# Improved expression by cytomegalovirus promoter/enhancer and behavior of vascular endothelial growth factor gene after myocardial injection of naked DNA

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## Abstract

**Direct injection of the vascular endothelial growth factor (VEGF) gene plasmid DNA into the myocardium was shown to induce development of new blood vessels to increase the circulation in the heart of patients with coronary artery diseases. However, such angiogenic gene therapy (via naked DNA) was limited by low level of gene expression. Furthermore, the temporal and spatial characteristics of VEGF gene transfer in the heart are not known. In this study, we demonstrated that a plasmid vector, containing the human cytomegalovirus immediate early (HCMV IE) promoter and enhancer, induces greater expression of gene in the rat heart monitored by gene fused to the chloramphenicol acetyl transferase (CAT) reporter, than four different viral and cellular promoters. Interestingly, expression of VEGF$_{121}$ protein showed an earlier peak, a shorter duration, and a wider distribution than that of CAT only. Therefore, a plasmid vector with an HCMV IE promoter/enhancer provides clear advantages over other previously developed plasmids. Furthermore,** expression profile of VEGF$_{121}$ gene may provide useful information in the design of angiogenic gene therapy in the heart.

**Keywords:** naked DNA, gene therapy, VEGF, heart

## Introduction

Angiogenic gene therapy to promote the development of supplemental collateral blood vessels in chronic myocardial ischemia has been exploited using direct myocardial administration of the vascular endothelial growth factor (VEGF) gene, in the form of naked DNA (Rosengart et al., 1999). Preliminary results using this strategy suggest that it may constitute an alternative treatment for patients with severe myocardial ischemia (Losordo et al., 1998; Symes et al., 1999) and heart failure (Carmeliet et al., 1999; Isner et al., 1999).

The success of gene therapy depends largely on the efficacy of gene delivery vectors. Although naked DNA is probably the safest and the most convenient form of gene delivery vector, its application has been limited by the attendant low level of gene expression (Verma et al., 1997). A potent eukaryotic expression vector will not only increase the efficacy and success of angiogenic gene therapy, but will also decrease the cost of therapeutic vectors in clinical application. Strategies to improve the efficiency of non-viral vectors include the development of enhanced expression plasmids. Enhancement of gene expression from plasmids might be achieved by altering the regulation of gene expression from the plasmid (Lee et al., 2000). Accordingly, we sought to investigate the potency, following direct injection into the myocardium, of vectors with different viral and cellular promoters.

Despite significant use of the VEGF gene injected as naked DNA into the patients heart (Losordo et al., 1998; Symes et al., 1999), the kinetics and distribution of VEGF protein after plasmid injection in the heart have not yet been defined. In previous reports, several laboratories have demonstrated the expression of naked DNA injected into the animal heart (Lin et al., 1990; Kitsis et al., 1991; Buttrick et al., 1992; von Harsdorf et al., 1993; Li et al., 1997). In these experiments, use was made of reporter genes, such as chloramphenicol acetyl transferase (CAT), $\beta$-galactosidase, or luciferase, which encode intracellular proteins with long half-lives. Following direct injection of naked DNA into animal hearts, these studies reported cases of long-term

expression, restricted to the area surrounding the needle injection site. However, these results do not permit any conclusions about the behavior of the VEGF gene, which is known to have a half-life of only a few minutes, and which encodes a signal sequence that facilitates secretion of the protein from intact cells (Ferrara *et al.*, 1992). Indeed, these facts suggest that VEGF might differ substantially in both its kinetics and its tissue distribution from proteins encoded by reporter genes.

Moreover, the VEGF transcript is normally spliced into at least five different forms (Neufeld *et al.*, 1999). Different isoforms of VEGF protein show different binding affinities for heparan sulfate, a component of the extracellular matrix. Of these, the isoform $VEGF_{121}$ binds least well to heparin, and diffuses into the systemic circulation (Park *et al.*, 1993). Therefore, investigation is required into the specific behavior, in terms of kinetics and spatial distribution, that characterizes $VEGF_{121}$ in the heart after gene delivery.

