

Review

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DNA vaccines: improving expression of antigens

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Abstract

DNA vaccination is a relatively recent development in vaccine methodology. It is now possible to undertake a rational step-by-step approach to DNA vaccine design. Strategies may include the incorporation of immunostimulatory sequences in the backbone of the plasmid, co-expression of stimulatory molecules, utilisation of localisation/secretory signals, and utilisation of the appropriate delivery system, for example. However, another important consideration is the utilisation of methods designed to optimise transgene expression. In this review we discuss the importance of regulatory elements, kozak sequences and codon optimisation in transgene expression.

Review

In 1990, the direct gene transfer of plasmid DNA into mouse muscle *in vivo* without the need for a special delivery system was demonstrated [1]. Furthermore, intramuscular inoculation with plasmid DNA encoding reporter genes induced protein expression within the muscle cells. This study provided evidence for the idea that naked DNA could be delivered *in vivo* to direct protein expression. Subsequently, a further study reported the gene expression a year or more after intramuscular injection of plasmid DNA [2]. Since these initial studies, many more experiments have been carried out to evaluate different factors that determine the efficiency of gene transfer and immunogenicity of plasmid DNA. Furthermore, plasmid DNA has been used to immunise against a variety of diseases (known as DNA vaccination). Alternatively, plasmid DNA has been used to treat genetic diseases and similar factors may affect the efficacy of this gene therapy.

DNA vaccines usually consist of plasmid vectors (derived from bacteria) that contain heterologous genes (transgenes) inserted under the control of a eukaryotic pro-

motor, allowing protein expression in mammalian cells [3]. An important consideration when optimising the efficacy of DNA vaccines is the appropriate choice of plasmid vector. The basic requirements for the backbone of a plasmid DNA vector are a eukaryotic promoter, a cloning site, a polyadenylation sequence, a selectable marker and a bacterial origin of replication [4]. A strong promoter may be required for optimal expression in mammalian cells. For this, some promoters derived from viruses such as cytomegalovirus (CMV) or simian virus 40 (SV40) have been used. A cloning site downstream of the promoter should be provided for insertion of heterologous genes, and inclusion of a polyadenylation (polyA) sequence such as the bovine growth hormone (BGH) or SV40 polyadenylation sequence provides stabilisation of mRNA transcripts. The most commonly used selectable markers are bacterial antibiotic resistance genes, such as the ampicillin resistance gene. However, since the ampicillin resistance gene is precluded for use in humans, a kanamycin resistance gene is often used. Finally, the *Escherichia coli* ColE1 origin of replication, which is found in plasmids such as those in the pUC series, is most often used in DNA

vaccines because it provides high plasmid copy numbers in bacteria enabling high yields of plasmid DNA on purification. This review describes the utilisation of methods designed to optimise transgene expression.

Regulatory elements

Various reports have described the strength of promoter/enhancers or other transcriptional elements in DNA vaccines (see Table 1) [5–20]. In general, virally-derived promoters have provided greater gene expression *in vivo* than other eukaryotic promoters. In particular, the CMV immediate early enhancer-promoter (known as the CMV promoter) has often been shown to direct the highest level of transgene expression in eukaryotic tissues when compared with other promoters. For example, in one study a plasmid expressing human immunodeficiency virus type 1 (HIV-1) Gag/Env under the regulation of the CMV promoter/enhancer was compared to a comparable plasmid utilising the endogenous AKV murine leukemia long terminal repeat [17]. Analysis of the immune responses in macaques injected with the plasmids showed that the CMV-containing plasmid elicited higher Gag- and Env-specific humoral and T-cell proliferative responses, reflecting the greater transcriptional activity of the CMV promoter. Furthermore, it has been demonstrated that inclusion of the CMV intron A improved the level of expression of transgenes expressed by the CMV promoter or other promoter/enhancers [21]. It is thought that the beneficial effect of introns on expression is primarily due to an enhanced rate of polyadenylation and/or nuclear transport associated with RNA splicing [22]. However, some widely used virally-derived promoters, such as the CMV promoter, may not be suitable for some gene therapy applications since treatment with interferon- γ or tumour necrosis factor- α may inhibit transgene expression from DNA vaccines containing these promoters [23,24]. Thus, alternatives to the CMV promoter have been sought. For example, the desmin promoter/enhancer, which controls expression of the muscle-specific cytoskeletal protein desmin, was used effectively to drive expression of the hepatitis B surface antigen priming both humoral and cellular immunity against the antigen [11]. These responses were shown to be of a comparable magnitude to those in mice immunised with comparable DNA vaccines containing the CMV promoter. Other tissue-specific promoters that have been studied include the creatine kinase promoter, also specific to muscle cells [5,25], and the metallothionein and 1,24-vitaminD(3)(OH)(2) dehydroxylase promoters, both of which are specific to keratinocytes [26].

