transfected cells are presented as relative values to that of 293T cells.

FIG. 3. (**A**) Gag protein expression levels in plasmid-transfected 293T cells analyzed by Western blotting. β-Actin was used as an internal standard. The position of Gag protein is indicated on the side with molecular weight. (**B**) Relative quantitative presentation of *Gag* mRNA and protein expression levels in transiently transfected 293T cells. The expression levels of *Gag* mRNA or protein are normalized to the expression level of pGag, which is set as 1. The average of two experiments with SD is indicated.

intronA and/or PREs were significantly higher than that observed in pGag-transfected cells. However, no significant differences were observed between these constructs carrying different PREs with or without intronA sequences. Therefore, our data confirmed previous findings that efficient expression of intronless genes from plasmid may require the presence of additional *cis*-acting regulatory modules within the expression cassettes, such as splice sites and hepadnaviral PREs (Callendret *et al.*, 2007).

Subsequently, the cell extracts were subjected to SDS-PAGE and Western blot analysis. It is quite interesting that the Gag expression level of pInGag was very low regardless of the presence of CMV intronA sequence (Fig. 3A, lane 3). However, to a large extent, the Gag expression was significantly enhanced when the constructs included PREs (Fig. 3A, lanes 4 and 5). More excitingly, Gag expression was remarkably enhanced by +HPRE/+intronA DNA plasmid (Fig. 3A, lane 6). To analyze the effect of PREs on transgene expression in RNA processing steps, we compared the relative level of *Gag* RNA and protein (Fig. 3B). Note that more obvious expression enhancement effect of PRE could be observed in Gag protein accumulation rather than total RNA in the transfected cells. This result further confirmed that PREs are very efficient for intronless gene expression at the post-transcriptional level.

Gag-specific humoral immune responses

pGag, pInGag, pHGag, pInHGag, pWGag, and pInWGag were intramuscularly immunized in Balb/C mice. Mice were injected three times with 3-week intervals. Each group was made up of eight mice, each received 10 μg or 40 μg DNA plasmid. Serving as negative control, a group of mice was injected with 40 μg of pCMV DNA.

Three weeks after the final immunization, the sera were collected to detect HIV-1 Gag–specific IgG responses by ELISA assay.

At the 40 μg dose, each of the *Gag* expression plasmids induced anti-Gag IgG response. pInGag Raised weak anti-Gag response that was much lower than that induced by
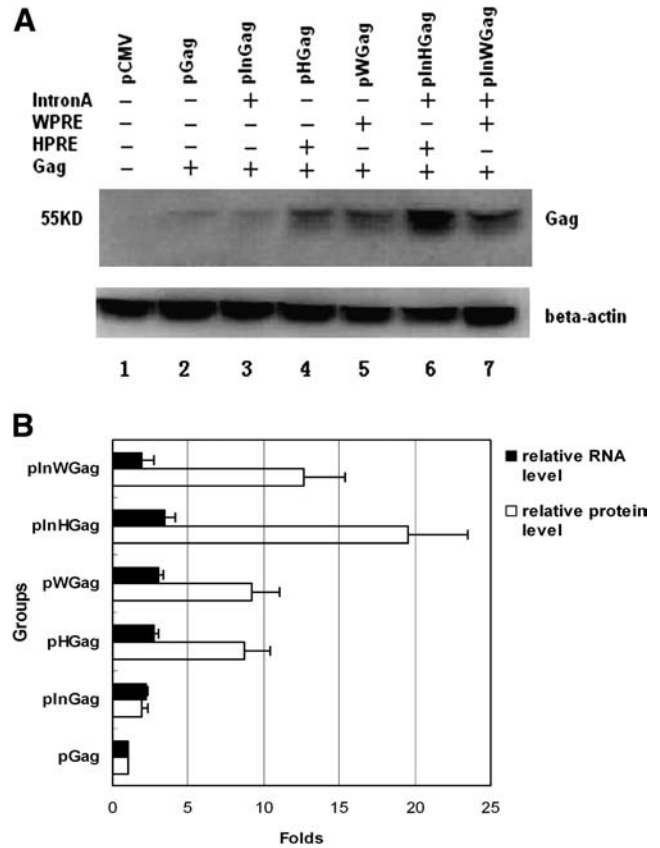
pWGag or pInWGag. pHGag And pInHGag elicited the highest antibody responses among all groups. At the dose of 10 μg of DNA, as demonstrated by Figure 4B, only pHGag and pInHGag induced satisfactory antibody responses. Parallel with *in vitro* gene expression data, both pGag and pInGag failed to induce any detectable anti-Gag antibodies. Somewhat to our surprise, the pWGag and pInWGag constructs could not induce anti-Gag IgG responses at the 10 μg dosage, although WPRE had enhanced the *Gag* gene expression *in vitro*.

