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Regulation of transgene expression in genetic immunization

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Abstract

The use of mammalian gene expression vectors has become increasingly important for genetic immunization and gene therapy as well as basic research. Essential for the success of these vectors in genetic immunization is the proper choice of a promoter linked to the antigen of interest. Many genetic immunization vectors use promoter elements from pathogenic viruses including SV40 and CMV. Lymphokines produced by the immune response to proteins expressed by these vectors could inhibit further transcription initiation by viral promoters. Our objective was to determine the effect of IFN-γ on transgene expression driven by viral SV40 or CMV promoter/enhancer and the mammalian promoter/enhancer for the major histocompatibility complex class I (MHC I) gene. We transfected the luciferase gene driven by these three promoters into 14 cell lines of many tissues and several species. Luciferase assays of transfected cells untreated or treated with IFN-γ indicated that although the viral promoters could drive luciferase production in all cell lines tested to higher or lower levels than the MHC I promoter, treatment with IFN-γ inhibited transgene expression in most of the cell lines and amplification of the MHC I promoter-driven transgene expression in all cell lines. These data indicate that the SV40 and CMV promoter/enhancers may not be a suitable choice for gene delivery especially for genetic immunization or cancer cytokine gene therapy. The MHC I promoter/enhancer, on the other hand, may be an ideal transgene promoter for applications involving the immune system.

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- Gene therapy
- Regulation
- Transcription
- Transgene

Introduction

During the past few years, the use of mammalian gene expression vectors for new methods such as genetic immunization has become increasingly important. Recent success with DNA vaccines indicates their enormous future potential in diverse fields such as infectious diseases, allergy and cancer (1-6). Depending on the antigen processing pathway, proteins produced through mammalian expression vectors can stimulate humoral or cellular immunity. Intracellular expression of a non-secreted antigen should specifically induce cellular immunity through presentation by the major histocompatibility complex class I (MHC I) molecules to cytotoxic T cells. During the immune response, many lymphokines are produced by the various cells involved, including IFN-γ, that can act

on the target cell carrying the transgene. Modulation of expression of the transfected gene could occur and, depending on the promoter used, increase or decrease transgene expression. Therefore, the proper choice of promoter/enhancer linked to the gene of interest is critical to the success of DNA vaccinations.

We used the mammalian MHC I promoter/enhancer, BL3-6prmtr (7), and compared transgene expression and effects of IFN-γ regulation to the popular viral promoter/enhancers of SV40 and CMV in various cell lines of several species. To date, almost all commercial mammalian expression vectors use viral promoter/enhancer sequences from pathogenic viruses such as simian virus 40 (SV40), or human cytomegalovirus (CMV). Although these promoter elements are from pathogenic viruses, they have become very useful due to high transcription initiation ability in most mammalian tissues (8). However, in genetic immunization applications involving human patients or animal husbandry, a mammalian promoter may be more desirable for easing public concern over transfecting DNA elements from pathogenic or tumor-causing viruses. BL3-6prmtr is the promoter for the cattle major histocompatibility complex class I gene. MHC I is expressed on nearly all tissues, is critical in immune system communication, and can be up-regulated by lymphokines such as IFN-γ (9).

The SV40, CMV, and MHC I promoter/enhancers have complex *cis* elements that bind varied cellular *trans*-factors (10-12). SV40 and MHC I promoters both have AP2 binding sites while CMV and MHC I promoters both have ATF binding sites. In common to all three promoters are AP1 and NFkB binding sites. Additionally, all three promoters have interferon response sequences. However, for CMV and SV40, factors binding to this region can inhibit transcription (13,14), whereas, for MHC I, interferon response factors enhance transcription (15,16).

Table 1 - Cell lines used for transfections.

All cell lines were provided by the American Type Culture Collection (ATCC).

