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Cloning and transcriptional activity analysis of the porcine cofilin 2 gene promoter

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ABSTRACT

Cofilins (CFL), including CFL1 and CFL2, are members of the family of actin-binding proteins in eukaryote. CFL2 is predominantly expressed in mammalian skeletal muscle and heart and is important to muscle fiber formation and muscular regeneration. To study transcriptional regulation of porcine *CFL2*, a 2.5 kb upstream sequence starting from the major *CFL2* transcriptional start site was cloned by genome walking. Twelve DNA fragments of the 5′ flank region of the porcine *CFL2* gene were further isolated from porcine genomic DNA via PCR and inserted into the luciferase reporter vector pGL4.10 to make 12 *CFL2* reporter constructs. All reporter vectors were transfected into C2C12, NIH3T3, or Hela cells and their relative luciferase activity measured after 48 h, respectively. Bioinformatics analysis suggested that there were two TATA-boxes at the −508 bp and −453 bp, as well as a GC-box and a CAAT-box in this sequence. Additional transcription factor binding sites including SP1, AP1, AP2, and GATA-1 sites were also predicted. The transcriptional activity of pGL4.10-1554 (1502 bp to +51 bp) was the strongest, and the promoter's active region was mapped to a region from −1502 bp to −1317 bp. Our data provide a foundation for future studies into transcriptional regulation of *CFL2*.

1. Introduction

Cofilin (CFL) is an actin-modulating protein which is widely distributed throughout muscle and non-muscle cells. In the mammal, a ubiquitously expressed cofilin (CFL1) and a skeletal muscle-specific cofilin (CFL2) have been described (Gillett et al., 1996; Ogawa et al., 1990; Ono et al., 1994). Many CFL homologs have been characterized biochemically and genetically in various organisms (Maekawa et al., 1984). The strongest expression level of *CFL2* was observed in skeletal muscle and in the heart, while *CFL1* expression level is strongest in the brain (Ono et al., 1994). CFL2 is an important regulator of actin dynamics (Maciver and Hussey, 2002; Papalouka et al., 2009; Thirion et al., 2001) and has recently been shown to be essential for muscle maintenance (Agrawal et al., 2012). The *CFL2* gene has been spliced into two transcripts, CFL2a and CFL2b, by alternative use of exon1a and exon1b in humans and in mice, and an expressed sequence tag homologous to murine CFL2 was mapped to human chromosome 14 (Gillett et al., 1996). The porcine *CFL2* gene was isolated in 2005 using candidate gene strategy methods and gene expression profile analytical chips (Song and Su, 2006; Song et al., 2008; Zhao et al., 2007). Porcine *CFL2* has two transcript variants: CFL2a and CFL2b, and the CFL2b amino acid sequence is 99% identical to the previously described murine CFL2, and 100% identical to human CFL2b. CFL2b in porcine muscle has a long transcript of 3012 bp, and a short transcript 1466 bp, whereas the human CFL2b short transcript is 1.65 kb and the long transcript is 3.3 kb. The mouse in the human cofilin, and CFL2b short transcript is 1.8 kb and the long transcript is 3 kb (Zhao et al., 2009).

In mammalian cells, the promoter is a complex region containing many binding sites of proteins that regulate gene expression. The promoter determines the transcriptional start point and the frequency with which the gene is transcribed, so promoter recognition is important for genome annotation (Lefaucheur et al., 2002). The genome-walking method can be used to identify unknown regions flanking a known DNA sequence, and using PCR to isolate unknown flanking regions is efficient, fast, and does not require a DNA construct or screening libraries. Several genome-walking methods have been developed for cloning the flanking regions of a gene with unknown restriction sites (Kilstrup and Kristiansen, 2000; Mishra et al., 2002; Nthangeni et al., 2005).