Our data also indicated that 10 μg dose of pInHGag induced much higher anti-Gag IgG response than 40 μg dose pInGag or pGag did ($p < 0.05$). Ten μg dose of pHGag elicited similar humoral response level compared with 40 μg dose of pGag (Fig. 4C).

Gag-specific cell-mediated immune responses

We next wanted to determine the effect that the DNA vaccine constructs, with PREs and/or CMV intronA, had on generation of Gag-specific T cell immune responses. Splenocytes from immunized mice were harvested 3 weeks after the third vaccination and stimulated with Gag peptide, and
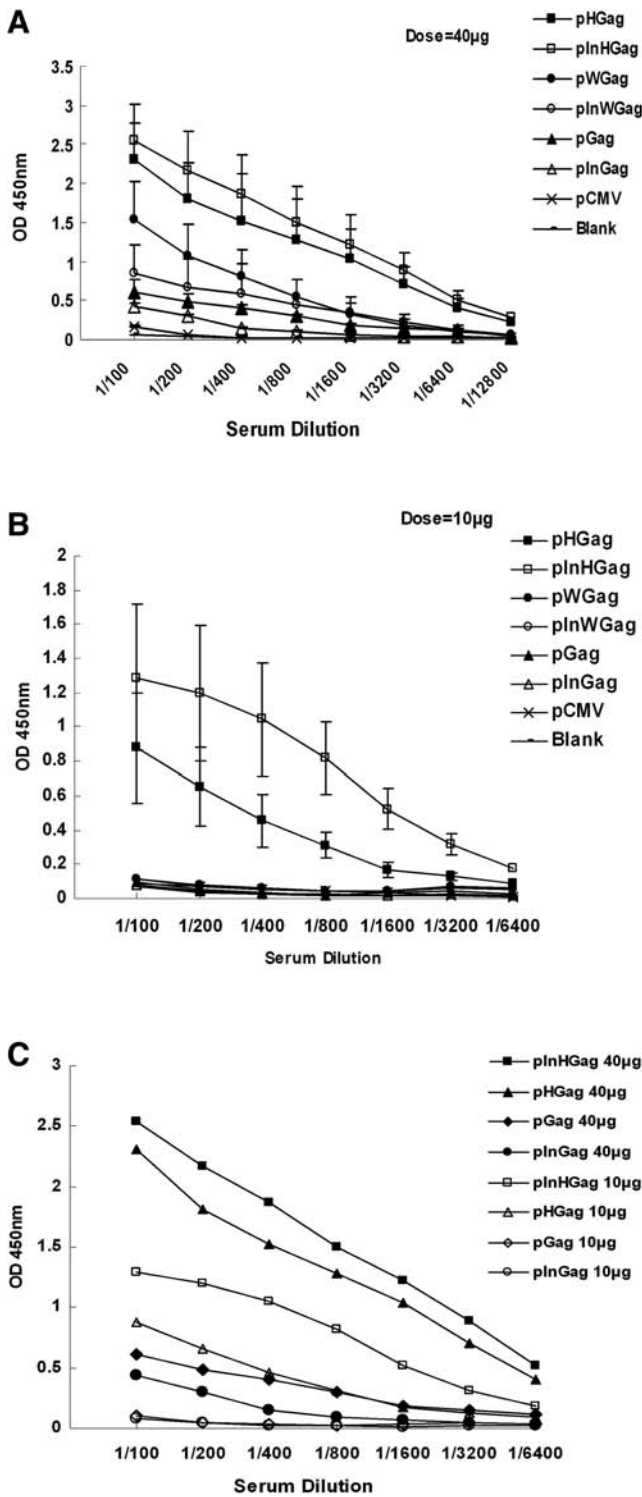
FIG. 4. Anti-Gag antibody responses in mice immunized with *Gag* DNA vaccine plasmids with or without PRE or/and CMV IE IntronA elements. Balb/c mice were injected three times intramuscularly at 3-week intervals with 10 