Since the rate of transcriptional initiation is generally increased by the use of strong promoter/enhancers, the rate of transcriptional termination may become rate-limiting [27]. In addition, the efficiency of primary RNA tran-

script processing and polyadenylation is known to vary between the polyadenylation sequences of different genes. Thus, the polyadenylation sequence used within a DNA vaccine may also have significant effects on transgene expression. For example, it was demonstrated that the commonly used SV40 polyadenylation sequence was less efficient than the minimal rabbit β -globin and bovine growth hormone polyadenylation sequences in mouse liver, although addition of a second SV40 enhancer downstream of the SV40 polyadenylation signal did increase expression to a level comparable to the other signals [10]. Therefore, it is possible that the strategy of inserting a second SV40 enhancer downstream of a SV40 polyadenylation sequence may be utilised in the construction of more efficient vectors.

Kozak sequences

Sequences flanking the AUG initiator codon within mRNA influence its recognition by eukaryotic ribosomes. As a result of studying the conditions required for optimal translational efficiency of expressed mammalian genes, the 'Kozak' consensus sequence has been shown to be important [28,29]. It has been proposed that this defined translational initiating sequence (-6 GCCA/GCCAUGG $+4$) should be included in vertebrate mRNAs located around the initiator codon [28]. It has also been suggested that efficient translation is obtained when the -3 position contains a purine base or, in the absence of a purine base, a guanine is positioned at +4 [29]. Prokaryotic genes and some eukaryotic genes do not possess Kozak sequences. Therefore, the expression level of these genes might be increased by the insertion of a Kozak sequence.

Codon usage

Codon bias is observed in all species, and the use of selective codons in genes often correlates with gene expression efficiency [30]. In general, taxonomically-close organisms, such as *E. coli* and *Salmonella enterica* serovar Typhimurium, for example, use similar codons for their protein synthesis whereas taxonomically-distant organisms, such as *E. coli* and *Saccharomyces cerevisiae*, utilise very different codons [31]. Mammalian codon usage is also different from that of microorganisms [32]. Nagata *et al.* [33] studied the effect of codon optimisation for mammalian cells of cytotoxic T-lymphocyte (CTL) epitopes derived from the intracellular bacterium, *Listeria monocytogenes*, and the parasite *Plasmodium yoelii*, and reported that the codon optimisation level of the genes correlated well with translational efficiency in mammalian cells.

The greatest deviation from random codon usage in an organism occurs in the most highly expressed genes as a result of selection for codons that maximise translational efficiency [34]. Minor tRNA species are avoided in highly expressed genes. Thus, differences between codon usage

Table 1: Comparison of promoters used in DNA expression studies *in vitro* and *in vivo*

Expressed antigen	Promoters/enhancers compared	<i>In vitro/in vivo</i> comparison	Reference
GFP	CMV, muscle-specific creatine kinase (CKM) promoter	Consistently higher levels of GFP expression were driven by the CKM promoter compared to CMV in mice.	[5]
LacZ	CMV, glial fibrillary acidic protein (GFAP) promoter, neuron-specific enolase (NSE) promoter	Injection of mice with the constructs containing the different promoters showed that GFAP is as efficient at driving lacZ expression as CMV.	[6]
CAT	HIV-1-LTR (long terminal repeat), RSV-TAR (transactivation response element)	HIV-1-LTR could be transactivated by tat in both stimulated and unstimulated cells; RSV-TAR was only transactivated in unstimulated cells.	[7]
CAT	CMV, RSV, SV40, murine leukemia virus (SL3-3) promoter	The CMV promoter was found to be stronger than any of the other promoters tested in muscle.	[8]
CAT	CMV, SV2	The CMV promoter was found to have greatest transcriptional activity.	[9]
Luciferase	CMV, RSV, SV40, PGK, hybrid β -actin promoter/CMV enhancer, CMV/IA (intron A)	The hybrid β -actin/CMV promoter/enhancer showed greater luciferase expression than RSV, SV40, PGK or CMV. CMV/IA also showed 2–6 fold <i>in vitro</i> and 1.5–3 fold <i>in vivo</i> higher luciferase expression than CMV.	[10]
Hepatitis B surface antigen (HBsAg)	CMV, desmin	The promoters performed equally well <i>in vitro</i> , and CTL and Th1 serum antibody responses against HbsAg in mice were of similar magnitude.	[11]
Hepatitis B envelope proteins	CMV, desmin	Greater <i>in vitro</i> expression of antigen was attributed to the desmin promoter. However, comparable humoral and cytotoxic immune responses were stimulated following i.m. injection of mice.	[12]
Rabies virus G protein	CMV, SV40	Comparable G antigen-specific antibody titres were stimulated in mice. Slightly higher T cell responses were observed from the CMV construct.	[13]
Influenza virus H5 hemagglutinin (HA)	CMV, β -actin	Constructs containing the CMV or β -actin promoters provided comparable protection against influenza in chickens.	[14]
Influenza virus H5 hemagglutinin (HA)	CMV, β -actin, RSV, SV40	Similar <i>in vitro</i> expression of HA. The greatest HA-specific antibody and protection against influenza in chickens was provided with the CMV construct.	[15]
Bovine herpesvirus glycoprotein D (gD)	RSV, CMV/IA	CMV/IA construct produced higher neutralising antibody titres against gD in i.d. injected cattle.	[16]
HIV-1 gag/env	CMV, AKV murine leukemia viral long terminal repeat	CMV showed 10–20 fold greater activity than AKV <i>in vitro</i> . Immunised macaques developed high humoral responses with the CMV construct only.	[17]
SV40 large tumour antigen	CMV, SV40	The CMV construct induced higher levels of antibody and protection in the murine experimental metastasis model than the SV40 construct.	[18]
<i>M. tuberculosis</i> apa + pro proteins	CMV, UbC	The CMV promoter was the most efficient tested.	[19]
Adenovirus E4 ORF3	CMV, RSV, SV40, UbC, EF-1 α	Following i.n. dosing to mice, constructs containing the UbC and EF-1 α promoters stimulated the most stable expression of antigen	[20]

