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BRIEF COMMUNICATION

Muscle-specific enhancement of gene expression by incorporation of SV40 enhancer in the expression plasmid

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Skeletal muscle is established as an ideal tissue for gene delivery to treat systemic diseases. However, the relatively low levels of gene expression obtained from using naturally occurring promoters, including the strong cytomegalovirus (CMV) enhancer/promoter (E/P), have limited the use of muscle as a target tissue. The relatively weak simian virus 40 (SV40) enhancer is known to have dual functions promoting localization of DNA to the nucleus and activating transcription. An SV40 enhancer incorporated either at the 5′ end of CMV E/P or the 3′ end of the polyadenylation site gave as much as a 20-fold increase in the level of exogenous gene expression in muscle in vivo, compared with expression observed with CMV E/P alone. The minimum requirement for this enhancement is a single copy of a 72-bp element of the SV40 enhancer, in combination with either the CMV E/P or skeletal actin (SkA) promoter. Enhancement of gene expression in muscle by this SV40 enhancer was also observed by using the powerful electroporation delivery. However, the SV40 enhancer does not increase the level of CMV E/P driven reporter gene expression in dividing tumor cells in vivo and in the dividing myoblast cell C2C12 in vitro. The data suggest that including this enhancer in the plasmid will enhance the level of gene production for muscle-based gene therapy. Gene Therapy (2001) **8,** 494–497.

Keywords: *SV40 enhancer; gene therapy; electroporation; muscle*

Skeletal muscle is an attractive tissue for somatic gene delivery because it is large, has good capacity for protein synthesis, is easily accessible for intramuscular injection, and has the ability to take up plasmid after intramuscular adminstration.[1,2] Intramuscular injection of exogenous plasmids has been shown to produce low levels of transgene expression in muscle cells that persist for more than 1 year.[1–3] Muscle can therefore function as a bioreactor for synthesis and secretion of proteins, leading to systemic therapeutic effects.[4] However, one major limitation of the use of muscle as a gene therapy target has been the relatively low levels of expression achieved with current plasmid expression systems.

The cytomegalovirus (CMV) enhancer/promoter (E/P) is generally considered to be the most powerful promoter for gene therapy in muscle, but interestingly the presence of multiple CMV enhancers does not improve gene expression further. Many efforts have been made, with some progress, to improve the level of exogenous gene expression in muscle through the development of novel gene expression systems. For example, a synthetic muscle-specific promoter shows more persistent expression than the CMV promoter.[5] The combination of a strong CMV enhancer and a muscle-specific promoter shows a slight improvement in expression, compared with the CMV promoter alone.[5] An alternative approach to enhance gene expression is to increase the efficiency of DNA uptake after intramuscular injection of plasmid by physical, chemical or biological delivery systems. Although adenoviral vectors may be effective in delivering gene into the muscle and produce high level of gene expression, the immunogenicity might be a concern.[6] Electroporation is a highly effective method for increasing expression by creating transient pores in plasma membranes through which plasmids can gain entry into cells.[7–9] Another attractive strategy is to incorporate into expression plasmids a sequence that facilitates their nuclear uptake in the cytoplasm.

Simian virus 40 (SV40) enhancer has been demonstrated to exhibit transcriptional activity and, more important, to facilitate nuclear transport of plasmids from cytoplasm *in vitro*.[10,11] The ability of the SV40 enhancer to facilitate nuclear transport has been clearly shown in various cell types in culture by a combination of microinjection and *in situ* hybridization,[10,11] but no *in vivo* study has been reported. In these previous studies, only the SV40 viral enhancer increased nuclear uptake of plasmid, whereas the CMV and Rous sarcoma virus (RSV) enhancers do not. It has been shown that the 72-base pair (bp) repeat in the SV40 enhancer is responsible for this function.[12] Here, we demonstrate that a single, dual-functional 72-bp element of the SV40 enhancer increases CMV promoter-driven gene expression by as much as 20-fold in murine tibialis muscle *in vivo*.

