

*Full Length Research Paper*

# Molecular cloning and sequence analysis of the cat myostatin gene 5' regulatory region

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**Cat is an important experimental and pet animal and plays a key role in keeping ecological balance. Myostatin is a negative regulator of skeletal muscle growth and development in mammals, but the gene sequence of it keeps unknown in cat. To better understand the structure and function of the myostatin promoter in cat, a 1409 bp fragment containing the 5'-regulatory region of the cat myostatin gene was cloned and sequenced (GenBank accession number is GU938462). Many potential transcription factor binding sites have been found by the bioinformatics analysis, such as TATA boxes, CAAT box, E-boxes, MEF2, MEF3, MTBF, PAX3, SMAD, HBOX, HOMF and TEAF motifs. Comparative analysis for some motifs showed both conservations and differences among cat, horse, porcine and human.**

**Key words:** Cat, myostatin 5'-regulatory region, molecular cloning, sequence analysis and comparison, transcription factor binding sites.

## INTRODUCTION

Myostatin, also named growth and differentiation factor 8 (GDF8), was first described by McPherron and Lee (1997). It is a member of the transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily and functions as a negative regulator of skeletal muscle development and growth in mammals. Natural mutations in the myostatin gene are responsible for the double muscling phenotype in several European cattle breeds and the gross muscle hypertrophy in a child (McPherron and Lee, 1997; Kambadur et al., 1997; Grobet et al., 1997; Schuelke, 2004), while administering myostatin can induce the cachexia in mice (Zimmers, 2002). Compared with the functional mechanism of the myostatin, little is known about the transcriptional regulation of the gene. Several earlier studies indicated that myostatin gene expression is regulated at the transcriptional level (Kambadur et al.,

1997; McPherron et al., 1997). The sequence of the 5' regulatory region in human, mouse, porcine, bovine, sheep, goat, horse and dog were cloned, and some of them have been characterised and investigated primarily (Ma et al., 2001; Spiller et al., 2002; Crisa et al., 2003; Salerno et al., 2004; Du et al., 2005, 2007; Allen and Du, 2008; Grade et al., 2009; Liu et al., 2010). Grade et al. (2009) identified a 260 bp long, evolutionary conserved region upstream of tetrapod myostatin and teleost myostatin b genes and the conserved fragments (as a minimal promoter) was able to drive the reporter gene expression in C2C12 cells. In spite of some level of conservation, many differences were also found among the animals (Du et al., 2005; Grade et al., 2009). Cat is an important experimental and pet animal, and plays a key role in keeping ecological balance. However, the cat myostatin gene sequence keeps unknown. To better understand the structure and function of the myostatin promoter in cat, we first cloned and sequenced a 1.409 kb fragment containing the 5'-regulatory region and part of the coding sequence of the cat myostatin gene (GenBank accession number is GU938462). Many potential transcription factor binding sites were identified by the bioinformatics tools to further investigate the transcriptional regulation of the

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**Abbreviations:** GDF8, growth and differentiation factor 8; TGF- $\beta$ , transforming growth factor  $\beta$ .