Species	Cell line	Tissue
Mouse	A20.J	B lymphoma
	C_2C_{12}	Myoblast
	G8	Myoblast
	P815	Mastocytoma
	RAW 264.7	Macrophage
Human	COLO 320HSR	Colon carcinoma
	LCL 721.221	B lymphoblast
	SW 837	Rectal carcinoma
Cow	BL3.1	B sarcoma
	MDBK	Kidney
Dog	D17	Osteocarcinoma
Monkey	Vero	Kidney
Rat	NMU	Mammary carcinoma
Sheep	FLK	Kidney

Comparison of luciferase expression driven by MHC I, SV40, and CMV promoter/enhancers

The luciferase gene driven by BL3-6prmtr (cattle MHC I), SV40, or CMV promoter/enhancer was used as a reporter gene transiently transfected in cell lines of various tissues from several species (listed in Table 1). Assays of luciferase production were used to compare the relative strengths of the MHC I promoter/enhancer to the viral promoter/enhancers transgene expression abilities in each cell line. Each of the three promoters was able to drive luciferase expression in all cell lines tested. However, BL3-6prmtr was, in many cases, weaker than either SV40 or CMV promoters. The data, summarized in Table 2, show that SV40 was stronger than BL3-6prmtr in 12/14 cell lines and CMV was stronger in 6/14 cell lines. Comparisons between SV40 and CMV promoter/enhancers could not be made because the vector constructs were not similar.

Effect of IFN-γ addition to transient transfected cell culture on transgene promoter

Luciferase production was positively or negatively altered by IFN-γ treatment depending on the promoter and cell line. Transiently transfected cell cultures were treated or not treated with rIFN-γ for 16 h and then assayed for luciferase production. Results demonstrate that BL3-6prmtr-driven luciferase production increased in all cell lines, whereas the SV40 or CMV promoter-driven luciferase production decreased in most cell lines (Table 2).

Using the mammalian expression vectors p6/Luc (MHC I promoter driven) and pcDNA3/Luc (CMV promoter driven), we established stable transfects of several cell lines to ascertain whether IFN-γ would affect chromosome integrated promoter/transgene expression differently than episomal transgene expression. We isolated neomycin-resistant clones of A20.J, P815, RAW 264.7, COLO 320HSR, SW 837, and BL3.1 cell lines and selected the highest luciferase-expressing clones of each transgene construct. IFN-γ treatment of these stable transfects produced similar results (Table 2) using the same IFN-γ dosage (500 U/ml) and measurement time point (16 h; data not shown).

Many gene delivery vaccination applications use muscle as the target tissue (17-19). Our results indicate that while SV40 and CMV promoters drive luciferase expression in myoblasts to higher levels than BL3-6prmtr, addition of IFN-γ drastically reduces SV40- and CMV promoter-driven expression while increasing BL3-6prmtr-driven expression. Figure 1 gives a graphic illustration of this change.

Kinetics of IFN-γ-treated stable transfect luciferase production

IFN-γ dosage and kinetics of luciferase

Table 2 - Effect of IFN-γ on luciferase transgene production driven by MHC I, SV40, or CMV promoters.

Note: Increased expression after IFN-γ treatment is in bold numbering. Decreased expression after IFN-γ treatment is in italics numbering. [a]Three transfections per cell line of each luciferase gene construct (in parentheses) driven by MHC I, SV40, or CMV promoters were done for each experiment. Transfections were performed using the cationic lipid method (LipofectAMINE; Life Technologies, Inc., Gaithersbourg, MD). At least three experiments were done for each cell line. Transfection efficiency was measured using internal controls for either ß-galactosidase (pGL6 and pGL2 Control) or CAT (p6/Luc and pcDNA3/Luc). [b]IFN-γ was added (+) or not added (-) to transient transfects which were assayed for luciferase production 16 h later. Human rIFN-γ was added to human, cow, dog, monkey, and sheep cell lines while mouse rIFN-γ was added to mouse and rat cell lines (see Table 1). Numbers represent mean picogram (pg) luciferase per transfection calculated using a luciferase standard curve. The coefficient of variation was less than 10% for each experiment.