or 40 μg plasmid DNA as indicated, or with 40 μg of empty plasmid DNA pCMV. Serum samples from Balb/C mice (eight/group) were collected for ELISA 3 weeks after the final vaccination. (**A**) 40-μg dose. (**B**) 10-μg dose. The open symbols represent 10 μg dose, and the solid symbols represent 40 μg dose in Figure 4 (**C**). (We omit some error bars in Fig. 4 to avoid overlap.)
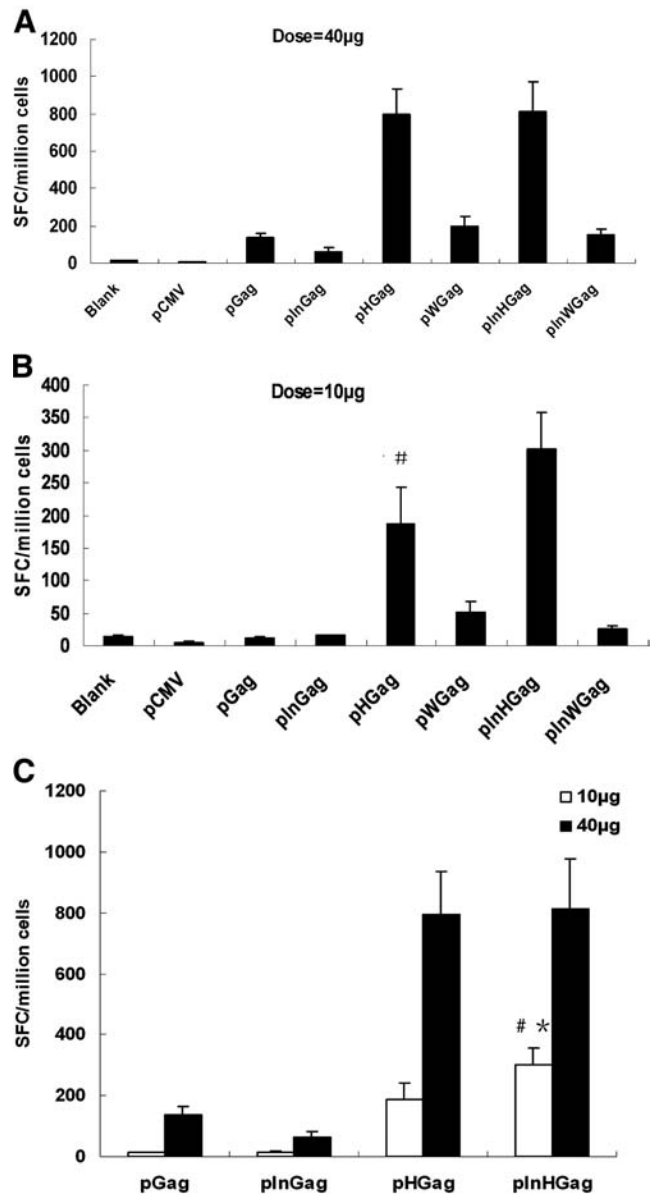

FIG. 5. Gag-specific IFN-γ T cells induced by DNA vaccination. Splenocytes were harvested and stimulated with Gag (No. 06042000010) peptide. The data are shown as means $\pm$ SD ($n = 8$ mice/group). (**A**) HPRE significantly improved Gag-specific T cell responses ($p < 0.01$). (**B**) #$p < 0.05$ compared with pWGag group. (**C**) #$p < 0.05$ compared with pGag group; *$p < 0.01$ compared with pInGag group.

