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Development of an efficient endothelial cell specific vector using promoter and 5' untranslated sequences from the human preproendothelin-1 gene

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Abbreviations: BAEC, bovine aortic endothelial cells; CPAE, calf pulmonary artery endothelial cells; HUVEC, human umbilical vein endothelial cells; ppET-1, preproendothelin-1

Abstract

We report here, that a vector constructed based on ppET-1 gene promoter and 5' untranslated region induced a high level of gene expression in endothelial cells and the specificity is even further enhanced under hypoxia-mimic conditions due to a natural hypoxia responsive element within the promoter region. A naked DNA vector that confers endothelial cell specific gene expression as well as efficient levels of gene expression was constructed with an endothelial cell specific naked DNA vector, pETlong, by using the full length promoter of the preproendothelin-1 gene and the entire 5' untranslated region upstream from the start codon. Inclusion of the entire 5' untranslated region in pETlong increased gene expression 2.96 fold as compared with that from pETshort, which contains only the promoter sequences. Reporter gene expression from pETlong was 7.9 fold higher as compared with that from CMV-driven promoter based vector in calf pulmonary endothelial cells. However, in nonendothelial COS cells, luciferase activity from pETlong was only 0.3 fold as compared with that of CMV-based vector. Similar results were observed in other nonendothelial cells. These results demonstrate that the pETlong drives gene expression in endothelial cells with high efficacy and specificity. We have examined hypoxia responsiveness of pETlong as the promoter region of the preproendothelin-1 gene contains hypoxia responsive elements. The activity of the pETlong vector was increased 1.6 fold under hypoxia-mimic conditions using cobalt chloride. The high levels of hypoxia-inducible expression in endothelial cells relative to the low levels of background expression in other cells shows that pETlong could be a useful tool for vascular targeting of vascular disease and cancer gene therapy.

Keywords: endothelial cell specific vector; gene therapy; naked DNA, preproendothelin-1; 5' untranslated region

Introduction

The success of gene therapy depends largely on the efficacy of gene delivery vector systems that can selectively and efficiently deliver genes to target organs with minimal toxicity (Niidome and Huang, 2002). Development of efficient endothelial cell selective vector systems will be invaluable to cancer gene therapy as well as other vascular diseases (Martin and Murray, 2000). Most frequently used strategies to develop such vectors are either using tissue targeting vector systems or tissue specific promoters (Varda-Bloom *et al.*, 2001). A few studies have reported construction of endothelial cell specific viral vectors using sequences from von-Willebrand factor, intracellular adhesion molecule 2, vascular endothelial growth factor receptor, or E-selectin (Modlich *et al.*, 2000; He *et al.*, 2001). However, one of the most frequently encountered problems with such tissue specific vectors using endogenous cell specific promoters is low levels of gene expression compared with widespread nonspecific viral promoters even when tissue specificity is achieved (Modlich *et al.*, 2000; Shibata *et al.*, 2000). In some cases, exogenous cytokine-inducible elements were inserted to 5' upstream of the promoters to overcome this low gene expression activity (Modlich *et al.*, 2000).

To develop highly efficient and endothelial cell specific expression plasmids, we chose to use the preproendothelin-1 (ppET-1) gene promoter because it's regulation and expression in endothelial cells are well studied (Wilson *et al.*, 1990; Bu and Quertermous, 1997;

Minchenko and Caro, 2000). A high specific vasoconstrictor, ppET-1, is produced by vascular endothelium in response to various signals such as injury, hypoxia, ischemia, and inflammatory mediators (Aversa *et al.*, 1997). Hypoxic responsiveness of ppET-1 is mediated through an inverted hypoxia responsive elements is located at -118 upstream of the transcription start site of the ppET-1 gene (Yamashita *et al.*, 2001). In our vector design, we have used not only the full length promoter sequences of ppET-1 gene but also the entire 5' untranslated region upstream from the start codon of the ppET-1 gene to improve the efficacy of ppET-1 gene promoter activity. Inclusion of intronic sequences to expression plasmid has been reported to enhance gene expression both *in vitro* and *in vivo*. Simari *et al.* showed that addition of 5' intronic sequences to cytomegalovirus immediate early promoter resulted in about 6 fold increase in gene expression (Simari *et al.*, 1998). Kay's group constructed a vector with hepatic factor IX intronic sequences expressing enhanced levels of proteins (Miao *et al.*, 2000). In this report, we show that our newly constructed vector based on ppET-1 gene promoter and 5' untranslated region drives high level of gene expression in endothelial cells and the specificity is even further enhanced under hypoxia-mimic conditions due to a natural hypoxia responsive element within the promoter region. Our results show that the vector could be a potentially useful tool for vascular targeting of vascular disease and cancer gene therapy.