The first aim of the present study was to examine the relative potency of eukaryotic expression vectors, with different promoters, in the myocardium after direct injection into the heart. The second aim was to investigate the behavior of $VEGF_{121}$ after direct naked DNA injection into the rat heart, and to compare this with the behavior of the CAT reporter gene product.

## Materials and Methods

### Structure of expression vectors

For the comparison of promoter potency, five plasmids were used in this study (Figure 1A). Plasmid pcDNA3.1 was purchased from Invitrogen and contains 587 base pairs (bp) of HCMV IE promoter. Plasmid pRSV contains the enhancer and promoter region of the 3' long terminal repeat (LTR) of the Rous sarcoma virus (RSV). We have constructed pCN, pEF1-$\alpha$, and pAct. For plasmids pCN, pEF1-$\alpha$, and pAct, the promoter region of pcDNA3.1 was replaced with each promoter, thereby making the backbone of each identical to pcDNA3.1. The construction and structures of pCN, pEF1-$\alpha$, and pAct have been described elsewhere (Lee *et al.*, 2000). Briefly, pCN contains the 600 bp HCMV IE promoter and its entire 5' untranslated region, consisting of 122 bp of exon 1, 827 bp of intron 1, and 16 bp of exon 2. Plasmid pEF contains 137 bp of the human elongation factor-1$\alpha$ (EF-1$\alpha$) promoter and its entire 5' untranslated region, consisting of 20 bp of exon 1, 945 bp of intron 1, and 31 bp of exon 2. Plasmid pAct contains 243 bp of human $\beta$-actin promoter and its entire 5' untranslated region, consisting of 76 bp of exon 1, 696 bp of intron 1, and 87 bp of exon 2. In both CAT and human $VEGF_{121,}$ the ATG codon was positioned at the site of the original start codons of the respective genes. The cDNA encoding human $VEGF_{121}$ was cloned from total RNA prepared from human placenta by RT-PCR. The primers used were 5'-ATGAACTTTCT-GCTGTCT-3' (sense) and 5'-TCACCGCCTCGGCTTGT CACATTTTTC-3' (antisense). The amplified cDNA was subcloned into the pCR2.1 vector (Invitrogen), and was confirmed by sequencing. Bioactivity of human $VEGF_{121}$ was confirmed by the Mile's permeability assay (Darren *et al.*, 2001).

### DNA preparation

Plasmids were prepared using the column method (Plasmid MegaKit; Qiagen, CA). All plasmid preparations contained a high percentage of supercoiled DNA (70-80%), and no RNA was detectable by gel electrophoresis. For injection, DNA was dissolved in 50 $\mu$l of normal saline.

### Animal experiments

All animal experiments were carried out in conform with the Guideline for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Male Sprague-Dawley rats weighing 300 grams were used for naked DNA injection. The rats were anesthetized with intra-peritoneal ketamine-xylazine (50 mg/kg and 2 mg/kg, respectively), intubated, and mechanically ventilated with room air. Under aseptic conditions, a thoracotomy was performed through the left fifth intercostal space, and the heart was exposed. Cardiac injections were made directly into the apex of the left ventricle. Fifty microliters of a DNA solution containing each plasmid were injected into the apical portion of the beating left ventricle, under direct visualization, using a 29-gauge needle. The DNA injection caused immediate subepi-cardial edema, with the tissue changing color from red to pink. The chest was then closed, and the animals were allowed to recover for specific periods before sacrifice. An antibiotic (0.1 g cefazolin) was injected intramu-scularly on the day of cardiac injection.

