CONSTRUCTION AND ANALYSIS OF TRUNCATED MUSCLE-SPECIFIC PROMOTERS

*Wang, B; *Zhou, LQ; *Kotchey, N; *Zhu, T; *Tian, MN; **Jiang, XC; *Li, J; +*Xiao, X
+ University of Pittsburgh, Pittsburgh, PA.
xiaox@pitt.edu

INTRODUCTION The tissue-specific promoters are highly desirable for the purpose of gene therapy in muscular disorders, but the full length muscle creatine kinase promoter (MCK) is not well suited for adeno-associated viral (AAV) vectors due to size constraints. Our aim is to develop multiple, greatly compact, highly active yet extremely tissue-specific promoters, of suitable size to be packaged into AAV viral vector containing the mini-dystrophin gene. Here we have constructed chimeric MCK promoters and compared the activity and tissue-specificity of new promoters in vitro and in vivo.

MATERIALS AND METHODS Construction of MCK Promoters and AAV Vector Production: The truncated chimeric promoters containing one, two, or three modified MCK enhancers (2R5S) along with the minimal MCK promoter driving reporter luciferase gene (Luc) were packaged into AAV vector. The serotype II AAV vectors were made according to a previously described method. Transfection of 293 Cell Line and Myotubes in vitro: Transfection of 293 cells was done by the calcium phosphate transfection method 1. Transfection of murine C6C12 myoblasts was performed by the Lipofectamine Plus Reagent Kit. The cells were monitored in day 1, 2, 3, and 4 to observe differentiation.

LacZ and AAV Vector Production: The truncated chimeric promoters containing one, two, or three modified MCK enhancers were packaged into AAV vector. The serotype II AAV vectors were made according to a previously described method. Transfection of 293 Cell Line and Myotubes in vitro: Transfection of 293 cells was done by the calcium phosphate transfection method 1. Transfection of murine C6C12 myoblasts was performed by the Lipofectamine Plus Reagent Kit. The cells were monitored in day 1, 2, 3, and 4 to observe differentiation.

Luciferase Assay and Dystrophin Immunostaining: For the in vitro and in vivo studies, the luciferase activities were determined by a Luminometer. Immunostaining against dystrophin was performed according to a previously described method. Creation of Lac Z and Human Mini-Dystrophin Transgenic Mice: The transgenic mice were generated to test dMCK promoter activity according to standard procedures. The transgenic mice were identified by PCR procedures. The PCR positive founders of Lac Z were bred to C57/BL10 or mdx mice at 3-month-old via a breeding process. The human-mini-dystrophin transgenic mice mated with homozygous mdx females to obtain a homozygous mdx background.

RESULTS For the in vitro C6C12 transfection experiment, the results showed that the levels of luciferase activity achieved by the chimeric promoters, especially the modified construct dMCK (double-modified enhancers) and tMCK (triple-modified enhancers), were significantly higher (> 10 fold) than the original truncated MCK promoter (regular enhancer) in differentiated C6C12 cells (Fig. 1).

For in vivo animal studies, both the dMCK and the CK6 promoters activities demonstrated the similar strength to the CMV promoter in the TA muscle, while the tMCK promoters activity was 4-fold than the CMV promoter (dMCK, tMCK, and CK6) showed significantly higher levels of luciferase expression compared with the original small MCK group (p<0.005). Particularly, the luciferase activity generated by the tMCK promoter was 18-fold higher than the original MCK promoter (Fig. 2).

The CMV promoter revealed a high level of luciferase gene expression in 293 cells and the liver when compared with MCK promoters (data not shown), indicating that the modified MCK promoters were highly active and also exceedingly tissue-specific.

The dMCK-LacZ transgenic mice strongly expressed LacZ in skeletal muscles at day 10. The results showed that whole bodies of transgenic LacZ mice all were strongly positive. The high levels of Lac Z gene expressions were observed in the different muscles such as soleus (HL), diaphragm muscle (DIA), intercostal muscle (ITM), hind limbs (HL), and facial muscles (FAC) (Fig. 3). To determine the activity of the dMCK promoter in mdx mice, a small animal model commonly used for DMD, the Lac Z transgenic mice bred with either C57/BL10 or mdx mice. Both strain showed dramatic expression of the Lac Z gene in the incision of the hind limbs at 20 days without difference.

In further studies, the 6-month-old Dys-Tg mdx mice demonstrated that the dMCK promoter has more activity in the fast skeletal muscles (GA, TA, QUAD, and ITM), but weak or absent in slow muscles (DIA and heart) (Fig. 5), which is in agreement with previous results.

DISCUSSION A short regulatory sequence with muscle-type-specific activities were found within the 352 bp C12 promoter region. The enhancer activates the MCK promoter only in differentiated muscle cells. Several of these constructs contain a mutagenized MCK enhancer that significantly increased gene transfer plus a truncated MCK promoter region. The CK6 promoter containing 2RSS enhancer and a proximal promoter extending from -358 to -7 bp relative to the transcriptional start site were used to drive a mini-dystrophin gene. However, we are still seeking an optimal small promoter that would satisfy the requirement for AAV vector delivery in muscle tissues. In these studies, we designed the two or three tandem modified MCK enhancers (2RSS) plus a minimal promoter region (-80 to +7 bp) to drive luciferase, LacZ, and human mini-dystrophin. The results confirmed that the new promoters with smaller size (500 to 750 bp) are suitable for gene vectors such as AAV, and can also benefit the gene therapy for DMD.

REFERENCES