Materials and Methods

Cell culture

BAEC (Bovine aortic endothelial cells), originally obtained from a local slaughterhouse were gifts from Dr. Inho Jo (Kim *et al.*, 1999). BAEC and COS cells were maintained in DMEM containing 1× antibiotic/antimycotic mix, 5 mM N-(2-hydroxyethyl)-piperazine-N-2-ethanesulfonic acid, and 0.37% sodium bicarbonate, supplemented with 10% fetal bovine serum (FBS). CPAE (Calf pulmonary artery endothelial cells) were purchased from the Korea Cell Line Bank and grown in RPMI with 20% FBS. HUVEC (Human umbilical vein endothelial cells) purchased from BioWhittaker, Inc (Walkersville, MD) were grown in endothelial cell growth medium kit 2 containing 2% FBS (Bio Whittaker, Inc.). All the cell lines were grown at 37°C in humidified 95% air/5% CO_2 and fed every 1-2 days. For hypoxia-mimic condition, cobalt chloride was added to cell cultures to a final concentration of 100 M for 24 h.

Plasmid construction

The full length ppET-1 gene promoter and its entire 5' untranslated region was cloned by PCR amplification of human genomic DNA, using a pair of primers, 5'-ACGCGTTCTGAAGTTAGCAGTGAT 3' and 5'-AAGCTTCCGTTCGCCTGGCGCA 3' for pETshort and 5'-ACGCGTTCTGAAGTTAGCAGTGAT 3' and 5'-AAGCTTTCTGAAAAAAAGGGATCA 3' for pETlong. These primers contain the *Mlu*I linker at the 5' end and the *Hin*dIII linker at the 3' end. The amplified cDNA was replaced with the promoter region of pcDNA3-Luc using *Mlu*I/*Hin*dIII site thereby making the backbone identical to pcDNA3 vector (Invitrogen Inc., Carlsbad, CA). pETshort plasmid contains 210 bp of ppET-1 gene promoter region. pETlong contains 210 bp of promoter sequences and its entire 5' untranslated region consisiting of 267 bp of exon 1. The start codon of the inserted gene was designed to coincide with the original ppET-1 gene.

Transient transfection

BAEC, CPAE, HUVEC, and COS cells were transfected by the calcium phosphate-DNA coprecipitation method. A total of 0.5 g of DNA in 25 l of $CaCl_2 \cdot H_2O$ (250 mM $CaCl_2$) was mixed with 25 l of 2× HBS (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 12 mM dextrose, 50 mM HEPES) with constant bubbling and within 5 to 10 min this solution was added to each well. HUVEC were transfected at 90% confluence; other cell lines were transfected at 70% confluence.

Luciferase assay

The cells were incubated in 24-well plates for about 24 h after transient transfection. The next day, transfected cells were washed with PBS and lysed with the Reporter Lysis Buffer (Promega, Madison, WI). Luciferase expression was assayed using a luciferase assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was determined by using an Auto Lumat LB9507 luminometer and expressed as relative light units. Transfections were performed in triplicate more than three times. Data are expressed as the mean ± SEM and shown for representative experiments.

Results

Construction of ppET-1 based expression vector

To develop endothelial cell specific expression plasmid for naked DNA gene therapy, we chose to use the promoter of human ppET-1 gene because its regulation and expression in endothelial cells are very