To study the mechanism of porcine *CFL2* transcription, we cloned the 5′-flanking region of the porcine *CFL2* gene using genome walking and analyzed the sequence with bioinformatics software (Neural Network

Abbreviations: CFL2, cofilin 2; dNTP, deoxyrinediamine triphosphate; BLAST, basic local alignment search tool; Taq, thermus aquaticus; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; IPTG, isopropyl-β-D-thiogalactopyranoside; EB, ethidium bromide; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid disodium salt; OD, optical density; *E. coli*, *Escherichia coli*; MyHC, myosin heavy chains; MyoD, myogenic determining factor; MRF, myogenic regulating factor.

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Promoter Prediction, CpG Island Search and TESS). We then inserted the promoter fragments into luciferase reporter vectors pGL4 to generate reporter constructs which were subsequently transfected into C2C12, NIH3T3, or Hela cells and their relative luciferase activity measured. Our data show that the active region of the *CFL2* promoter is from −1502 bp to −1317 bp. Also, various transcription factor binding sites were found in the 5′-flanking region of porcine *CFL2*. This work will provide a launch point for future investigations into the relationship between *CFL2* expression regulation and muscle development.

2. Materials and methods

2.1. DNA preparation

The genomic DNA of adult porcine skeletal muscle was prepared using standard protocols according to the manufacturer's instructions for the Universal Genomic DNA Extraction Kit Ver. 3.0 (TaKaRa).

2.2. Cloning of the 5′ flanking region of CFL2 by genome walking

The 5′ flank region of *CFL2* was cloned using a Genome Walking Kit (TaKaRa). The genome walking fragment was amplified by using universal primers (AP1–AP4) and the gene-specific primer SP1–SP3 (SP1 5′-AGAAAGAGCAGTTCCTCTGT-3′, SP2 5′-CAAAGGCAAAACTGACTCTG-3′ and SP3 5′-AAAGATGTGTAGGGGTCCTC-3′) which were designed according to the porcine *CFL2* CDS sequence (GenBank EU561660 and EU561661). In the first PCR amplification, the SP1 adaptor and AP1–AP4 primers were used, with 1 μl (containing ca. 1 μg of DNA) of the genomic DNA as template. Cycles were as follows: denaturation at 94 °C for 1 min, 98 °C for 1 min, 5 cycles of 30 s at 94 °C, 1 min at 65 °C, and 2 min at 72 °C, then 94 °C for 30 s, 25 °C for 3 min and 2 min at 72 °C and at last, 45 cycles of 94 °C for 30 s, 1 min at 65 °C, 72 °C for 2 min, followed by a 10-min extension at 72 °C, after which the products were stored at 4 °C until the second amplification. Next, 1 μl of the first PCR product was used as the template, and the primers were SP2 and AP1–AP4. Analysis of PCR products was carried out by electrophoresis of 10 μl of the reaction mixture on a 1% agarose gel. The purified product of the nest PCR was cloned into a pMD18-T vector and sequenced with M13 primers from both strands by TaKaRa Biotechnology (Dalian, China).

2.3. Bioinformatics analysis

The 5′-flanking region of the *CFL2* sequence was assembled with DNAMAN software. The core active area of this promoter was predicted using the online tool, Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html). CpG islands were predicted using CpG Island Searcher (http://cpgislands.usc.edu/) with the lower limit values of Obs/Exp >0.6 and a GC content >50%. Putative binding sites of the main transcription factors in the promoter region of the porcine *CFL2* gene were predicted using the TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME) and TFSEARCH.