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The CMV promoter is a strong promoter, and we were interested in using it to test whether the SV40 enhancer is able to improve the level of gene expression driven by this promoter in muscle. The plasmid contains an efficient 5′ untranslated region (UTR), a short but efficiently spliceable synthetic intron to enhance gene expression, and a 3′ hGH (human growth hormone) UTR that is efficient for protein secretion in muscle.[13] A single copy of the 72-bp repeat of the SV40 enhancer was cloned in the 5′ end of the CMV promoter and injected into mouse tibialis muscles as described in the legend of Figure 1b. Five mice were used for testing each gene construct. Significant enhancement of expression by the SV40 element was observed at both 2 and 7 days after injection ($P < 0.05$). The average secreted alkaline phosphatase (SEAP) level at day 2 was 3.5 ng/ml for mice treated with plasmid pAP1529 containing the 72-bp element of SV40 enhancer. This value is five-fold higher than that of control mice injected with plasmid pAP1166, which does not contain the SV40 element. A 20-fold difference was detected between groups 7 days after injection. This experiment was repeated, demonstrating the same results. These results clearly demonstrate that a single copy of a 72-bp repeat of the SV40 enhancer is able to significantly increase gene expression from the strong CMV promoter in muscle.

To show that the SV40 enhancer can increase gene expression driven by other promoters and that both the sense and antisense orientations are effective, a 72-bp repeat of the SV40 enhancer was cloned into the 5′ end of SkA promoter in both the forward and reverse orientations. The plasmid backbone is the same as the one described above. Luciferase was used as the reporter gene. At 48 h after injection of the plasmid DNA to 10 hind limb tibialias muscles of five CD1 mice for each gene construct, the 72-bp enhancer element in either the forward or reverse orientation increased luciferase expression (Figure 2, $P < 0.05$). When the enhancer was in the forward orientation (pLC1507), a stronger effect was observed, with expression 19-fold higher than that seen with control plasmid (pLC1072) containing no SV40 enhancer. The reverse orientation (pLC1508) showed a lower enhancement in gene expression than was observed with the forward orientation, but was still 6.6-fold higher than that of the control plasmid.

Electroporation delivery has been shown to be a very useful method for gene delivery in muscle.[14–16] *In vivo*, electroporation increases gene expression by at least two orders of magnitude over intramuscular injection alone, using either secreted or non-secreted reporter genes. To test whether inclusion of the SV40-enhancer element in the gene expression constructs was still able to enhance the level of gene expression after electroporation of plasmid DNA into muscle, three different constructs were used, each containing or lacking the SV40 72-bp sequence. Five animals were used for each gene construct. The luciferase and the SEAP constructs are shown in Figures 1a and 2a, respectively. Interleukin-2 (IL-2) constructs are described in Figure 3a, which contains a full-length SV40 enhancer (245-bp). The control IL-2 plasmid (pIL0905) contains both a strong CMV promoter and a constitutive CMV enhancer. A SV40 enhancer was cloned at the 5′ end of the strong CMV enhancer, replacing one CMV enhancer. The plasmid backbones for all three constructs were same with all the expression

