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Improved Expression of Vascular Endothelial Growth Factor by Naked DNA in Mouse Skeletal Muscles: Implication for Gene Therapy of Ischemic Diseases

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We have constructed an expression vector, pCK, that is able to drive high levels of gene expression in the skeletal muscles of mice. pCK contains not only the full length immediate-early (IE) promoter of human cytomegalovirus but also its entire 5′ untranslated region upstream from the start codon of the IE gene. In addition, pCK contains the kanamycin resistance gene, but lacks nucleotide sequences unnecessary for its function as a gene delivery vector, allowing the plasmid size to be 3.7 kb. pCK produced significantly higher levels of vascular endothelial growth factor 165 both *in vitro* and *in vivo* than the control vector, the structure of which is similar to naked DNA vectors employed in previous gene therapy trials. pCK would not only significantly increase the therapeutic effects of naked DNA gene therapy but also dramatically cut down the costs for production and treatment. © 2000 Academic Press

Key Words: **naked DNA gene therapy; ischemic diseases; VEGF; muscles.**

Gene therapy is an innovative form of medicine (1). Among several gene delivery vectors currently used in gene therapy trials, naked DNA alone or in combination with others such as liposome, electroporation, or gene gun is employed in almost 25% of approved clinical protocols. Naked DNA, when used alone, is probably the safest and the most convenient form of gene delivery vectors. However, its approach has been limited because of its low stability and low efficiency in entering the nucleus, resulting in low levels of gene expression (2). Despite such drawbacks, naked DNA gene therapy using vascular endothelial growth factor 165 ($VEGF_{165}$) gene has been shown to be effective in ischemic diseases including coronary artery disease and peripheral arterial occlusive disease (3, 4).

However, a major limiting factor for its large scale clinical application remains the expression level of the therapeutic protein at a given amount of DNA injected to the target site. In previous gene therapy trials, 4 mg DNA in total was applied to a patient, 2 mg each (500 μg per site, 4 injections) with an interval of 4 weeks (3). This is a relatively large amount of DNA and as such, it may contribute to high production costs. 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 proteins and also yield the highest possible copy number in *E. coli.*

To develop a high efficiency expression plasmid for naked DNA gene therapy, especially for ischemic diseases, we chose to use the major immediate-early (IE) promoter of human cytomegalovirus (HCMV) because it has been well known that this control region drives high levels of gene expression in a variety of mammalian cells. Here in this report, we show that our newly constructed vector based on HCMV drives higher level of gene expression both *in vivo* and *in vitro* than DNA vectors employed in previous gene therapy trials.

MATERIALS AND METHODS

Plasmids. pCI-neo was purchased from Promega and pcDNA3.1 was from Invitrogen. pCN contains the 600 bp HCMV IE promoter and its entire 5′ untranslated region consisting of 122 bp exon 1, 827 bp intron 1, and 16 bp exon 2 (5). The start codon of the inserted gene was designed to coincide with that of the original IE gene of HCMV. The full length IE promoter of HCMV and its entire 5′ untranslated region was cloned by PCR amplification of pEQ276 (5), using a pair of primers, 5′ ACGCGTTGACATTGATTATTG 3′ and 5′ AAGCTTCGTGTCAAGGACGGT 3′. These primers contain the *Mlu*I linker at the 5′ end and the *Hin*dIII linker at the 3′ end. This amplified fragment was inserted to the *Mlu*I/*Hin*dIII sites of pcDNA3.1, resulting in pCN. The backbone of pCN is therefore identical to pcDNA3.1. pEF contains 137 bp of the human elongation factor 1α (EF1α) 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 (6). This 1.2