### Measurement of CAT and VEGF

Rats were killed at the indicated times following cardiac injection. The atria and great vessels were trimmed, and the hearts were washed in ice-cold phosphate-buffered saline. Except when indicated, the apical one third of the heart was homogenized in 2 ml ice-cold homogenization buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% Na-deoxycholate, 2% NP-40, 0.2% sodium dodecyl sulfate, 1 mM PMSF). Homogenates were then centrifuged at 6,000 $g$ for 10 min at 4°C. Supernatants were collected to measure the levels of CAT and human VEGF, using commercially available ELISA kits (CAT, Boehringer Mannheim, Switzerland; VEGF, R&D Systems, MN). Compared to the classical $^{14}$C-based CAT assay, the
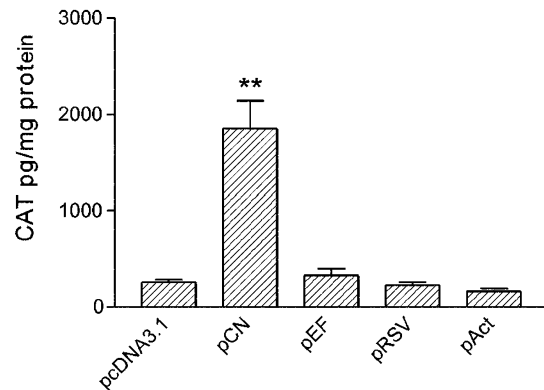
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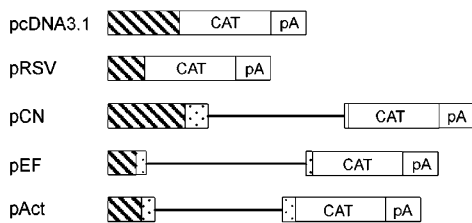


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**Figure 1.** (A) Structure of expression vectors. Hatched box, promoter; dotted box, exon; line, intron; pA, polyA tail. (B) Comparison of the levels of gene expression of the five promoters coupled to the chloramphenicol acetyl transferase (CAT) gene. Thirty micrograms of DNA were injected into rat hearts *in vivo*. Three days after injection, rats were sacrificed, and their hearts assayed for CAT expression by ELISA. **$P < 0.01$ *vs.* other promoters. Values are given as mean ± SEM (n varies from 8 to 15 for each promoter).

CAT ELISA provides more accurate measurements for the actual amount of CAT protein synthesized, rather than merely measuring the CAT enzyme activity, which can decrease during long-term storage. The CAT ELISA also allows direct comparison of data from different sets of experiments, and is equal in sensitivity to the radioactive CAT assay. The VEGF ELISA kit is specific to human VEGF. Antibody used in the kit has no cross reactivity against murine VEGF. Protein was quantified using a calorimetric assay (Bio-Rad, CA). Results are expressed as picograms of CAT or VEGF per milligram protein ± standard error of the mean (SEM).

### Statistics

Results are expressed as the mean ± standard error of the mean (SEM). The means of groups were compared by an unpaired two-tailed Student's *t-test*. Statistical significance was assumed if the null hypothesis could be rejected at the 0.05 level.

## Results

### Comparison of *in vivo* gene expression in the rat heart using different promoters

Initially, we determined the relative strength of five promoters linked to the CAT reporter gene. In these experiments, 30 μg each of pcDNA3.1-CAT, pCN-CAT, pEF-CAT, pRSV-CAT, or pAct-CAT was injected into the apex of the rat heart. The rats were killed three days after injection, and levels of CAT gene expression were analyzed by ELISA. The data, which are shown in Figure 1B, demonstrate that pCN was about sevenfold more greater than those expressed with other promoters ($P < 0.05$). The ranking of potency of gene expression

was pCN >> pEF > pcDNA3.1 > pRSV > pAct. However, apart from pCN, there was no significant difference in gene expression between viral (pRSV, pcDNA3.1) or cellular (pEF, pAct) promoters.

### Expression of pCN-CAT injected into the myocardium

To determine the dose-response of CAT gene expression after direct intracardiac injection, increasing doses of naked DNA in 50 μl saline were injected into the heart. Proteins were extracted from the injected heart three days later, and levels of CAT were determined. As depicted in Figure 2A, an injected amount as small as 1 μg DNA resulted in significant CAT expression. CAT expression increased in a dose-responsive manner in the range of 1-10 μg DNA, before reaching a plateau at doses over 10 μg, reflecting saturation kinetics in DNA uptake, transcription, or both. Injection of even higher amounts of total DNA resulted in a decrease in the expression of CAT ($P < 0.05$ compared to the peak level of CAT gene expression). The reasons for this reaction is not known at this time. However, we conjecture that high levels of foreign bacterial protein might interfere with transcription, with translation, or with both.