in a heterologous gene and the host organism may affect expression. To improve expression of human immunodeficiency virus type 1 gp120 from a DNA vaccine vector, André *et al.* generated a synthetic gp120 sequence in which most of the wild-type codons were replaced with codons from highly expressed human genes. The resulting construct showed increased *in vitro* expression of gp120 compared to the wild-type sequence. In addition, significantly increased antibody titres and CTL reactivity were observed following administration of the vector containing the synthetic sequence. Similarly, a DNA vaccine vector encoding a synthetic epitope of listeriolysin O with mammalian codon usage showed higher translation efficiency than a vector containing the wild-type sequence in murine cells [36]. Furthermore, the first DNA vaccine was capable of inducing specific CD8⁺ T cells able to confer partial protection against challenge with *L. monocytogenes* where the second DNA vaccine could not. A number of other studies have reported that increased immune responses may be obtained by DNA vaccination with a transgene sequence with optimised codon usage. [36–40].

Conclusions

In this review the methodologies by which antigen expression has been optimised to date, i.e. optimisation of vector and transgene sequences, have been discussed. It is clear that transgene expression may be increased through the use of optimised promoters and polyA sequences. However, in some circumstances it may be necessary to optimise DNA vaccines to produce reduced transgene expression. For example, the weaker SV40 promoter has been used rather than the CMV promoter to drive expression of antigens that induce cell death upon overexpression [13]. Tissue-specificity is also considered important. Such tissue-specific expression systems may be able to produce stable expression by reducing the probability of inducing an immune response to the transgene. It may be possible to design vectors for gene therapeutic purposes that avoid inducing unwanted immune responses against the encoded antigen by using tissue-specific promoters [41]. Restricting the site of expression of genes should minimise the risks related to aberrant expression of a gene product. Furthermore, it should be possible to develop

expression systems where gene products are only expressed in the critical cell types for DNA vaccination or gene therapy, for example, dendritic cells (DCs). As a better understanding of the proteins whose expression is limited to DCs is obtained, novel expression systems will be generated. Finally, through increased knowledge of the regulation of expression of antigens, it is now possible to produce multivalent systems whereby multiple antigens may be expressed from a single DNA vaccine vector [42].

It is clear that the optimisation of antigen expression is an important consideration in DNA vaccine vector design. However, it is important to recognise that other aspects of vector design may influence the efficacy of the vaccine/gene therapy. A rational approach to improve the efficacy of DNA vaccination or gene therapy would optimise the: (i) vector backbone DNA sequence; (ii) transgene sequence; (iii) co-expression of stimulatory sequences; (iv) delivery system used for the vector; (v) targeting of the vector for appropriate immune stimulation.

The backbone of a DNA vaccine vector could be further modified to enhance immunogenicity via the manipulation of the DNA to include certain sequences, so that the DNA itself will have an adjuvantising effect. DNA vaccine vectors contain many CpG motifs (consisting of unmethylated CpG dinucleotides flanked by two 5' purines and two 3' pyrimidines) that, overall, induce a Th1-like pattern of cytokine production [43], and are thought to account for strong CTL responses frequently seen following DNA vaccination [44]. It is possible to augment responses to DNA vaccine vectors by incorporating CpG motifs into the DNA backbone of the plasmid [45]. Alternatively, immune responses may be modulated or enhanced by the co-expression of stimulatory molecules or cytokines [46,4] or through the use of localisation or secretory signals [47–49], or ligand fusions [50–54] to direct antigens to sites appropriate for immune modulation. Finally, a variety of routes of administration of DNA vaccines have been studied, including intramuscular, intradermal, subcutaneous, intravenous, intraperitoneal, oral, vaginal, intranasal and, more recently, non-invasive delivery to the skin (reviewed by Gurunathan *et al.* [4]).

The approaches outlined above will together allow for the rational and optimised design for DNA vaccines and gene therapy vectors. The ability to improve antigen expression through the use of optimisation of regulatory elements, kozak sequences and codon usage is highlighted in this review, as part of this rational approach.

Competing interests

None declared.

Authors' contributions

HSG, KAB and RWT produced the manuscript together. All authors read and approved the final manuscript.

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