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kb region was obtained by PCR amplification of genomic DNA prepared from peripheral blood lymphocytes of a healthy individual. As in the case of pCN, the PCR primers contained *Hin*dIII and *Mlu*I linker at the 5′ and 3′ ends, respectively. The amplified fragment was inserted to the *Mlu*I/*Hin*dIII site of pcDNA3.1, resulting in pEF. Thus, the backbone of pEF is identical to pcDNA3.1. Like pCN, the ATG codon of the reporter gene is positioned to the site of the original start codon for EF1α. pACT contains 243 bp of the human β-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 (7). This 1.0 kb region was obtained by PCR as described above. As in the case of pEF, the PCR primers contained *Hin*dIII and *Mlu*I linker at the 5′ and 3′ ends, respectively. The amplified fragment was inserted to the *Mlu*I/*Hin*dIII site of pcDNA3.1, resulting in pACT. The backbone of pACT is therefore identical to pcDNA3.1. Like pEF, the position of ATG codon of the reporter gene is similar to that of the start codon for the β-actin gene. pCK was constructed by PCR-amplifying the region from the IE promoter to the poly A signal sequence of pCN, and ligating the amplified fragment with *Afl*III/*Apo*I fragment of pZERrO-2 (Invitrogen). CAT or $VEGF_{165}$ was inserted to the *Hin*dIII/*Xba*I site of each expression vector plasmids.

VEGF cloning. cDNA encoding $VEGF_{165}$ was cloned from total RNA prepared from human vascular muscle cells by RT-PCR. PCR primers were 5′AAGATGAACTTTCTGCTGTCT3′ and 5′TCTAGATCACCGCCTCGGCTTGTCACATCT3′ (8, 9). The amplified cDNA was initially cloned into the pCR2.1 vector (Invitrogen) and its nucleotide sequence was confirmed by sequencing.

Cell culture. C2C12 cells were purchased from ATCC (No. CRL-1772) and grown in Dulbecco's modified Eagle medium supplemented with 10% calf serum as suggested. To evaluate expression of the exogenously added gene, C2C12 cells were transfected with plasmids using Fugene6 (Boehringer Mannheim). Forty-eight hours after transfection, supernatants were taken and levels of $VEGF_{165}$ expression were determined by ELISA by the commercially available kit (R&D System). In case of CAT expression, cells were lysed and CAT assays were performed as described previously (10).

Animal model. One hundred microgram of plasmid in 100 μl PBS (11) was injected into the anterior tibialis muscle of unanesthetized male Balb/C using an insulin syringe with a 27-gauge needle. All protocols were approved by the Seoul National University Animal Care and Use Committee. Two days later, the injected areas were excised and homogenized in total protein extraction buffers (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP-40, 0.2% SDS, 1 mM PMSF). The supernatants containing total protein were subjected to CAT or $VEGF_{165}$ assays as described above.

Statistics. Results were expressed as mean ± SEM. Statistical significance was analyzed by nonparametric Wilcoxon 2 test. The data set was considered significantly different when P value < 0.05.

RESULTS

Construction of HCMV-based expression vector. To develop a high efficiency expression plasmid for naked DNA gene therapy, we chose to use the major IE promoter of HCMV because it has been well known that this control region drives high levels of gene expression in a variety of mammalian cells, including vascular smooth muscle and skeletal muscle cells (12–14). We constructed pCN in a way that it contains not only the full length IE promoter of HCMV but also its entire 5′ untranslated region consisting of the entire exon 1 and intron 1, and a part of exon 2 (Fig. 1). In addition, pCN was designed in a way that the start codon of the inserted gene coincides with the ATG codon of the original IE gene of HCMV unlike many other HCMV promoter-based expression vectors. This 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 (15, 16).

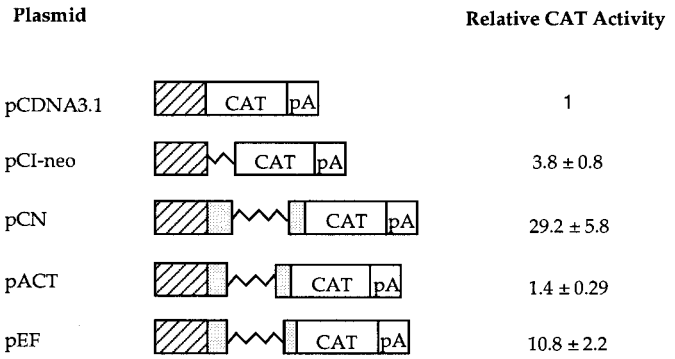

FIG. 1. Comparison of the levels of CAT gene expression from various promoters. C2C12 cells were transfected with the indicated vectors using Fugene6. Forty-eight hours after transfection, cells were harvested and CAT assays were performed. Hatched box, promoter; dotted box, exon; wavy line, intron; pA, poly A tract.