We have also tested the time course of CAT gene expression after direct intracardiac injection. The rats were injected with 10 μg of pCN-CAT, and then killed at various intervals; data are plotted in Figure 2B. CAT expression was detectable one day after injection, with a peak at day five, and a subsequent decline in expression through to day 30. Significant levels of CAT were detectable even 30 days after a single injection.

### Expression of pCN-VEGF$_{121}$ injected into the myocardium
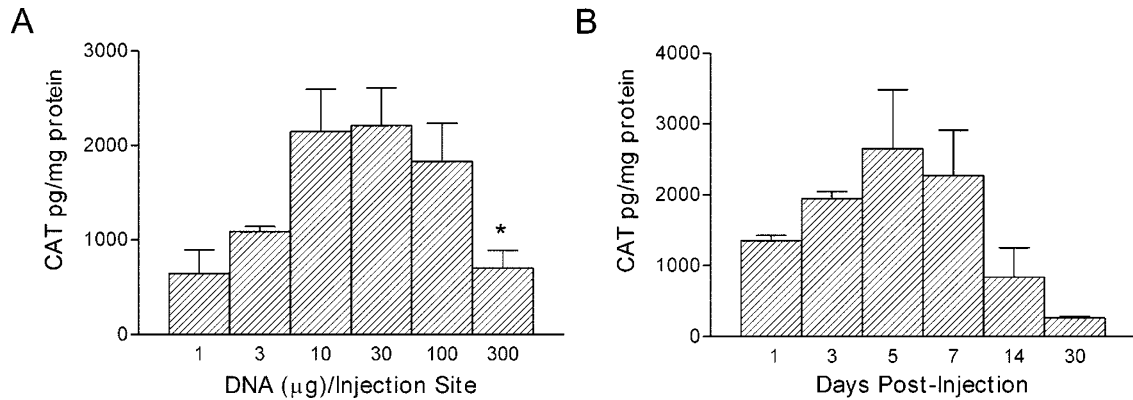
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**Figure 2.** Bar graphs showing dose-response data and time-course of the expression of injected pCN-CAT. (A) Different concentrations of DNA were injected in 50 μl normal saline (NS). Three days after injection, hearts were excised and levels of CAT protein were determined by ELISA. *$P < 0.05$ compared to the value for 30 μg. Values are given as mean ± SEM ($n = 3$ for each dose). (B) Ten micrograms of plasmid were injected into the rat heart, and the heart assayed for CAT by ELISA at each time point. Values are given as mean ± SEM ($n = 3$ for each time point).
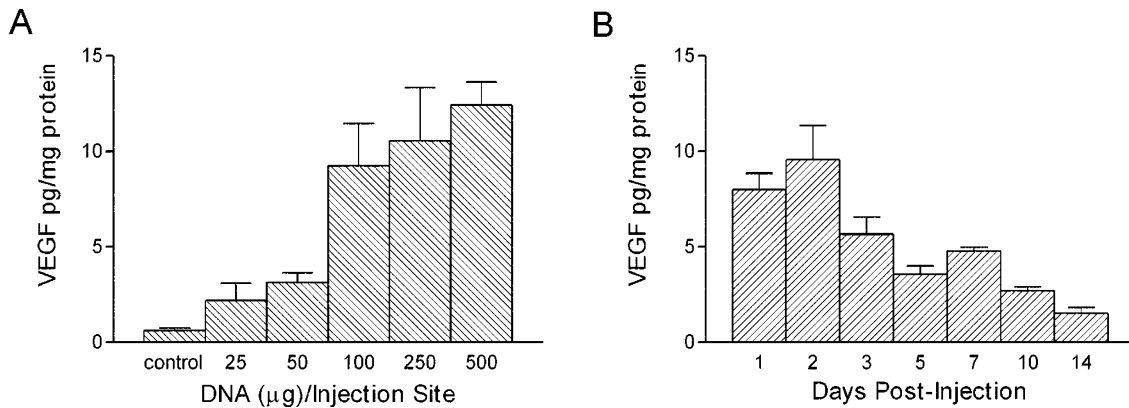
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**Figure 3.** Dose-response and time-course of the expression of injected pCN-VEGF$_{121}$. (A) Different concentrations of DNA were injected in 50 μl saline. Two days after injection, hearts were excised and levels of VEGF protein were determined. pCN-CAT was used as a control. Values are given as mean ± SEM ($n = 3$ for each dose). (B) Two hundred fifty micrograms of plasmid were injected into the rat heart, and the hearts were assayed for VEGF using ELISA at each time point. Values are given as mean ± SEM ($n = 3$ for each time point).