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-1212 AGACCTTACC CCAATCCCT E Box7GCAGGTC TGCCCTCTGG TCAAAATGAG AAACTGGCAA
-1152 AGGGGTGCAA AE Box7CCAATCGTA GTATTGGGGA ATGTTAAAAA CGGACACCCC TTTATTATGA
-1092 TGCTCTTTCA CTTTTATGTG CTGACAGTAT TTTGATATAA TTTACATAGA ATGGATACTA
-1032 CGCTTTTTAC TTTTGE Box7CCAAT GGAAATCTGA GGCAAAGTGT GCTTGCGTGC TGCATTGCCA
-972 GTCGTAE Box7AAAC CCATTGCTT CTTATTCTGA GTTCAGAGAT TTTTCTACT AGCTGACATT
-912 ATCCTCTTGG TAATAAACAA TGAAGAAACA CATCTCTGA ATAATACTAA TCTGCAGCTT
-852 TACAAGAGGA AAGAAGTAA TAGTAATCAG CCAAAAATTG AGCACCATT TCATAGTAAT
-792 GAAAAACATT AAATTATTTA AAAATAATTC E Box6CATGTGTAAT ATAACATTAT AATAAGTTGA
-732 TTTTTAACGT GTGCTAAGAA TTTAAACAGG AAAACAAGCC TCTCAAATTT TAGCTE Box6CCAAT
-672 E Box6CTTTTACTA GTAACACTAT CTTAGCTTTT CTTTAAATTC AGGTCTTCTT AACTTAAATT
-612 CATATTTCCC TAATTACAGG GCACTTAAAC ATAATTTAAT ACAGTAAATA AAATTTATTT
-552 TACTTE Box5CAAAT GCTTGCCTAA ATAACAGATA CTCATTTTAA TTTTAAAGGA AGTAACATT
-492 CAACTTTTAA AGCATGAAGT GTAAAACATA GATTTACTTA TTTAAATTAT AACCTGAGTT
-432 E Box4TCACATGTAA AGATTAAGAA GATTTAAGTG TAGTTTCTAT TATTGTTAAAC ATAGACTTTA
-372 TTTTTTTTTT E Box3AAATGTCACA TATATCTTTC ATTATTGTGA GATTTATTTT TTTTATGAAG
-312 TAGTCGAATG AATCAGCTCA CCCTTGACTG TAACAAAATA CTGTTTGGTG ACTTGTGACA
-252 GACAGGGTIT TAACCTCTGA CAGCGAGATT CATTGTGGAG CAAGAGCAAT BoxCCAAT CATAGATCC
-192 TGACGCAE Box2CACT TGCTCATCA AAGTTGGAATATA BoxTATAAAAAGCC E Box1ACTTGGAAATA CAGTATA BoxTATAAAA
-132 GATTCACCTGG TGTGGCAAGT TGCTCTCAG ACTGTACAGG CATTAAAATT GTGCCTGGCA
-72 TTAE Box2CTCAAAA GCAAAAGAAA AGTAAAAGA AGAAATAAGA ACAAGGAAAA AGATTGTATT
-12 GATTTTAAAA +1TCATGCAGAA ACTGCAATC TATGTTTATA TTTACCTGTT TATGCTGATT
+49 GTTGCTGGTC CAGTGGATCT AAATGAGAAC AGTGAGCAAA AAGAAAATGT GGAAAAAGAG
+109 GGGCTGTGCA ATGCATGTAC TTGGAGACAA AACACTAAAT CTTCAGAAT AGAAGCCATA
+169 AAAATTCAGA TCCTCAGTAA ACTTCGCCT

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**Figure 1.** Sequence and sequencing analysis of the myostatin 5'-regulatory region in cat.

myostatin gene in cat.

## MATERIALS AND METHODS

### Polymerase chain reaction (PCR) condition

A 1.409 kb myostatin gene fragment containing the promoter sequence, 5' UTR and part of the coding sequence was amplified by polymerase chain reaction (PCR) from the cat (in China) genomics DNA. Primers were designed based on regions conserved in other animals as follows: sense primer, 5' AGACCTTACCCCAAATCCCTG 3' and anti-sense primer, 5' AGGCGAAGTTTACTGAGG 3'. The PCR reaction was performed in a 25  $\mu$ l reaction containing 220 ng of cat genomics DNA, 1 $\times$ Taq reaction buffer, 5 nmol dNTPs, 20 pmol of each primer and 0.25 units of Taq DNA polymerase (Takara). The PCR program was carried out for an initial 5 min 94°C denaturing step, 30 cycles (each cycle included 30 s at 94°C, 30 s at 56.2°C and 1.5 min at 72°C) and a final 10 min extension at 72°C in a G-STORM T-gradient thermocycler.

### Sequencing and analysis

The myostatin fragment was cloned into pMD18-T Vector (Takara) and

sequenced with an ABI automated DNA sequencer after the agarose gel extraction using gel extraction kit (BioDev). Primers used for sequencing the entire 1409 kb fragment were as follows: RV-M primer, 5' AGCGGATAACAATTTACACACAGG 3'; M13-47 primer, 5' CGCCAGGGTTTTCCAGTCACGAC 3'. Database searches and sequence alignments were performed using the NCBI (National Center for Biotechnology Information) and DNAMAN software. The transcriptional response elements were analyzed by the MatInspector software (Genomatix Software). The analysis criteria was selected as follows: the maximum core similarity (1.0) and the higher matrix similarity (>0.80).

## RESULTS

### Cloning and characterization of the cat myostatin promoter

The 1.409 kb myostatin gene fragment containing the 5'-regulatory region in cat was amplified, cloned and sequenced (Figure 1). It was deposited in the Genbank (the accession number is GU938462). The MatInspector analysis revealed that there are many potential

**Table 1.** Distribution and description of selected transcriptional response elements of the cat myostatin 5'-regulatory region.