In vitro assessments of pCN with other vectors. We compared our newly constructed vector, pCN, with other commercially available IE promoter-based vectors, pcDNA3.1 and pCI-neo, for their levels of gene expression by transient transfections using the bacterial CAT sequence as a reporter gene. The former vectors lack introns, while the latter contains heterologous intron sequences. Expression from pCN was 10–30 fold higher than that from commercially available vectors (Fig. 1). In addition, we have constructed vectors driven by promoters from housekeeping gene such as human EF1α (pEF) and β-actin (pACT). These vectors containing housekeeping gene promoters are constructed in a way similar to pCN so that they contain not only the full length promoter but also its entire 5′ untranslated region consisting of the entire exon 1 and intron 1, and a part of exon 2. CAT expression from pCN was the highest among all these tested vectors (Fig. 1). Based on these and other previous results (17), it appears that high level gene expression from pCN results from increase in transcription.

Construction of pCK. pCN contains the neomycin resistance gene under the control of the SV40 early promoter in its plasmid backbone. In order to make pCN better fit in the context of naked DNA gene therapy, we have modified backbone of pCN, eventually constructing pCK (Fig. 2). In pCK, the β-lactamase gene was replaced with the gene conferring the resistance to kanamycin because residual ampicillin in final DNA solution might cause allergic reactions to some

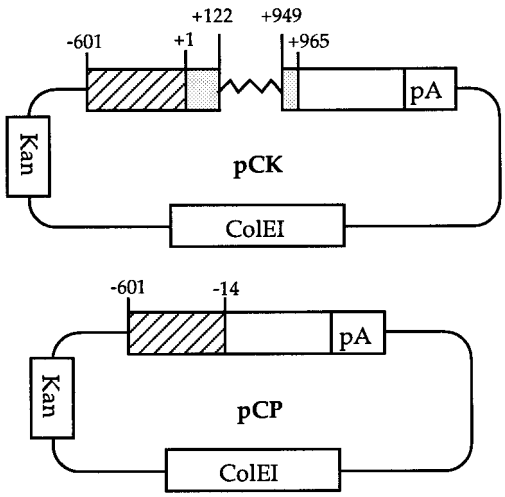

FIG. 2. Structure of pCK and pCP. pCK and pCP constructed as described under Materials and Methods. Hatched box, promoter; dotted box, exon; wavy line, intron; pA, poly A tract; Kan, kanamycin resistance gene; ColEI, *E. coli* origin of replication.

patients undergoing gene therapy trials. Furthermore, all possible nucleotide sequences unnecessary for a vector to function as a gene delivery vehicle were removed from the plasmid in order to minimize the size of the plasmid to 3.7 kb, resulting in a relatively high copy number in *E. coli.* We have also constructed a control vector (pCP) whose backbone is identical to that of pCK except that it lacks the untranslated leader sequences of the major IE region of HCMV (Fig. 2).

In vitro gene expression from pCK. We first tested the level of $VEGF_{165}$ expression *in vitro* using the mouse muscle cell line. C2C12 cells were transfected with either plasmid expressing $VEGF_{165}$ from pCK (pCK-VEGF) or pCP (pCP-VEGF), and culture supernatants were taken to measure the level of $VEGF_{165}$ 48 h posttransfection using the commercially available ELISA kit. All transfections were performed in triplicate per experiment more than three times. $VEGF_{165}$ expression from pCK was reproducibly 30- to 40-fold higher than pCP (Fig. 3A). To confirm the results, we repeated the experiments using the bacterial CAT gene. Again, pCK produced a minimum of 30-fold higher levels of CAT than pCP (Fig. 3B).