To determine the efficiency and kinetics of expression of the VEGF gene injected into rat myocardium, we injected a constant volume of 50 μl containing an increasing amount of pCN-VEGF$_{121}$ plasmid DNA, ranging from 25 - 500 μg per injection site, into each rat heart. The rats were killed two days after injection, proteins were extracted from the heart, and the level of VEGF gene expression was analyzed by ELISA. The data shown in Figure 3A indicate that, contrary to the pattern of expression of the CAT gene, there was a linear dose-response relationship between the level of VEGF gene expression and the quantity of plasmid DNA injected between 25 and 500 μg per injection site.

To examine the duration of expression from the VEGF$_{121}$ gene delivered as naked DNA, we injected a single dose of 250 μg of DNA into the heart and analyzed by ELISA the level of VEGF produced for the next 14 days (Figure 3B). Peak VEGF$_{121}$ expression occurred two days after injection, and then slowly declined. Significant levels of VEGF were detectable even 14 days after the single injection.

## Spatial extent of expression of VEGF at the protein level in cardiac muscle

To determine the spatial extent of expression of injected genes in the heart, we injected the apical portions of individual hearts with a single 50 μl bolus of pCN-CAT (30 μg), or pCN-VEGF$_{121}$ (250 μg). After injection of each DNA construct, the rats were killed on the day of peak expression. The hearts were cut into four sections from apex to base, and these sections were then assayed individually for the presence of protein, using ELISA. Data are shown in Figure 4. The relative amount of protein extracted from each section showed quite striking differences between the two genes. Over 99% of the expression of the CAT gene was restricted exclusively to the apical section that included the injection site (Figure 4A). As described in Figure 4B, the level of VEGF$_{121}$ protein showed a concentration gradient from the apex to the base, *i.e.*, 44% VEGF protein in the apical section,
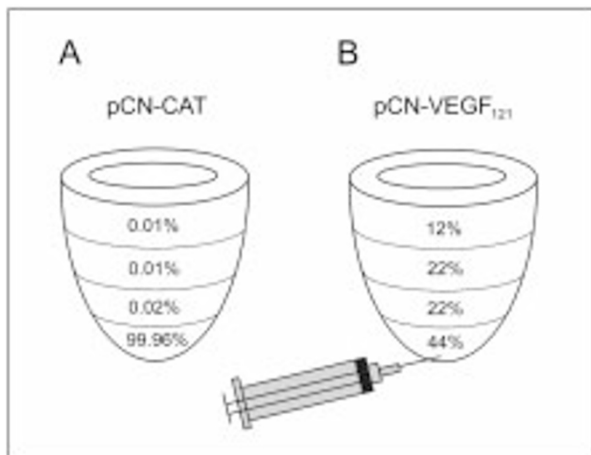
**Figure 4.** Spatial distribution of expression of (A) CAT protein (30 μg pCN-CAT) and (B) VEGF$_{121}$ protein (250 μg pCN-VEGF$_{121}$), following a single injection into the rat heart. Plasmid DNA was injected into the apex of the individual heart. Three days (CAT), or two days (VEGF$_{121}$) after injection of each construct, rats were sacrificed and hearts were excised. The hearts were divided into four sections from apex to base. Using ELISA, each section was then assayed individually for the presence of protein. Values are given as the mean of three rats, expressed as a percentage of total protein content of the entire heart.

22% in the middle sections, and 12% in the basal section, reflecting secretion and diffusion of the VEGF$_{121}$ protein.