IFN-γ production was detected by ELISPOT analysis. As shown in Figure 5A, when mice were immunized with high-dose (40 μg) vaccines, all DNA vaccine constructs could elicit Gag-specific T cell responses. However, mice vaccinated with pHGag (+HPRE/−intronA) or pInHGag (+HPRE/+intronA) generated significantly higher Gag-specific T cell responses than mice vaccinated with other plasmid DNA without the HPRE element ($p < 0.01$). This result was consistent with that of the humoral immune responses.

From the data presented (Fig. 5B), it is clear that only pInHGag, pHGag, and pWGag could elicit Gag-specific cellular immune responses with 10 μg dose ($p < 0.05$). pInHGag

DNA vaccine generated a significantly higher number of Gag-specific T cells (301 SFC/million splenocytes) than any other DNA vaccine without HPRE element did ($p < 0.01$). In addition, the number of Gag-specific T cells induced by DNA vaccines was 186 SFC/million splenocytes for pHGag and 51 SFC/million splenocytes for pWGag, indicating that pHGag elicited significantly higher ($p < 0.05$) cell-mediated immune responses than pWGag. It was clearly demonstrated that HPRE strongly enhances the immunogenicity of *Gag* DNA vaccine in mice, whereas WPRE has less effect.

At 10 μg low dosage, pInHGag induced a much higher Gag-specific cell immune response than 40 μg of pGag ($p < 0.05$) or pInGag ($p < 0.01$) did (Fig. 5C). Mice immunized with low-dose pHGag DNA plasmid generated similar levels of Gag-specific T cells compared with mice administrated with high-dose (40 μg/mice) pGag DNA plasmid ($p > 0.05$).

Discussion

Since the early 1990s, DNA vaccination has become an important approach against infectious diseases, malignant tumors, and allergy. Many efforts have been made to design and develop better therapeutic and prophylactic DNA vaccines. Most DNA vaccine optimization strategies have been focused on three areas: (1) to enhance *in vivo* delivery and transfection of DNA vaccine plasmids, (2) to develop both chemical and genetic adjuvants regulating the magnitude and balance of immune responses, and (3) to improve gene expression through optimizing the gene regulatory elements or coding sequences.

Among all these efforts, higher antigen expression strength is pursued mainly through optimization of the codon usage of the antigen gene and optimization of the gene expression regulatory elements at the transcription level. A few studies have demonstrated that some PREs such as the HPRE and the WPRE, when combined with appropriate promoter/enhancer elements, can also play an important role in the enhancement of transgene expression from retroviral vector through promoting the nuclear export of intronless transcripts (Zufferey *et al.*, 1999; Gruh *et al.*, 2008). Moreover, the WPRE has been shown to improve gene expression from nonviral vectors and enhance the immune efficacy induced by DNA vaccination (Pertl *et al.*, 2003). As another better known gene expression enhancement approach, the involvement of RNA-splicing donor and receptor elements in the gene sequences has been considered to enhance gene expression both *in vitro* and *in vivo* through improving the efficiencies of several key steps in gene expression process, including the cytoplasmic transport of the spliced transcripts (Chapman *et al.*, 1991; Norman *et al.*, 1997; Le Hir *et al.*, 2003).

However, some questions on the optimization of gene expression at the posttranscriptional level remain unanswered. First, the role of HPRE on gene expression and immunogenicity induced by DNA vaccine remains to be demonstrated. Second, the comparison of expression-enhancing effect of different PREs such as the WPRE, HPRE, and IntronA, especially in the context of a DNA vaccine, has not been reported. Third, whether PREs could substitute for CMV intronA or have a synergistic effect with it is unknown. Finally, previous studies have demonstrated that codon usage has become an important consideration in the design of

gene-based HIV-1 vaccines (Wang *et al.*, 2006). It is not clear, as compared with codon-optimized DNA vaccine, whether DNA vaccine carrying HIV gene with wild-type sequence could elicit comparable level of immune responses when the PRE and/or intron elements were introduced.