In vivo gene expression. To examine the level of gene expression from pCK *in vivo,* 100 μg of the expression plasmid dissolved in 100 μl PBS was injected into the anterior tibialis muscle of Balb/C mice (n = 10 to 15 mice per experiment, total n = 41 from three experiments). PBS was used instead of water because when DNA was dissolved in water, the level of gene expression was significantly decreased (data not shown). Two days after injection, the muscle was homogenized in an extraction buffer, total protein obtained, and ELISA performed using the same amount of protein. The level of $VEGF_{165}$ expressed in the muscle ranged from 15 to 1400 pg and from 0 to 110 pg in Balb/C injected with pCK and pCP, respectively. It is worth noting that 24 of 41 mice injected with pCK-VEGF expressed a level of $VEGF_{165}$ higher than 110 pg, the highest amount ever produced from mice injected with pCP-VEGF (Fig. 4A). The average $VEGF_{165}$ expression from pCK and pCP expressed as mean ± SEM was 55.2 ± 5.73 and 228 ± 47, respectively. These results include data from three different experiments done at different times. Statistical analysis using Wilcoxon 2 test between the two groups revealed that the difference was indeed significant (P = 0.0001). We have repeated experiments with the CAT reporter gene. When 100 μg of pCK-CAT in 100 μl PBS was

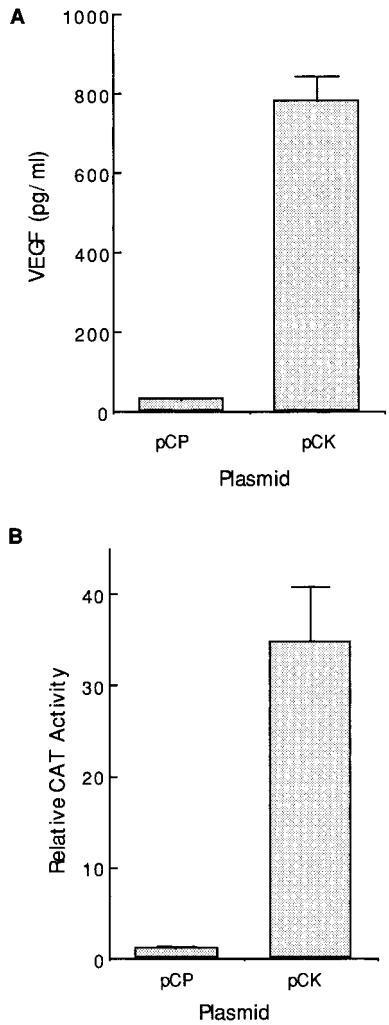

FIG. 3. Comparison of the levels of gene expression *in vitro.* (A) $VEGF_{165}$ expression from pCP and pCK in C2C12 cells by transient transfection assays. Forty-eight hours after transfection, supernatants were taken and levels of gene expression were determined by ELISA. (B) The level of CAT gene expression in C2C12 cells. Transfections were performed in triplicate more than three times. One representative result is shown in this figure.

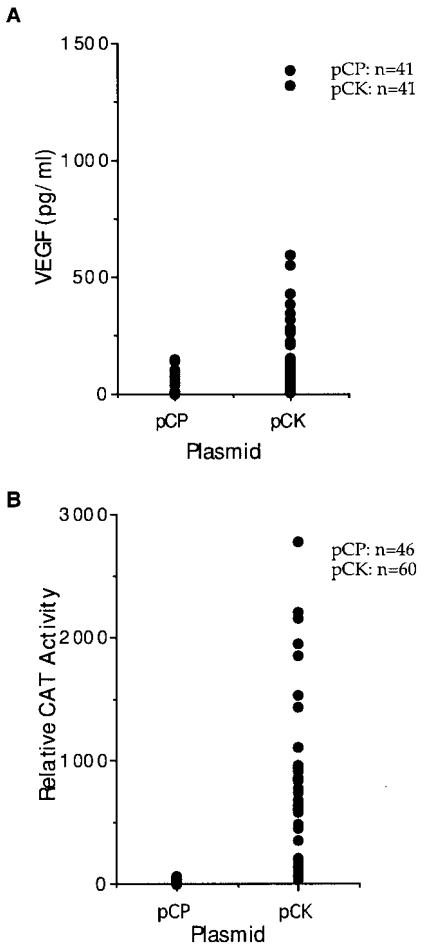

FIG. 4. Comparison of the level of gene expression *in vivo.* (A) $VEGF_{165}$ expression in mice. Two days after DNA, the injected areas were excised and subjected to ELISA assays. Ten to 15 mice were used per experiment and three separate sets of experiments were carried out resulting total of 41 mice. One apparent closed circle may represent more than 2 mice. Results from three sets of experiments are combined and shown here as mean ± SEM. (B) CAT expression in mice. Experiments were performed as described except that the CAT gene was used. Ten to 20 mice were used per experiment and three separate sets of experiments were carried out (n = 46 for pCP and n = 60 for pCK). Similar data were obtained in all experiments and results from three experiments are combined here for analysis. Fold of induction was expressed as mean ± SEM.