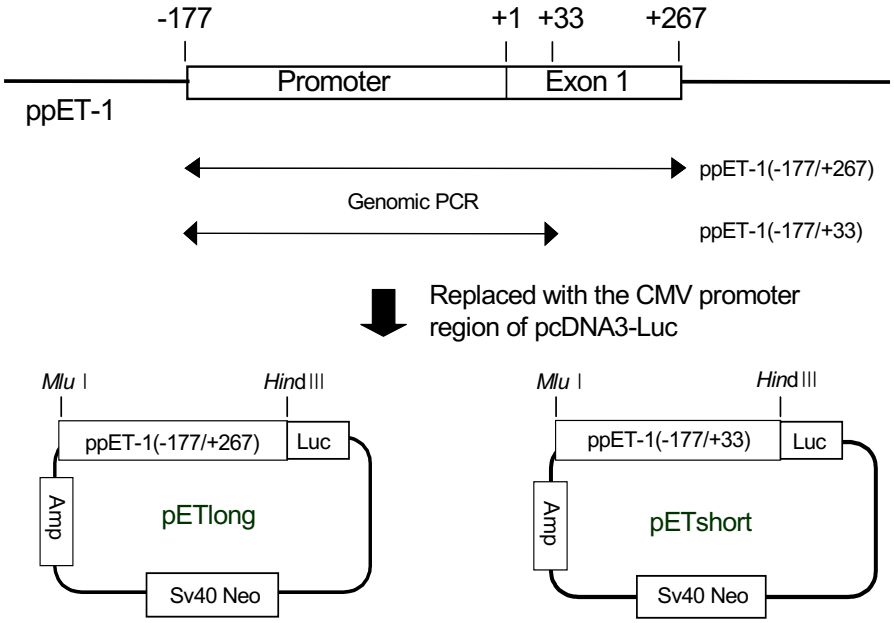

Figure 1. Structures of pETlong and pETshort. pETlong and pETshort were constructed as described under Materials and methods.

well characterized. Previous studies have adopted the -177 bp to +33 bp (relative to transcriptional start site) region of the ppET-1 gene for construction of an endothelial cell specific retroviral gene therapy vector (Mavria *et al.*, 2000). DNA fragments containing the same region (-177 to +33) and a longer fragment down just upstream to the start codon (-177 to +267) were amplified by PCR from human genomic DNA. Both the fragments were replaced by CMV promoter in pcDNA3-Luc to construct pETlong and pETshort (Figure 1). pETlong consists not only the promoter but also its entire 5' untranslated region of ppET-1 gene. In most cases, 5' untranslated regions and introns have not been used in expression vectors because of their large sizes and absence of dramatic enhancer elements. However, several data demonstrate that 5' untranslated regions, introns, or 3' untranslated regions can contribute to increase in gene expression (Brinster *et al.*, 1988; Palmiter *et al.*, 1991; Liu *et al.*, 1995).

Effect of 5' untranslated sequences in pETlong

First, we have examined the effect of the entire 5' untranslated sequences of ppET-1 gene on gene expression level. We have compared pETlong with pETshort for their levels of luciferase gene expression by transient transfection in nonendothelial COS cells. Relative luciferase units from pETlong which includes 5' untranslated leader sequences was 46,522 ± 8,538 which is 2.96 fold higher than pETshort (15,720 ± 1,389) in COS cells (Figure 2A). Similar results were obtained in BAEC (Figure 2B).

Endothelial cell-specific expression of pETlong

We next proceeded to investigate the expression profile in endothelial cells. Three different endothelial cells were transiently transfected with pETlong plasmids and levels of luciferase activity were measured 24 h after transfection. Gene expression levels were normalized by luciferase activity from parallel transfected control pcDNA3 plasmid for transfection efficiencies. We did not cotransfected control β-galactosidase plasmids that promoter interference might occur. Rather, we prepared, transfected same amounts of either pETlong or pcDNA3-Luc each in triplicate, and assayed four different cells simultaneously. Reporter gene expression from pETlong was 8 (± 0.80) fold higher as compared with that from CMV-driven promoter based vector in CPAE. However, in nonendothelial COS cells, luciferase activity from pETlong was only 0.3 (± 0.01) fold as compared with that of CMV-based vector. Expression patterns between endothelial cells were similar. HUVEC and BAEC expressed about 3 fold lower levels of luciferase compared with CPAE but still about 8 fold more than COS cells (Figure 3).