Cell line	MHC I				SV40		CMV	
	(pGL6)[a]		(p6/Luc)		(pGL2 Control)		(pcDNA3/Luc)	
	(-)[b]	(+)	(-)	(+)	(-)	(+)	(-)	(+)
A20.J	30	**41**	50	**81**	826	*409*	126	*16*
BL3.1	40	**54**	7	**10**	8	*5*	4	*2*
C_2C_{12}	19	**52**	20	**61**	403	*83*	182	*87*
COLO	56	**107**	30	**53**	28	*11*	11	*2*
D17	38	**40**	14	**16**	88	*68*	30	*22*
FLK	36	**251**	99	**234**	215	**354**	59	**89**
G8	28	**96**	9	**17**	810	*209*	53	*23*
721.221	34	**39**	19	**20**	43	*18*	18	*17*
MDBK	8	**9**	8	**10**	10	*9*	13	*8*
NMU	24	**63**	91	**176**	250	*75*	154	*34*
P815	28	**30**	4	**5**	57	*34*	4	*2*
RAW	20	**74**	12	**25**	50	**176**	9	**11**
SW837	8	**9**	9	**10**	16	*12*	8	*7*
Vero	34	**62**	14	**35**	208	*150*	10	*7*

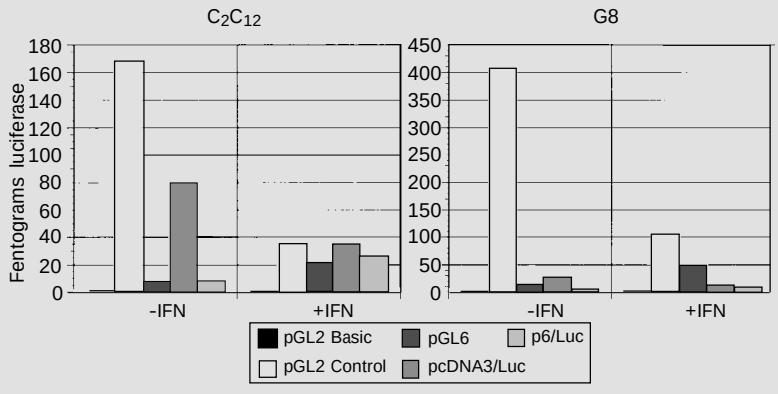

Figure 1 - BL3-6prmtr, SV40, and CMV promoter-driven luciferase production in transfected myoblasts. The mouse myoblast cell lines, C_2C_{12} and G8, were transfected with luciferase gene constructs driven by no promoter (pGL2 Basic), the SV40 promoter/enhancer (pGL2 Control), the CMV promoter/enhancer (pcDNA3/Luc), or the cattle MHC class I promoter/enhancer (pGL6; p6/Luc). Transfectant cell cultures were either untreated (-IFN) or treated (+IFN) with IFN-γ for 16 h and then assayed for luciferase production. Three experiments of three transfections of each luciferase construct were performed. Measurements represent the mean relative light units (RLU) with coefficient of variation of less than 10%.

expression driven by CMV or MHC I promoters in these stable transfects were measured. These data showed an optimal IFN-γ dosage of 100-500 U/ml for both CMV and MHC I promoters for highest luciferase production at all time points (data not shown). The kinetics of luciferase transgene production showed that the amount of CMV-driven luciferase product peaked at approximately 8 h and then dropped to below baseline (untreated) levels at 16-24 h, while the MHC I-driven luciferase product continued to rise through 48 h. Figure 2 shows these results using stably transfected human colon carcinoma (COLO 320HSR) and mouse macrophage (RAW 264.7) cell line clones.

Effect of mixed lymphokine addition to transfected cell culture on transgene expression

An *in vivo* immune response that was either initiated or promoted by the transgene would release a multitude of lymphokines, along with IFN-γ, that may have additive, synergistic, or antagonistic effects on the transgene promoter. For example, tumor necrosis factor-alpha (TNF-α), another lymphokine produced during an immune response, has been shown to inhibit CMV transcription but acts synergistically with IFN-γ in enhancing MHC I transcription (20,21). Using the supernatant from mitogen-stimulated T cells as a source of immune response lymphokines, we tested transgene expression in response to these mixed lymphokines as well as IFN-γ in a transiently transfected human colorectal carcinoma cell line. The results, shown in Figure 3, indicate that the T cell blast supernatant increased transgene expression driven by MHC I promoter to an even greater extent than IFN-γ alone. Viral promoter-driven expression was up-regulated by the mixed lymphokines compared to IFN-γ-treated cells but not to the level of untreated cells.