injected into the anterior tibialis muscle of Balb/C mice, levels of CAT expression from pCK (n = 20 per experiment, total n = 60 from three experiments) were much greater than those from pCP (n = 10 to 20 per experiment, total n = 46 from three experiments) in almost all mice tested (Fig. 4B). The average CAT activity from three sets of experiments expressed as mean ± SEM was 11 ± 1.8 and 707 ± 87.5 for pCP and pCK, respectively (P = 0.0001). These results demonstrated that pCK efficiently expressed exogenously added genes *in vivo.*

Dose dependency of gene expression. We have also tested effects of plasmid concentrations on the expression level of the exogenously added gene. Balb/c mice were injected with increasing doses of DNA dissolved in 100 μl PBS (n = 10 per dose per experiment), proteins extracted from the injected site two days later and analyzed by ELISA or CAT assays. The results from three experiments were combined for analysis (Fig. 5; n = 30 per dose in total). The level of $VEGF_{165}$ expression from pCK was increased up to 250 μg DNA and somewhat decreased at a higher concentration (Fig. 5A). The difference was statistically significant between dosage groups ($P < 0.05$ in all cases). On the other hand, CAT expression from pCK appeared not to be dose-dependent (Fig. 5B), due presumably to the high stability of this bacterial protein (18).

Time course of gene expression. To examine how long the $VEGF_{165}$ gene delivered by naked DNA could be expressed, we injected 100 μg of DNA to the skeletal muscles of Balb/c mice and analyzed the level of $VEGF_{165}$ produced for 16 days by ELISA (n = 10 per time point per experiment). Data from three experiments were combined for analysis, resulting in total

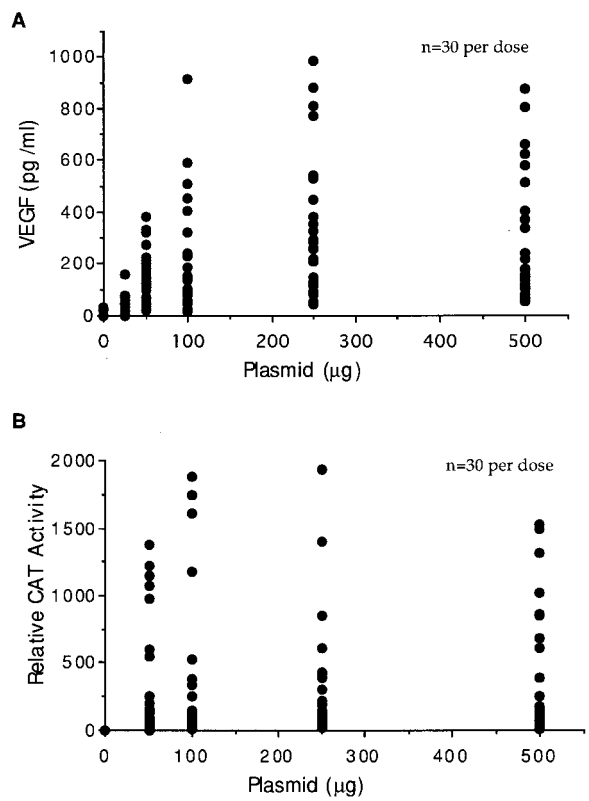

FIG. 5. Effects of plasmid concentration on the level of gene expression *in vivo.* Two days after DNA injection, muscles of mice were excised and levels of $VEGF_{165}$ (A) and CAT activity (B) were determined (n = 10 per dose per experiment). Three independent set of experiments were performed at different times and similar data were obtained in all experiments. Results from three experiments are combined here. Expression is displayed as mean ± SEM. Statistical analysis was performed using Wilcoxon two-sample test.