Matrix family	Detailed family information	Matrix	Detailed matrix information	Position
VTBP	Vertebrate TATA binding protein factor	VTATA.01(TATA box)	Cellular and viral TATA box elements	-166/-150(+); -142/-126(+)
		VTATA.02	Mammalian C-type LTR TATA box	-1088/-1072(-); -329/-313(-)
		MTATA.01	Muscle TATA box	-720/-704(-); -644/-628(+); -455/-439(+)
CAAT	CCAAT binding factors	CAAT.01(CAAT box)	Cellular and viral CCAAT box	-210/-196(+)
		NFY.04	Nuclear factor Y (Y-box binding factor)	-1145/-1131(+); -1021/-1007(+); -680/-666(+)
MEF2	myocyte-specific enhancer binding factor 2	MEF2.06	Myocyte-specific enhancer factor 2	-782/-760(+)
		MEF2.01	Myocyte-specific enhancer factor 2	-565/-543(-)
MEF3	MEF3 (myocyte enhancer binding factor 3) binding sites	SIX.01	Binding sites for Six1, Six4 and Six5	-445/-433(-)
HMTB	Human muscle-specific Mt binding site	MTBF.01	muscle-specific Mt binding site	-1201/-1193(-); -535/-527(-)
SMAD	Vertebrate SMAD family of transcription factors	SMAD.01	Sma- and Mad-related proteins	-1186/-1178(+)
PAX3	PAX-3 binding sites	PAX3.01	Pax-3 paired domain protein, expressed in embryogenesis	-204/-186(-)
HBOX	Homeobox transcription factors	MEOX1.01	Mesenchyme homeobox 1	-1062/-1044(-)
		EVX1.01	Even-skipped homeobox 1	-836/-818(-)
		GSH1.01	Homeobox transcription factor Gsh-1	-806/-788(+); -605/-587(-); -565/-547(-); -347/-329(-)
		EN1.01	Homeobox protein engrailed (en-1)	-625/-607(-); -594/-576(-); -457/-439(-); -418/-400(+)
HOMF	Homeodomain transcription factors	GSH2.01	Homeodomain transcription factor Gsh-2	-609/-591(-); -608/-590(+)
		MSX2.01	Muscle segment homeo box 2, homologue of Drosophila (HOX 8)	-609/-591(+)
TEAF	TEA/ATTS DNA binding domain factors	MSX.01	Homeodomain proteins MSX-1 and MSX-2	-522/-504(+)
		TEAD.01	TEA domain-containing factors, transcriptional enhancer factors 1, 3, 4, 5	-1129/-1117(-)

transcription factor binding sites located in the cat myostatin gene upstream sequence. According to previous works, we focused on analyzing and discussing the motifs listed in Table 1, including some general core promoter elements (TATA box and CAAT box), several muscle-specific transcription factor binding sites (MTATA,

MTBF, MEF2 and MEF3) and some elements playing important role in myogenesis (PAX3, SMAD, HBOX and HOMF, TEAF). In addition, according to the core sequence "CANNTG", we found seven potential E-boxes (motifs existed in many muscle-specific genes promoter) within 1.212 kb of the cat myostatin gene upstream





**Figure 2.** Alignment analysis of the myostatin 5'-regulatory region sequence among cat, horse, porcine, human, bovine, sheep and mouse.

sequence (Figure 1).

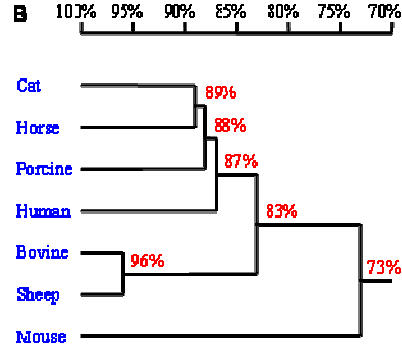
**Comparative analysis of the myostatin promoter sequence and motifs**

Comparison of the 1.212 kb myostatin 5'-regulatory region sequence among seven animals revealed a high degree of conservation during evolution (Figures 2 and 3). The similarity between cat and horse (NW-