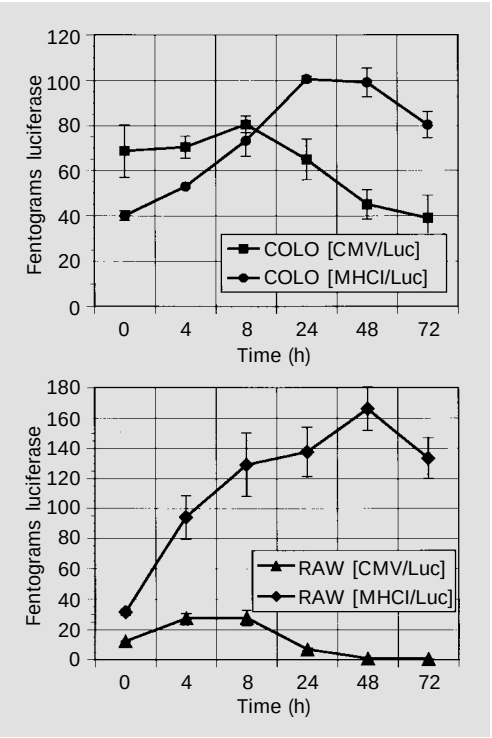

Figure 2 - Kinetics of luciferase transgene expression in stably transfected cell lines. Human colon carcinoma (COLO 320HSR) or mouse macrophage (RAW 264.7) cell clones with a chromosome-integrated luciferase transgene driven by either the CMV promoter (CMV/Luc) or the MHC I promoter (MHC I/Luc) were cultured in 24-well plates and treated with IFN-γ (500 U/ml) for 0, 4, 8, 24, 48, and 72 h. Cells were harvested in 100 µl lysis buffer at these time points and lysate was stored at -70ºC until assayed for luciferase. Graphs show the mean relative light units (RLU) and standard deviations of four experiments.

Discussion

Foremost in DNA vaccine delivery vector design should be the proper choice of promoter/enhancer to drive expression of the transferred gene. This choice is dependent upon target cell type as well as the functional goal of the transgene. DNA vaccine applications are designed to affect immune function to protect against a pathogenic agent, or lymphokine gene transfer to fight cancer. These applications stimulate the immune system resulting in production of many lymphokines that can directly act on the transfected target cell and thus indirectly act on the transgene promoter. At least one of these lymphokines, IFN-γ, is a potent antiviral agent, and has been shown to inhibit viral transcription of SV40 and CMV (13,14), although many promoters for transgene expression are from SV40 or CMV (2,17-19,22-25). Our objective was to determine what effect IFN-γ had on transgene expression driven by SV40, CMV, or MHC I promoter/enhancers using cell lines of various tissues

to ascertain the best choice for gene delivery vector promoter in immunoregulatory applications.

Our results show a down-regulation effect of IFN-γ on both SV40 and CMV promoter/enhancer-driven transgene in most cell lines tested and an up-regulation effect of IFN-γ on the MHC I promoter/enhancer-driven transgene in all cell lines tested. We note, however, that even with IFN-γ down-regulation, viral promoter-driven expression was frequently greater than that of IFN-γ up-regulated MHC I promoter-driven expression. Nonetheless, these negative selective pressures may shorten the expressible life of the transgene. For example, we noticed in our stably transfected cell clones that luciferase expression driven by BL3-6prmtr stayed at the same level for many cell passages whereas luciferase expression driven by CMV promoter showed a "dampening" of expression soon after cloning. This theory has yet to be tested.