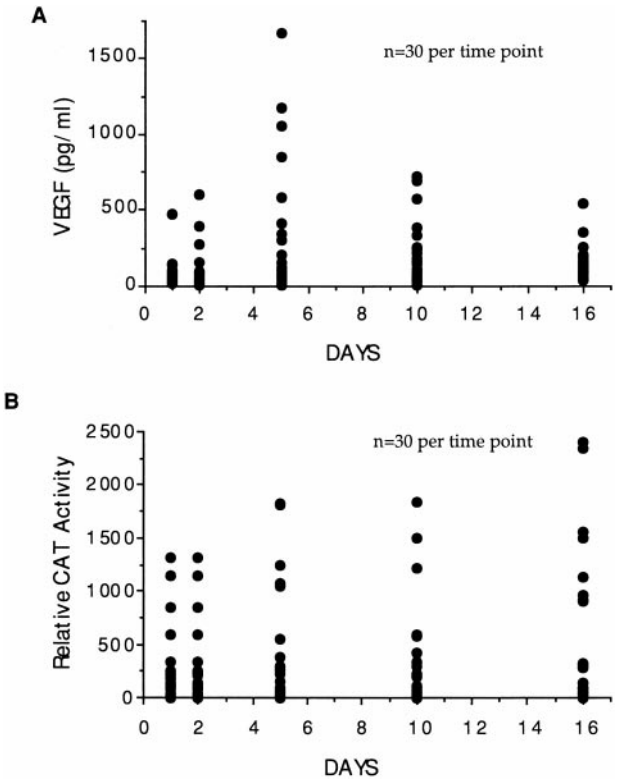

FIG. 6. Time course of gene expression after a single injection of pCK expressing $VEGF_{165}$ (A) or CAT plasmid (B). The anterior tibialis muscle of Balb/C mice after injection of plasmid were collected at each time points and assayed for $VEGF_{165}$ or CAT activity. Results from three experiments are combined for analysis. The level of expression is presented as mean ± SEM. Statistical analysis was performed using Wilcoxon two-sample test.

sample size of 30. $VEGF_{165}$ expression was detected 1 day after injection. Significant levels of $VEGF_{165}$ expression were detected even at 16 days (Fig. 6A). CAT activity was also readily detected 1 day after injection and high levels were maintained throughout the experiment (Fig. 6B; $n = 10$ per time point per experiment, total $n = 30$ from three experiments). There was no statistically significant difference in CAT expression between time points except for day 0, suggesting that the highly stable CAT protein was maintained at high levels for a long time (18).

DISCUSSION

The above results showed that our newly developed vector pCK efficiently expressed the exogenously added gene *in vivo* as well as *in vitro.* In transient transfection assays, pCK reproducibly produced much higher levels of both $VEGF_{165}$ and CAT than all expression vectors tested so far, including commercially available HCMV IE promoter-based plasmids and those using housekeeping gene promoters. The expression of the $VEGF_{165}$ gene lasted at least up to 16 days following the single injection. Because a half-life of $VEGF_{165}$ is known to be only a few minutes (19), our DNA must exist in a stable manner. Indeed, we have found pCK-VEGF in injected sites by PCR at 16 days (data not shown). However, the injected plasmid was not detected by PCR in other organs we tested, including testes, heart, liver, brain, and spleen, indicating that injected DNA does not move to other sites (20). No $VEGF_{165}$ was found in sera of all mice tested by ELISA, suggesting a highly localized expression of $VEGF_{165}$ when delivered by naked DNA (data not shown). In the case of $VEGF_{165}$, the protein level peaked at 250 μg of plasmid. Taken together, pCK is believed to produce a high level of $VEGF_{165}$ at a lower concentration of the plasmid and possibly for a longer time than the plasmids used in other studies.

We believe that pCK provides clear advantages over other previously developed plasmids, especially in the case of gene therapy for ischemic diseases. For example, in one clinical trial, almost 2 mg plasmid with a structure similar to pCP was injected into a patient (3). Based on our data, a minimum of 4-fold lower amounts of plasmid may be used to obtain the same therapeutic effects. Therefore, the pCK vector would not only significantly increase therapeutic effects at a given dose but also lower the costs of production and thus treatment. Our vector should be useful for gene therapy for any diseases that can be treated with a reasonable level of gene expression in a transient manner in a localized area.

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