In the current study, we observed the enhancing effect of both the HPRE and WPRE, on antigen gene expression in transfected 293T cells, which is consistent with the results of some previous studies (Xu *et al.*, 2003; Guang and Mertz, 2005; Klein *et al.*, 2006; Mähönen *et al.*, 2007). These PRE elements were able to functionally substitute for the HIV-1 Rev responsible element and mediate CRM1-dependent nuclear export pathway of intronless transcripts (Roth and Dobbelstein, 1997, Popa *et al.*, 2002). However, the DNA vaccine constructs carrying HPRE showed significantly higher immunogenicity than those carrying WPRE. pInHGag Plasmid in general showed the strongest immunogenicity among all constructs. Immune responses induced by 10 μg pHGag were greater than those induced by 40 μg pGag (Figs. 4C and 5C). To our surprise, neither pWGag nor pInWGag plasmid elicited detectable level of anti-Gag antibody. Only weak cell-mediated immune responses could be detected in the pWGag group. This result is quite different from the previous reports (Garg *et al.*, 2004; Callendret *et al.*, 2007). The underlying mechanism that makes the difference remains to be revealed.

The hCMV intronA was demonstrated to be able to enhance expression of heterologous genes *in vitro*, especially when combined with the hCMV IE promoter/enhancer elements (Chapman *et al.*, 1991). Other studies indicated that the effects of introns on expression were attributed primarily to their ability to alter the nucleocytoplasmic distribution of mRNAs by RNA splicing and enhancing the rate of RNA polyadenylation (Huang and Gorman, 1990; Lu and Cullen, 2003; Nott *et al.*, 2003). In contrast, mRNAs of intronless genes could be efficiently processed if they contain *cis*-acting RNA elements such as the HPRE or WPRE. The current study showed that HPRE or WPRE had a greater effect on *Gag* gene expression *in vitro* than CMV intronA. Further, pInHGag carrying both the HPRE and IntronA elements induced the best immune responses among all groups, suggesting synergistic effect on gene expression.

It is known that the HIV type 1 (HIV-1) *Gag* and *Env* gene have inhibitory sequences that affect the accumulation and utilization of their mRNAs, but the inhibitory effect can be overcome by the RRE interaction (Hadzopoulou-Cladaras *et al.*, 1989; Hammarskjöld *et al.*, 1989; Schwartz *et al.*, 1992). Codon-usage optimization of these genes can lead to Rev-independent expression and has been proved a very effective DNA vaccine optimization strategy. In our present study, we have found that HPRE, which has a similar regulatory pathway to HIV-1 RRE, enhanced the *in vitro Gag* expression and immunogenicity of *Gag*-based DNA vaccine in Balb/C mice. We also observed that 10 μg of pInHGag that carries both the intronA and HPRE induced robust anti-Gag IgG antibody response that was comparable to that induced by the same amount of codon-optimized *Gag* DNA vaccine (data not shown).

In summary, the current study has provided valuable information on the vector optimization strategies employing 5′intronA and 3′HPRE elements that could obviously improve *HIV-1* gene expression and immunogenicity of a *Gag*-based

DNA vaccine. Our data also demonstrated that DNA vaccine carrying an HIV gene with native codon usage could achieve impressive *in vitro* expression and *in vivo* immunogenicity in a *Rev*-independent manner if the elements were appropriately optimized at the posttranscriptional level. Further, our data highlight the application of this alternative gene optimization strategy in the high-throughput screening experiments involving native an HIV genes or the production of lentiviral vectors where the codon-optimized gene is not available.

Disclosure Statement

This article was authored by Jing Sun, Dingfeng Li, Yanling Hao, Yuwei Zhang, Wenling Fan, Jingjing Fu, Yunzhang Hu, Yong Liu, and Yiming Shao.

All authors have directly participated in the planning, execution, or analysis of this work. All authors have read and approved this version of the article.

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