In fact, studies using the stable transfects showed an ever increasing amount of luciferase from MHC I promoter-driven transgene over 48-72 h while amounts of luciferase from transfect cultures of CMV promoter-driven transgene were near or below baseline levels at this time. These experiments showed an initial increase in CMV promoter-driven production peaking at around 8 h and then dropping to below baseline levels. Also, after 48 h, MHC I promoter-driven transgene product levels began to drop. We believe that these results were due to the cells being plated at 70% confluence at the beginning of the experiment, and to the fact that the medium was not changed through the 72-h period. Therefore, the slight increase in CMV promoter-driven luciferase production may reflect increased cell numbers to confluence rather than an IFN-γ-induced enhancement. The IFN-γ inhibitory effect on the CMV promoter and enhancing effect on the MHC I promoter probably occurred within 4 h. The reason MHC I promoter-driven luciferase production began dropping by 48-72 h may have been due to lack of fresh medium (growth factors) in the culture and perhaps the beginning of cell death. Since luciferase is a fairly stable protein, these data indicate that as long as IFN-γ is present, the MHC I promoter

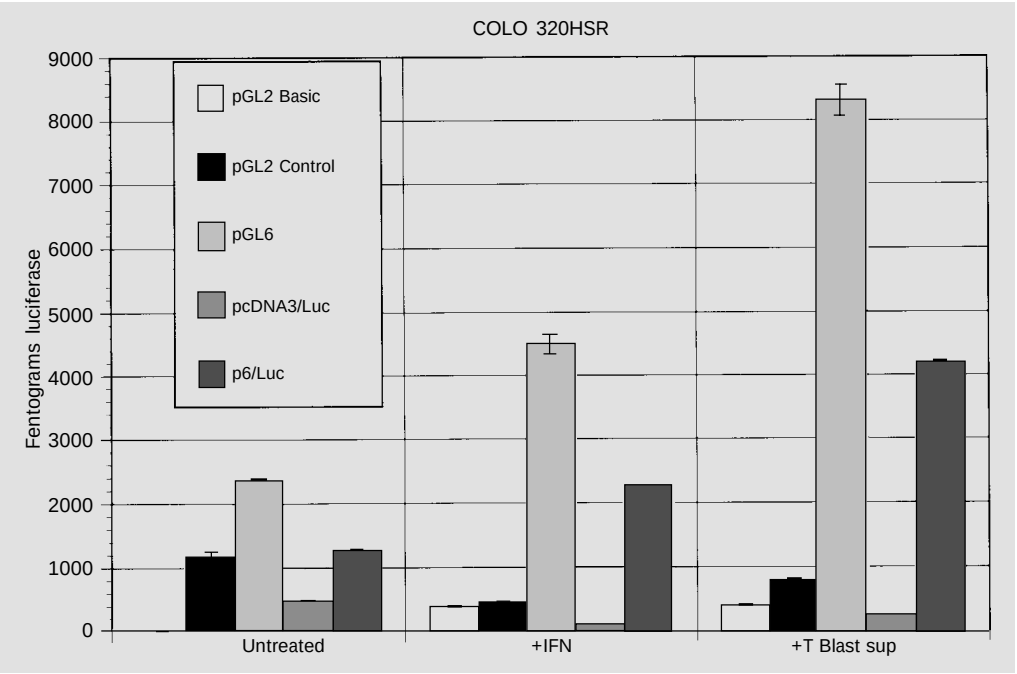

Figure 3 - Effect of IFN-γ and T cell blast supernatant (sup) on BL3-6prmtr, SV40, and CMV promoter-driven luciferase production in transfected human colorectal carcinoma cells. The human colorectal carcinoma cell line COLO 320HSR was transfected with luciferase gene constructs driven by no promoter (pGL2 Basic), the SV40 promoter/enhancer (pGL2 Control), the HCMV promoter/enhancer (pcDNA3/Luc), or the cattle MHC class I promoter/enhancer (pGL6; p6/Luc). Transfected cell cultures were untreated or treated with either IFN-γ (+IFN) or 10% supernatant from 3d mitogen-stimulated T cells (+T Blast sup) and then assayed for luciferase production. Three experiments of three transfections of each luciferase construct were performed. Measurements represent the mean relative light units (RLU) with a coefficient of variation of less than 10%.

is up-regulated and the CMV promoter is down-regulated.