## Discussion

Both the development of a potent vector to deliver the VEGF gene, and an understanding of the kinetics and distribution of VEGF gene expression, are potentially of profound importance in angiogenic gene therapy. Two observations result from this study. Firstly, we have demonstrated that a vector with the HCMV IE promoter/enhancer induces more potent gene expression in the myocardium than do other vectors with viral or cellular promoters. This finding is noteworthy, given the low efficiency of transfection by naked DNA. Secondly, using the above vector, we have defined the behavior of the therapeutic gene, VEGF$_{121}$, injected into the rat heart, with respect to the dose-response effects, and the temporal and spatial distribution of gene expression, all of which are quite different from those of the reporter gene (CAT). Our results, therefore, provide useful information applicable to angiogenic gene therapy in patients with coronary artery disease.

Gene therapy has been proposed or investigated for a number of cardiac diseases, including the treatment of congestive heart failure and therapeutic angiogenesis (Yla-Herttuala *et al.*, 2000). Preliminary results utilizing this approach as the sole therapy for patients with chronic myocardial ischemia suggest that direct injection of VEGF as naked DNA into cardiac myocytes may improve collateral blood flow and reduce the frequency

of, and threshold for, myocardial ischemia (Losordo *et al.*, 1998; Symes *et al.*, 1999). Therapeutic angiogenesis is an innovative approach to the treatment of ischemic diseases, whereby the administration of the VEGF gene facilitates the "biological revascularization" of obstructed vascular segments, and the reperfusion of ischemic tissues (Rosengart *et al.*, 1999). Moreover, it has been suggested that in certain cases of heart failure, the angiographically occult intramural coronary vasculature consists the locus of the disease as well as the cure (Isner and Losordo, 1999). In this case angiogenic gene therapy may have therapeutic implications in the treatment of heart failure.

Naked DNA is probably the safest and the most convenient form for the delivery of gene vectors (Kay *et al.*, 1997). Moreover, since the myocardium takes up DNA more efficiently than other tissue types, gene injection into the rat heart is quite efficient, and results in levels of gene expression up to 50-fold higher than similar experiments in skeletal muscles (Lin *et al.*, 1990; Kitsis *et al.*, 1991; Buttrick *et al.*, 1992). However, gene expression resulting from direct naked DNA injection into the myocardium is lower, by orders of magnitude, than that seen with adenovirus infection of the myocardium (Kass-Eisler and Leinwand, 1997). Therefore, the best way of making naked DNA gene therapy affordable is to use an expression system that will drive the highest possible level of therapeutic protein expression.

To develop a high efficiency expression plasmid for naked DNA gene therapy, the relative strengths of five different promoters were determined: HCMV IE pro-moter/enhancer, HCMV IE promoter, RSV LTR, EF 1-α, and β-actin, all fused to the CAT reporter gene. The pCN vector with the HCMV IE promoter/enhancer was app-roximately sevenfold more active than vectors carrying other viral and cellular promoters. In previous reports, viral promoters such as HCMV IE and RSV, and tissue-specific promoters such as α-MHC, were tested in the myocardium (Lin *et al.*, 1990; Kitsis *et al.*, 1991; Buttrick *et al.*, 1992; von Harsdorf *et al.*, 1993; Li *et al.*, 1997). It was reported that viral promoters were more potent than tissue-specific promoters in the heart (Buttrick *et al.*, 1992; von Harsdorf *et al.*, 1993). The adult-type EF-1α protein is one of the most abundant proteins in eukaryotic cells (Slobin, 1980). Even though the promoter of the human EF-1α gene has well-recognized, strong promoter activity in various types of cells (Wakabayashi-Ito and Nagata, 1994), pCN was more potent than pEF in the myocardium. However, we cannot rule out the possibility that cellular promoters (pEF or pAct) induce longer-term gene expression than viral promoters.

The plasmid pCN was constructed to contain, not only the full length IE promoter of HCMV, but also its entire 5' untranslated region, comprising all of exon 1 and intron 1, and a part of exon 2. In addition, our vector was designed in such a way that the start codon of the

inserted gene coincides with the ATG codon of the original IE gene of HCMV, which is not the case in many other HCMV promoter-based expression vectors (Petitclerc *et al.*, 1995). This strategy is based on a variety of data suggesting that IE genes, such as IE1 and IE2, are expressed at very high levels during HCMV infection, and that such high levels of gene expression are determined not only by the promoter, but also by the untranslated leader sequence of the coding region (Chapman *et al.*, 1991; Simari *et al.*, 1998).