Effect of hypoxia on pETlong

Yamashita *et al.* have identified a HIF-1 binding site at -118 bp upstream of the transcription start site (Yamashita *et al.*, 2001). We have examined whether expression from pETlong responds to hypoxia in CPAE. Cells were transiently transfected with pETlong and incubated with cobalt chloride (100 M) for 24 h. As shown in Figure 4, the relative luciferase units

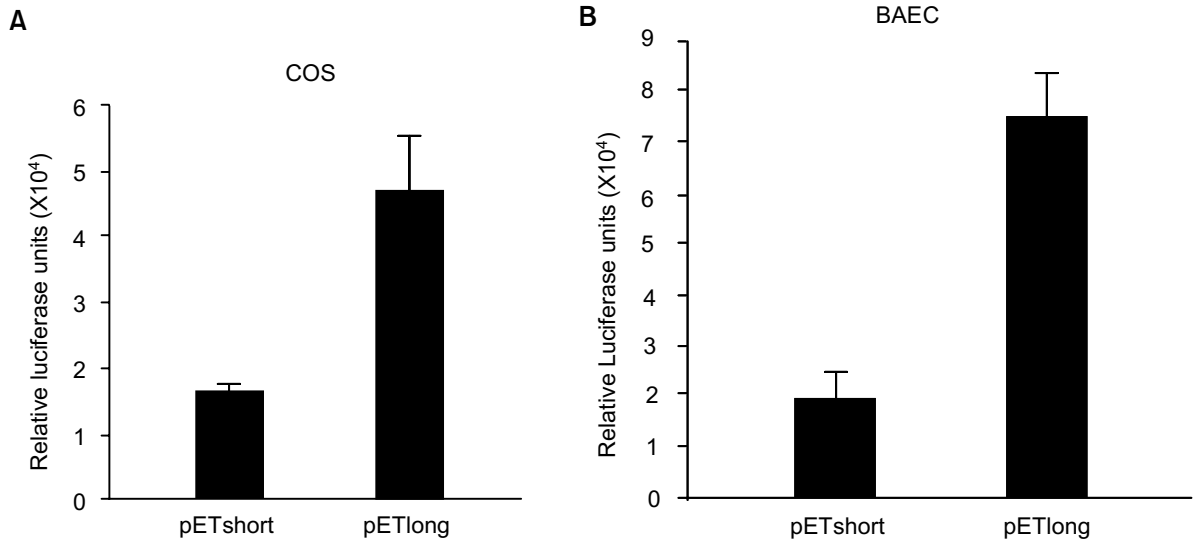

Figure 2. Comparison of levels of luciferase reporter gene expression between pETshort and pETlong vectors. Constructs were transfected into COS cell (A) and BAEC (B) by calcium phosphate. An approximately 3 fold increase in luciferase production was observed in cells transfected pETlong when compared with pETshort. Transfections were performed in triplicate more than three times. One representative result is shown in this figure. Data are expressed as the mean±SEM.

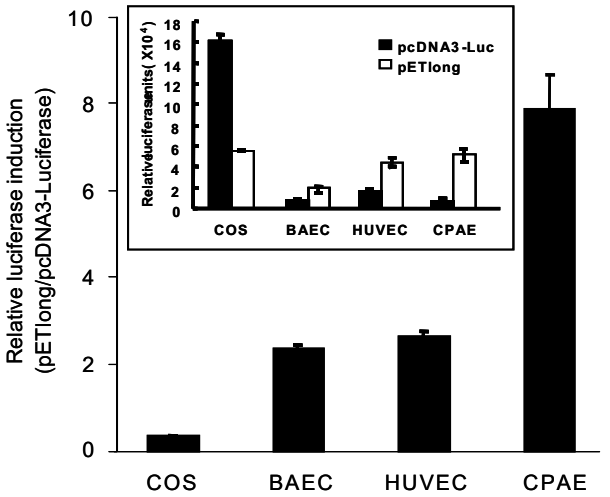

Figure 3. Endothelial cell specificity of the pETlong. A panel of endothelial (BAEC, CPAE, and HUVEC) and non-endothelial cell lines (COS) were transfected with pETlong and pcDNA3-Luciferase for determination of luciferase expression. Endotherial cells specificity of pETlong was assessed by ratio of pETlong expression level/pcDNA3-LUC expression level. The inset shows the raw luciferase data from one experiment. Transfections were performed in triplicate more than three times. One presentative result is shown in this figure. Data are expressed as the mean±SEM.