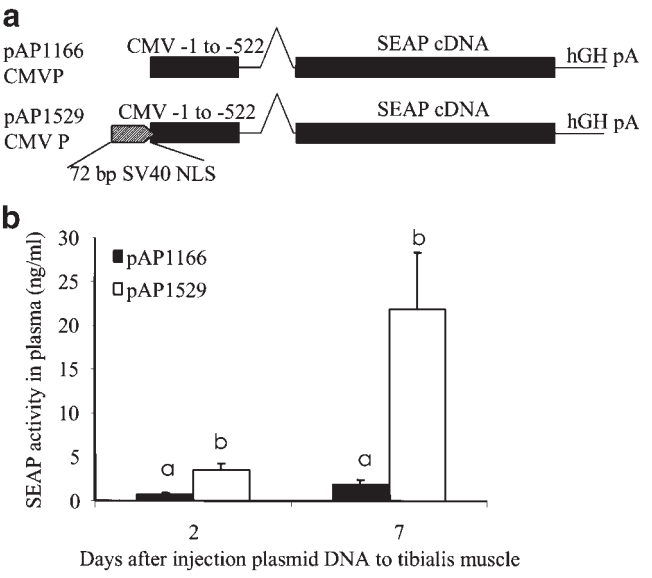

Figure 1 *Structure of pAP1166 and pAP1529 gene constructs and the effect of a single 72-bp element of the SV40 enhancer on CMV E/P-driven gene expression in vivo. (a) SEAP gene expression plasmid structure. pAP1529 is identical to pAP1166, except for the addition of the 72-bp element of SV40 enhancer. Constructs pAP1166 and pAP1529 were constructed in the Valentis plasmid backbone[13] (Valentis, Burlingame, CA, USA), which includes 107 bp of 5′ UTR (UT12), a 117 bp of 5′ synthetic intron (ivs8), a kanamycin resistance gene, and a PUC12 backbone. The constructs pAP1166 and pAP1529 contain a CMV E/P and SEAP reporter gene. A single-copy 72-bp element of the SV40 enhancer was generated by annealing two oligonucleotides (5′-AAGCTTATGC TTTGCATACT TCTGCCTGCT GGGGAGCCTG GGGACTTTCC ACACCCTAAC TGACACACAT TCCACGCTG GTTGGTACCT GCA-3′ and 5′-P-GGTACCAACC AGCTGTGGAA TGTGTGTCAG TTAGGGTGTG GAAAGTCCCC AGGCTCCCCA GCAGGCAGAA GTATGCAAAG CATAAGCTTT GCA-3′), containing sequence of –112 to –255, which is relevant to the SV40 early promoter transcription start site. This DNA fragment was subcloned into pAP1166 5′ of the CMV promoter Sse8387 site to generate pAP1529. The CMV promoter/enhancer spans –1 to –522, which is relevant to the transcription start nucleotide. (b) Comparison of SEAP expression in muscle between pAP1166 (closed bars) and pAP1529 (open bars) at days 2 and 7 after direct injection of 12.5 μg of plasmid in each tibialis muscle of CD-1 mice. All plasmids were manufactured with use of the Qiagen Giga EndoFree Plasmid Kit,[20] and DNA plasmid was formulated in 5% pvp.[21] Six- to 8-week-old male CD-1 mice (Charles River) (29–31 g) were maintained under National Institutes of Health guidelines with a 12-h day/night cycle. Each tibialis muscle was injected through the skin with a 28.5-gauge needle and a 25-μl formulation containing 12.5 μg of plasmid. Five mice were used for each group. Fifty μl of blood was collected intraorbitally from each mouse, and the serum was assayed for SEAP activity with the Phospha-Light chemiluminescent reporter assay (Tropix, Bedford, MA, USA) and an EG&G Berthold MicroLumat LB96 luminometer (Wallac, Gaithersburg, MD, USA) in accordance with the manufacturer's instructions. Five mice were used for testing the gene expression of each gene construct. Experimental data were analyzed by one-way analysis of variance, with measurement of relative light units or yield in weight as the main effect. Means of individual treatment were compared using Student's t test when the main effect was significant. Statistical significance was defined as $P < 0.05$. The other experiments described in Figures 2 and 3 were subjected to the same statistical analysis. The different alphabetical letters on the top of each bar indicate statistically significant differences.*

elements described above. The effects of the combination of electroporation and the presence of the SV40 sequence are shown in Figure 3b–d. In combination with electroporation, the expression levels of all reporter genes containing the SV40-enhancer element, either a 72-bp repeat or a full-length (245-bp) enhancer, were about two-fold