2.4. Stepwise truncation of the 5′-upstream region of the porcine CFL2 gene

To produce the luciferase construct harboring DNA fragments of different sizes, twelve DNA fragments of the 5′-flank region of the pig *CFL2* gene were isolated from porcine genomic DNA using PCR, including 222 (nt −170 to +51, using primers P1 and F), 383 (nt −331 to +51, using primers P2 and F), 482 (nt −430 to +51, using primers P3 and F), 666 (nt −614 to +51, using primers P4 and F), 912 (nt −860 to +51, using primers P5 and F), 1021 (nt −968 to +51, using primers P6 and F), 1093 (nt −1040 to +51, using primers P7 and F), 1305 (nt −1253 to +51, using primers P8 and F), 1369 (nt −1317 to +51, using primers P9 and F), 1554 (nt −1502 to +51, using primers P10 and F), 1680 (nt −1628 to +51, using primers P11 and F), and 1826 (nt −1774 to +51, using primers P11 and F). Primer sequences are shown in Table 1. Stepwise truncations were digested with two restriction enzymes *Kpn*I and *Nhe*I. The PCR cycle was initiated by denaturing at 94 °C for 2 min followed by 35 cycles of 30 s at 94 °C, 30 s at 53 °C, and from 45 to 90 s (time was determined according to each fragment's length) at 72 °C each with a final extension step of 10 min at 72 °C. The products were purified using a DNA Gel Extraction Kit (AxyPrep, America). All recombinant constructs were purified and sequenced; and their quantity and quality were routinely checked using agarose gel electrophoresis and $OD_{260/280}$ readings.

Table 1
Primer used for *CFL2* promoter reporter construction.

Name	Sequence (5′ to 3′)[a]
P1	CGGGGTACCAACATTTCAGGGATGGGACAGC
P2	CGGGGTACCTATGAGGAAACGGCACTGGCTA
P3	CGGGGTACCCGATCCGATCCGTTAGTTGTGA
P4	CGGGGTACCCCTTTGCCTTCCGCTACCTAAT
P5	CGGGGTACCCCGCCATTTTAACCTGATTTTG
P6	CGGGGTACCGGCACTATGGTAAGGCGGGAGC
P7	CGGGGTACCCTCCTTCTGCTCGCATCGCC
P8	CGGGGTACCGTAGAGGCCTCCTCGCCAGGT
P9	CGGGGTACCCTCGGGGAAGCAGTGTAGAAAC
P10	CGGGGTACCCCCCCCCATAGTGTTTATTCTC
P11	CGGGGTACCCCAAGGAATGTTACTACCCAAT
P12	CGGGGTACCAGAGATTGCAGTTGCCAAGACG
F	CTAGCTAGCGCCCAGGGAGGATTCACTTTAG

[a] Restriction sites are underlined.

2.5. Luciferase constructs

The purified products were cloned into the pMD18-T vector (TaKaRa) to produce recombinant constructs (pMD18-T −222 to pMD18-T −1826) which were confirmed with sequencing. The twelve fragments were excised from the constructs pMD18-T −222 to pMD18-T −1826 with *Kpn*I and *Nhe*I, and then subcloned into the pGL4.10 luciferase reporter vector (Promega) directly upstream of the luciferase translation start site to produce pGL4.10 −222 to pGL4.10 −1826. In the luciferase experiments, pGL4.74 Renilla was used as an internal control for transfection efficiency. Promoter activity was normalized to pGL4.74 Renilla and was represented as the average of data from three replicates; the empty luciferase vector pGL4.10 [luc2] without treatment was arbitrarily assigned a unit of 1, and results are expressed as multiples of the pGL4.10 [luc2] vector.

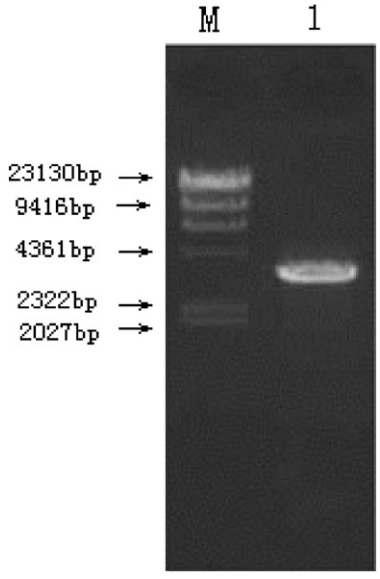

Fig. 1. Results of AP3-3rd test by bacteria liquid PCR. M: λ-Hind III digest DNA Marker 1: AP3-3rd PCR product.