We have shown that the expression of injected CAT constructs exhibits features of saturation kinetics at doses above 10 µg, and even results in decreased levels of expression at higher doses. To some extent, these phenomena of saturation and decreased gene expression have been demonstrated before, when very high doses of CAT-coding naked DNA were injected into the canine myocardium (von Harsdorf *et al.*, 1993), or when gene expression was enhanced by electroporation of the luciferase gene into rat liver (Heller *et al.*, 1996).

The temporal characteristics of pCN-CAT expression presented here are consistent with previous reports of gene injection in mouse, rat, and dog hearts (Buttrick *et al.*, 1992; von Harsdorf *et al.*, 1993; Li *et al.*, 1997). This implies that, even though pCN induces higher levels of gene expression, the temporal pattern of gene expression is no different from that found with other viral promoters used previously. Gene expression after injection of plasmid DNA is generally stable over time, which is one of the advantages of this method when compared to the more transient gene expression following recombinant adenovirus injection (Kass-Eisler and Leinwand, 1997). However, this pattern of expression, assayed at the protein level, is likely to overestimate the duration of expression of the injected DNA, because in most cell types the CAT protein has a long half-life of more than 50 h (Thomson *et al.*, 1991). Therefore, the pattern of CAT gene expression shown in Figure 2B not only reflects the activity of the driving promoter, but also reflects the half-life of the expressed protein as well.

Despite the clinical use of VEGF gene transfer as naked DNA, there are no published reports of dose-response effects, or of the temporal or spatial characteristics of VEGF expression following DNA injections into the heart. Since assays for a reporter gene do not necessarily reflect the behavior of the therapeutic gene, we wished to address whether the expression of VEGF and CAT exhibited similar behaviors. In contrast to the patterns of reporter gene expression, which were dose-limited, very stable over four weeks, and restricted to a limited area of the left ventricle, the kinetics of $VEGF_{121}$ expression were quite different. The level of protein extracted from hearts was lower by orders of magnitude than that of CAT protein. While CAT gene expression showed a decrease of protein with higher doses of plasmid, there was a dose-response relationship be-

tween the level of VEGF gene expression and the quantity of plasmid DNA injected for doses between 10 and 200 µg per injection site, indicating that VEGF protein has no deleterious effect on gene expression. We suggest that this is the case, either because the level of VEGF is low, or because VEGF is a eukaryotic protein whereas CAT protein is of bacterial origin. Furthermore, VEGF exhibited an earlier peak of protein expression than CAT, and a rather rapid decline of gene expression over time, reflecting the short half-life of the $VEGF_{121}$ protein. However, expression of $VEGF_{121}$ was readily detected by ELISA in all hearts injected with 100 µg of DNA. Expression of the exogenous gene lasted a surprisingly long time, as significant levels of VEGF were detectable even 14 days after a single injection. Since the half-life of VEGF is known to be only a few minutes (Ferrara *et al.*, 1992), our DNA must exist in a relatively stable form.

The spatial distribution of CAT gene expression showed a pattern similar to that found in previous studies (Lin *et al.*, 1990; Buttrick *et al.*, 1992). The histological distribution of cell transfection has been addressed in several reports, either by analysis of expression in different areas around the injection site (Buttrick *et al.*, 1992), or by the use of constructs containing the lacZ gene (Lin *et al.*, 1990). Apparently, only cells in the immediate vicinity of the needle tract are transfected. Therefore, VEGF naked DNA could only transfect the apical portion of the heart surrounding the injection site. However, VEGF could be detected in other parts of the heart, reflecting the secretory and diffusible nature of the $VEGF_{121}$ protein. This finding has clinical implications, because it means that the injection sites of VEGF DNA need not exactly match the ischemic lesions.

In conclusion, in angiogenic gene therapy for the patients with coronary artery diseases, plasmid vectors with the HCMV IE promoter/enhancer will provide clear advantages over other previously developed plasmids. Furthermore, knowledge of the kinetics and distribution of VEGF expression will also prove to be useful in this context.

## Acknowledgements

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