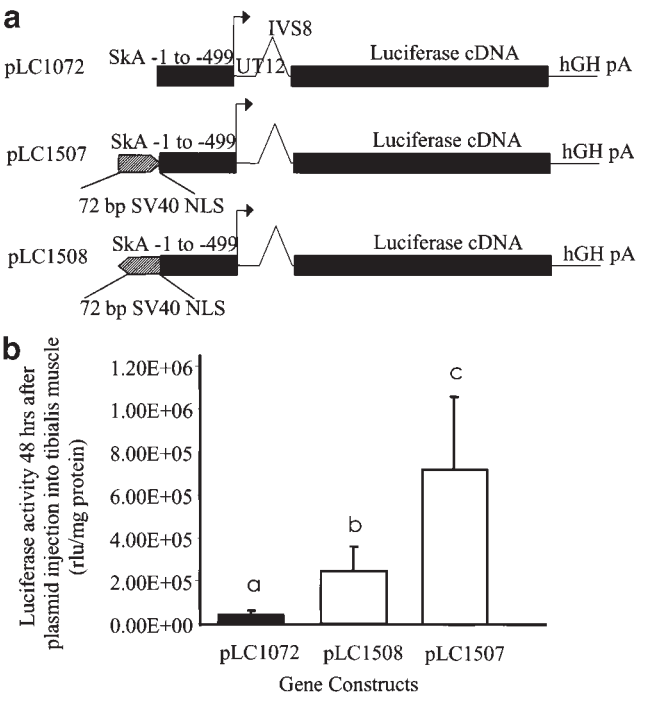

Figure 2 *Structure of luciferase expression gene constructs under control of SkA promoter and the effect of a single 72-bp element of SV40 enhancer with different orientations on gene expression in vivo. (a) Gene structures. All three constructs are identical, except that pLC1507 contains an SV40 72-bp enhancer element in the forward orientation and pLC1508 contains the same element in the reverse orientation. The construction of the 72-bp enhancer element of SV40 was same as that described in the Figure 1 legend. The reporter gene is luciferase, and the SkA promoter spans –1 to –499, which is relevant to the transcription start nucleotide. (b) Enhancement of luciferase gene expression by the SV40 72-bp element 48 h after direct injection of 12.5 μg of plasmid in each tibialis muscle of CD-1 mice. The plasmid preparation and injection procedures were the same as those described in the legend of Figure 1b. The protein extraction from the muscle and luciferase assay was described previously.[20] Five mice were used for testing the gene expression of each construct. The different alphabetical letters on the top of each bar indicate statistically significant differences ($P < 0.05$).*