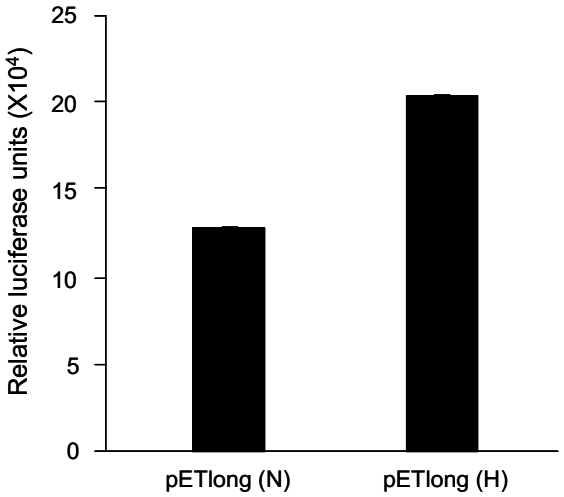

Figure 4. Effect of hypoxia on luciferase activity in CPAE expressing pETlong vector. Compared to normoxic control (N), there is a 1.6 fold increase in luciferase production from cells exposed under hypoxia-mimic conditions (H) for 24 h. Transfections were performed in triplicate more than two times. One presentative result is shown in this figure. Data are expressed as the mean±SEM.

of pETlong under hypoxia-mimic conditions was 199,000 ± 370 which is 1.6 fold higher compared with normoxia (124,000 ± 524) in CPAE. These data suggested that expression from pETlong is inducible in hypoxia.

Discussion

The major obstacle for a success of gene therapy is the lack of efficient gene delivery vehicles (Verma and Somia, 1997). Nonviral vectors based on simple plasmid DNA are considered to be safe, simple, and easier to use. However, in most cases the duration

of expression is shorter compared with viral vectors. Long-term persistent gene expression was often considered to be the major advantage of using viral integrating vectors. However, recent studies showed that gene expression could persist more than a year even with the episomal plasmid vectors with tissue specific promoters (Chen *et al.*, 2001; Miao *et al.*, 2001). Nonviral vectors containing a modified endogenous alpha-1 antitrypsin promoter region produced not only tissue specific expression but also elevated, sustained level of gene expression for at least 1.5 years (Miao *et al.*, 2001). For both viral and nonviral vectors, localized, tissue specific expression is desirable that it would dramatically reduce its side effects by localizing the expression of the therapeutic genes. Production of sustained gene expression by tissue specific nonviral vector systems is probably by the reduction of an immune response to the transgene (Niidome and Huang, 2002). Therefore, development of tissue specific expression nonviral vectors is important for protection of healthy unaffected organs and for duration of gene expression as well.

The lumen of all blood vessels and heart is covered by endothelial cells (Schlaeger *et al.*, 1995). These cells are often targets for gene therapy because they are very closely involved in pathological status such as angiogenesis, atherosclerosis, tumor growth, myocardial infarction, and limb ischemia. Several reports have shown development of endothelial cell specific vector systems, each aiming ultimately for safe and efficient expression vector for human application. Ma *et al.* have delivered oligonucleotides using special formulation of lipids (Ma *et al.*, 2002). He *et al.* have examined FLk-1 and thrombomodulin promoters in endothelial cells of transgenic pigs that vascular endothelium is the most immediate barrier between the xenogeneic donor organ and host immune systems (He *et al.*, 2001). Modlich *et al.* have developed endothelial cell specific promoter based retroviral vectors by the use of hypoxic and cytokine-inducible enhancers (Modlich *et al.*, 2000). Here in this study, we demonstrate that our newly developed vector pETlong efficiently expressed the exogenously added gene in endothelial cells in *in vitro*. pETlong was constructed to contain not only the full length promoter of ppET-1 gene but also its entire 5' untranslated region. In addition, our vector was designed in such a way that the start codon of the original ppET-1 gene. Expression from pETlong were approximately 8 fold higher than those from commercially available pcDNA3 in CPAE cells. In addition, luciferase activity from pETlong was approximately 3 fold higher compared with pETshort, which harbors same promoter regions of a retroviral vector reported by Mavria *et al.* (2000) in COS cells. This strategy is based on a variety of data that high levels of gene expression are determined not only by the promoter but also by the untranslated leader sequences of the coding region (Lee *et al.*, 2000; Jin *et al.*, 2002). Expression from pETlong was far greater than that from CMV-based vector in endothelial cells and was further enhanced under hypoxia-mimic conditions. pETlong would not only be useful for gene therapy but also would be invaluable for various gene transfer studies targeting endothelial cells.

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