Although the type of cell or tissue is important in determining whether IFN-γ as well as other immune response lymphokines will enhance or inhibit transgene expression driven by the viral promoters, our results indicate that most probably expression will be down-regulated. The MHC I gene, on the other hand, is a critical immune system communication molecule and has been shown to have enhanced transcription due to lymphokines produced during an immune response (9). The use of T cell blast supernatant-treated transfected cell lines confirmed the enhanced expression of BL3-6prmtr-driven transgenes, similar to the results shown in Figure 3, for all cell lines tested, including COLO 320HSR, LCL 721.221, BL3.1, and FLK (data not shown).

The MHC I promoter/enhancer was used to drive luciferase in two different promoters and, as seen in Table 2, caused different luciferase expressions. Vectors pGL2 Control (SV40 driven) and pGL6 (MHC I driven) were standard reporter gene vectors typical for use in transient transfection assays. Vectors pcDNA3/Luc (CMV driven) and p6/Luc (MHC I driven) were engineered from a mammalian expression vector containing a separate, selectable *neomycin* gene independently driven by the SV40 promoter/enhancer. This vector is typical for use in stable transfections. Even though the same MHC I promoter/enhancer drove expression of the same *luciferase* gene, the lower expression results of p6/Luc compared to pGL6 could be due to transcription factor competition by the SV40 promoter or some inhibitory effect by the *neomycin* gene product, as others have shown (26,27). The fact that many selectable marker genes in mammalian gene expression vectors are under viral promoter control should be another consideration in choosing the best gene delivery vector for immune response applications. Whether the transgene was integrated in the genome or episomal did not alter the general effect of IFN-γ on a particular cell line according to our stable or transient transfection results using pcDNA3/Luc and p6/Luc.

We still need to elucidate whether these data represent effects of an actual immune response to the transgene typical in DNA vaccination. Intramuscular DNA vaccination of mice with rabies virus glycoprotein gene driven by SV40 or MHC I promoter developed a comparable antibody response (28). However, co-inoculation of mouse IFN-γ plasmid and either of the SV40- or MHC I-driven rabies vaccine vectors resulted in a reduced antibody and T helper cell proliferation response in these mice. Cattle vaccinated with bovine herpesvirus-1 glycoprotein D (gD) gene driven by CMV or MHC I promoter delivered by the biolistic process resulted in a greater gD-specific antibody response to the CMV/gD vaccine compared to the MHC I/gD vaccine (data not shown). In both of these examples the measurement of the DNA vaccine response was based on antibody titer or T helper cell proliferation – immune responses that depend upon MHC II antigen presentation. Therefore, the protein produced by the transgene had to be secreted by the transfected cell, taken up and processed by antigen presenting cells, and presented in the context of MHC II to B and T helper cells. To truly assess the efficacy of the MHC I versus the viral DNA vaccine promoters *in vivo*, studies must be done on the long-term or memory response. Our data suggest that, although MHC I is a weaker promoter than the viral promoters, immune response cytokines could eventually shut down the viral promoter while augmenting the MHC I promoter. Thus there may be long term protection with DNA vaccines driven by the MHC I promoter. Additionally, studies must be done on cytotoxic T cell responses of MHC I versus viral promoter-driven vaccines. Because the cytotoxic T cell response involves antigen presentation by MHC I (most likely in the transfected

cell), the lower but longer term expression of MHC I promoter may induce a superior cytotoxic T cell response compared to viral promoter-driven vaccines.

We believe the MHC I promoter/enhancer, BL3-6prmtr, would be a better choice for gene delivery vector promoter in immunoregulatory applications than the widely used viral promoters of SV40 and CMV. This belief is based on the generally inhibitory effect of IFN-γ, as well as other lymphokines, on SV40 and CMV promoter-driven transgene expression, and the amplified effect of IFN-γ and other lymphokines on MHC I-driven transgene expression. Many researchers using viral promoters to express transgenes have been frustrated by the short-lived expression of these transgenes. Our results offer an explanation for these observations. Admittedly, the viral promoters of SV40 and CMV are generally stronger than the MHC I promoter, BL3-6prmtr. Nevertheless, our data suggest that for long-term sustained transgene expression necessary for an optimal immune response, the mammalian MHC I promoter/enhancer could be a preferable option.

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