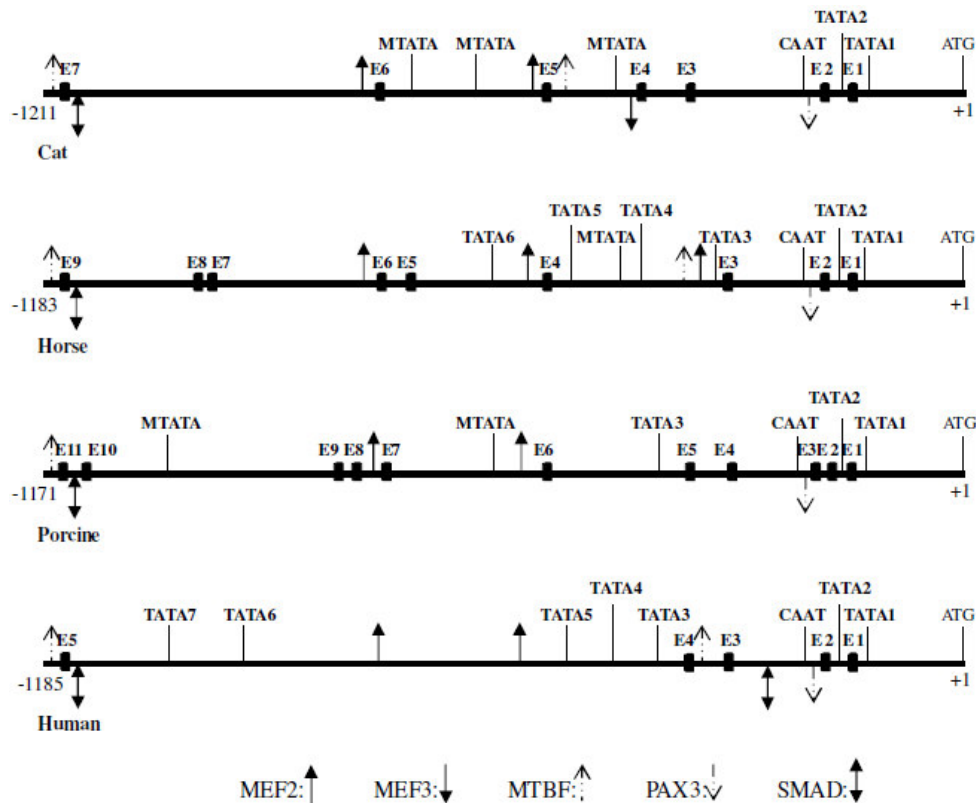
001867384), porcine (AY527153), human (NG-009800), bovine (AJ438578), sheep (AY918121) or mouse (AY204900) myostatin gene upstream regions were found to be 89.4, 87.6, 87.1, 82.4, 80.9 and 74.3%, respectively. It is remarkable that the conservation degree is higher in two positions (1 to 70 and 874 to 1215 bp from the left) among these animals (Figure 2). The first conserved sequence (1 to 70 bp) probably corresponds to a mammal enhancer of the myostatin gene while the second conserved region (874 to 1215 bp) contains the

**A. Homology matrix of 7 sequences**

Cat	100%								
Horse	89.4%	100%							
Porcine	87.5%	87.9%	100%						
Human	87.1%	88.0%	86.2%	100%					
Bovine	82.4%	83.9%	88.5%	81.5%	100%				
Sheep	80.9%	82.4%	86.7%	80.2%	95.7%	100%			
Mouse	74.3%	75.3%	74.2%	76.1%	69.6%	68.5%	100%		



**Figure 3.** Homology matrix(A) and tree(B) of the myostatin 5'-regulatory region sequence among cat, horse, porcine, human, bovine, sheep and mouse.



**Figure 4.** Comparison of some important motifs along 1.211kb myostatin 5'-regulatory region sequence among four mammals.

myostatin promoter and 5'UTR.