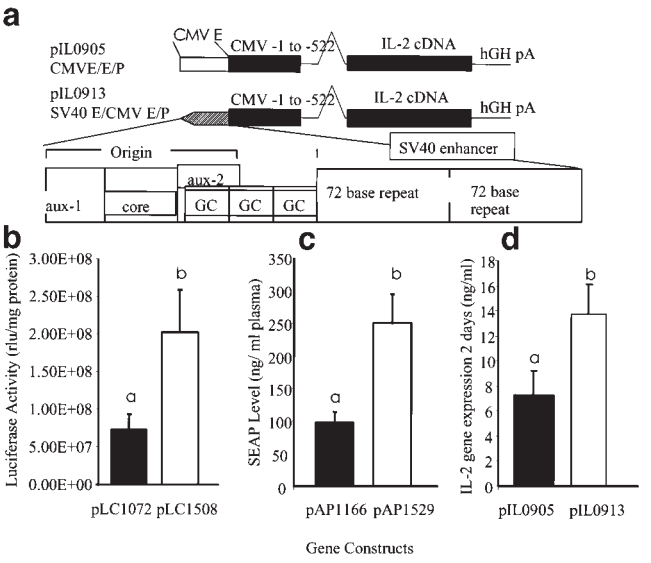

Figure 3 *Structure of IL-2 expression gene constructs and the effect of the combination of SV40 enhancer and electroporation on gene expression in vivo. (a) Structure of IL-2 gene expression constructs. The two constructs are identical, except that pIL0905 contains an extra CMV enhancer (–220 to –522 relative to transcription start nucleotide) and pIL0913 contains a full-length (245-bp) SV40 enhancer. The full SV40 enhancer was obtained from pSEAP construct purchased from Clontech (Palo Alto, CA, USA), and the CMV enhancer was derived from pAP1166. (b) Luciferase gene expression with or without SV40 enhancer delivered by electroporation 2 days after direct injection of 12.5 μg of plasmid in each tibialis muscle of CD-1 mice. For the electroporation injection, the hind leg was positioned between two stainless steel parallel plate caliper electrodes 1.5 cm² in area, which were noninvasively placed in contact with the skin, and then electric pulses were applied to the leg 2 min after the injection of plasmid DNA with a 28.5-gauge needle. The electric condition was 25 ms in duration and 375 V/cm in field intensity, and involved two pulses. The electrodes were connected to an Electro Square Porator (T830, BTX, San Diego, CA, USA). Five mice and 10 tibialias hind limb muscles were used for testing the gene expression of each construct. The different alphabetical letters on the top of each bar indicate statistically significant differences ($P < 0.05$). (c) SEAP gene expression with or without an SV40 enhancer delivered by electroporation. The procedures of administration plasmid by electroporation, blood collection and SEAP assay were the same as described above. The different alphabetical letters on the top of each bar indicate statistically significant differences ($P < 0.05$). (d) IL-2 gene expression with or without an SV40 enhancer delivered by electroporation. The procedures of plasmid administration by electroporation, blood collection and muscle extract preparation were the same as described above. The level of IL-2 in serum and muscle extract supernatant was determined by an IL-2 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions. The assay results were read by a microspectrometer (Microplate Bio Kinetics Reader EL340, Bio-Tek Instruments, Winooski, VT, USA). The different alphabetical letters on the top of each bar indicate statistically significant differences ($P < 0.05$).*

greater than those without the SV40 element ($P < 0.05$). A similar result was observed in tibialis muscle *in vivo* by electroporation delivery of GFP (green flurorescence protein) expression plasmid with or without the full length SV40 enhancer (data not shown).

The data derived from the use of different reporter genes – GFP, SEAP, luciferase, and IL-2 – clearly demonstrate that the SV40 enhancer increased gene expression in muscle *in vivo*. To our knowledge, this is the first report demonstrating that a combination of SV40 enhancer and CMV E/P enhances gene expression in muscle 20-fold, compared with the CMV E/P alone. A single 72-bp repeat element of the SV40 enhancer is sufficient to yield an enhanced gene expression comparable to the enhancement of nuclear plasmid uptake obtained *in vitro* with the full-length sequence, suggesting that it is not necessary to use the whole SV40 fragment.[11,17] This is supported by our *in vivo* result (Figure 3), in which a two-fold increase was observed in muscle with either a full-length SV40 enhancer (pIL0913) or a 72-bp fragment (pAP1529 and pLC1508). Moreover, the 72-bp sequence does not contain the origin of replication of SV40, suggesting that the increase in gene expression was not related to potential gene replication in muscle.

Electroporation is a powerful delivery technique that has recently been utilized for plasmid-based gene therapy, increasing expression about 100-fold for any given reporter gene expression plasmid tested.[14–16] Under these conditions of much higher basal expression, the net effect of the SV40 sequence on expression was still observed, although greatly reduced at about a two-fold effect. With regard to the reduced effect of SV40 enhancer by electroporation delivery, we speculate that (1) electroporation augmented direct shuffling of DNA plasmid into the

nucleus by creating transient pores on the membrane of nucleus, thus, making the nuclear localization enhancer no longer important; and (2) the high concentration of DNA plasmid in cytoplasm by electroporation delivery saturated the nuclear translocation mechanism.

Although the mechanism of action of the SV40 enhancer in increasing gene expression in muscle, even with the strong CMV E/P, is speculative, several lines of data suggest the involvement of nuclear localization. First, *in vitro* data are consistent with an enhancement of nuclear localization of DNA.[12,18] Second, the SV40 enhancer does not increase expression levels in dividing tumor tissue *in vivo* and murine muscle-derived dividing cells *in vitro*; in contrast there is a significant enhancement of gene expression levels in the post-mitotic, terminally differentiated muscle. Third, the increase in expression caused by the SV40 enhancer can be observed in the presence of the powerful CMV E/P. It is unlikely that further enhanced gene expression by SV40 enhancer is due to transcriptional activation because CMV E/P is very strong.[19]

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