The conservation and variation of some important regulatory motifs among four animals' myostatin gene 5'-regulatory region were compared (Figure 4). TATA-boxes 1, 2 and CAAT-boxes along the myostatin 5'-regulatory region are conserved in both position and sequence among four mammals. However, other TATA-boxes and MTATA motifs were not entirely the same for the four mammals analysed. As far as the E-boxes along the

myostatin gene 5'-regulatory region are concerned, the number is different and the position was not entirely the same among the four mammals. Out of the seven E-boxes (E) in 1.211 kb of the cat myostatin 5'-regulatory region, three E-boxes are conserved in position and sequence among the four mammals. E-boxes 1, 2 and 7 of the cat are at almost the same positions as E1, E2 and E9 of the horse, as E1, E3 and E11 of the porcine and as E1, E2 and E5 of the human. However, other E-boxes

were not entirely the same for the four mammals. In addition, some important motifs such as PAX3 (-204/-186bp), MEF2 (-782/-760 bp, -565/-543 bp), SMAD (-1186/-1178 bp), MTBF (-1201/-1193 bp) of the cat myostatin 5'-regulatory region were found in similar positions of other three animals. Of particular interest was the presence of MEF3 motif along the cat myostatin 5'-regulatory region different from other animals.

## DISCUSSION

The detection of some transcription factor response elements within 1.211 kb cat myostatin gene upstream sequence (Table 1), suggested these potential regulatory motifs may play important roles in the regulation of the cat myostatin expression and therefore on muscular development. The high conservation of some motifs such as several E-boxes, MEF2, MTBF, PAX3 and SMAD among four animals further implied the importance of these motifs.

Some motifs (E-boxes, MEF2, MTBF and SMAD) along the myostatin 5'-regulatory region have been found and identified in human, mouse, bovine or sheep. The E-box is one of the sequence motifs for binding of the basic helix-loop-helix myogenic regulatory factors (MRFs) including the MyoD, Myf5, myogenin and MRF4 transcription factors (Apone and Hauschka, 1995; Catala et al., 1995; Ceccarelli et al., 1999). Many muscle-specific genes such as the myosin light chain have multiple E-boxes in their promoter region to cooperatively regulate gene transcription (Rao et al., 1996). The importance of some E-boxes along the myostatin promoter in bovine, human and sheep were testified by experiments (Spiller et al., 2002; Ma et al., 2003; Du et al., 2007). MEF2 (MEF2A, MEF2B, MEF2C and MEF2D) are required for the muscle-specific genes transcription and the myoblasts differentiation during myogenesis (Olson et al., 1995; Akkila et al., 1997). The obvious positive effect of the MEF2 motif on the human or sheep myostatin promoter activity was testified by electrophoretic mobility shift assay (EMSA) or mutational analysis (Ma et al., 2003; Du et al., 2007). The function of the MTBF motif was also tested in sheep (Du et al., 2007). Allen et al. (2007) demonstrated that SMAD transcription factors promoted the transcription of the myostatin gene by binding to the SMAD site of the mouse myostatin promoter. Obviously, the presence of multiple E-boxes, MEF2, MTBF and SMAD along the cat myostatin 5'-regulatory region suggests the importance of the motifs for the transcriptional regulation of the myostatin gene in cat.

In addition, the importance of other transcriptional response elements and corresponding transcriptional factors for regulating the myogenic genes transcription were testified by some researchers. For example, Pax3 can activate the myogenic determination gene Myf5, leading to the formation of skeletal muscle (Lagha et al.,

2008). Six1 and Six4 homeoproteins can transactivate myogenin by binding to the MEF3 site of myogenin promoter (Spitz et al., 1998). During embryogenesis, some members of the Msx homeoprotein family function in regulating the skeletal muscle differentiation. Msx1 and H1b can bind to the regulatory element of MyoD and cooperate to inhibit muscle differentiation in cell culture (Lee et al., 2004). Meox1 is a transcription factor that plays a role in the commitment of cells to the skeletal muscle lineage and is essential for specification of mesodermal cells into the muscle lineage (Petropoulos et al., 2004). TEA domain (TEAD) transcription factors play important regulatory roles in muscle gene expression by binding to MCAT elements (muscle C, A and T sites, conserved sequence: CATTCC) located in the promoter/enhancer region of several cardiac, smooth and skeletal muscle genes (Yoshida, 2008; Tsika, 2008). The presence of PAX3, MEF3, MSX, MEOX and TEAD motifs along the myostatin gene 5'-regulatory region in animals including cat implies that the motifs may regulate the skeletal muscle by affecting the transcription of myostatin gene.

It is noticeable that some of other regulatory motifs (not shown in Table 1) revealed by MatInspector analysis may also be crucial. Taken together, many motifs analyzed by the bioinformatics might play potential roles for regulating the myostatin transcription of cat and other animals. It is indispensable to further investigate the roles of the motifs identified in this study by mutational analysis or EMSA for better understanding of the transcriptional regulation of the myostatin gene.

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