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Some Motifs Were Important for Myostatin Transcriptional Regulation in Sheep (Ovis aries)

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Many motifs along the 1.2 kb myostatin promoter (MSTNpro) in sheep have been found by the MatInspecter program in our recent study. To further verify the role of the motifs and better understand the transcriptional regulation mechanism of the myostatin gene in sheep, the reporter gene EGFP (enhanced green fluorescent protein) was selected and the wild-type (*W*) vector MSTNPro^{W}-EGFP or motif-mutational (*M*) vector MSTNPro^{M}-EGFP were constructed. The transcriptional regulation activities were analyzed by detecting the fluorescence strength of EGFP in C2C12 myoblasts transfected with the vectors. The results showed that E-box (E) 3, E4, E5 and E7, particularly E3, E5 and E7, had important effects on the activity of the 1.2 kb sheep myostatin promoter. In addition, we also detected several other important motifs such as MTBF (muscle-specific Mt binding factor), MEF2 (myocyte enhancer factor 2), GRE (glucocorticoid response elements) and PRE (progesterone response elements) along the sheep myostatin promoter by the mutational analysis.

Keywords: C2C12, Motifs, Myostatin, Sheep, Transcriptional regulation

Introduction

Myostatin (MSTN), also known as growth/differentiation factor-8 (GDF-8), a member of the transforming growth factor-β (TGF-β) superfamily, is expressed almost exclusively in skeletal muscle and acts as a negative regulator of skeletal muscle growth and development in mammals (McPherron *et al*., 1997; McPherron and Lee, 1997; Kambadur *et al*., 1997; Grobet *et al*., 1997; 1998; Rios *et al*., 2004). The highly homologous sequences of myostatin protein's C-terminal active region in human, rat, murine, porcine, chicken, turkey, bovine and sheep showed that the function of myostatin were extremely conserved throughout evolution (Lee and McPherron, 2001; Gu *et al*., 2004) although myostatin exhibited more differences in the structure, distribution and role for fish and Drosophila species (Roberts and Goetz, 2001; Kerr *et al*., 2005; Lee-Hoeflich *et al*., 2005). However, there were higher differences for the promoter region among animals compared to the coding region (Gu *et al*., 2004; Du *et al*., 2005). Particularly, the variations of some regulatory motifs along the myostatin promoter (MSTNpro) existed among several mammals, such as E-boxes (E), myocyte enhancer factors 2 (MEF2), muscle-specific Mt binding sites (MTBF), glucocorticoid response elements (GRE) and progesterone response elements (PRE) (Du *et al*., 2005). In addition, little research has been conducted about the regulation of the myostatin gene in comparison to its biological function. Therefore, it is necessary to investigate the role of some motifs along the myostatin promoter in sheep (AY918121) although some preliminary studies on the myostatin promoter in mouse, human and bovine have been carried out (Ma *et al*., 2001; Spiller *et al*., 2002; Salerno *et al*., 2004). To further verify the motifs along the myostatin promoter in sheep (Fig. 1) found by the MatInspecter program (a program used for identifying the potential transcription factor binding sites) in our recent research (Du *et al*., 2005) and better understand the transcriptional regulation mechanism of the myostatin gene,

Abbreviations: ActR II, activin type II receptors; CMV, cytomegalovirus; E, E-box; EGFP, enhanced green fluorescent protein; GDF8, growth/differentiation factor 8; GRE, glucocorticoid response elements; h, hour; kb, kilo base; M, mutation; MB, myoblasts; MEF2, myocyte enhancer factor 2; min, minute; MRFs, muscle regulatory factors; MSTN, myostatin; MSTNpro, myostatin promoter; MTBF, muscle-specific Mt binding factor; Myf5, myogenic factor 5; MyoD, myogenic differentiation 1; PCR, polymerase chain reaction; PRE, progesterone response elements; RU-486, mifepristone; S, second; TGF-β, transforming growth factor β; W, wild-type; X, any motif.

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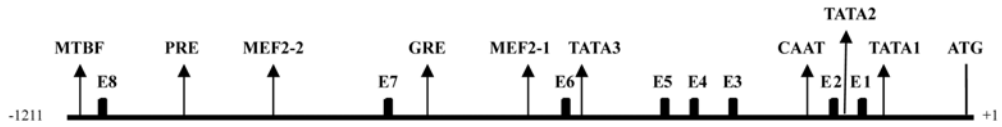

Fig. 1. Some important motifs along the 1.2 kb myostatin promoter in sheep. The 1.2 kb sheep myostatin promoter region consists of three TATA boxes (TATA), a CAAT box (CAAT) and eight putative E boxes (E). The myocyte enhancer factor 2 binding sites (MEF2), muscle-specific Mt binding site (MTBF), glucocorticoid response element (GRE) and progesterone response element (PRE) during the promoter were also indicated.

the wild-type (*W*) vector MSTNPro^{W}-EGFP and motif-mutational (*M*) vector MSTNPro^{M}-EGFP were constructed. The transcriptional regulation activities were analyzed by detecting the fluorescence strength of the reporter gene EGFP (enhanced green fluorescent protein) in C2C12 myoblasts transfected with the vectors.

Materials and Methods

Construction of MSTNPro^{W}-EGFP and MSTNPro^{M}-EGFP vectors. The 1.2 kb wild-type myostatin promoter fragment was amplified by polymerase chain reaction (PCR) from the plasmid (T-MSTNpro) containing the 1.5 kb myostatin promoter region of sheep (Small Tail Han sheep in China) (Du *et al.*, 2005). The PCR was performed in a 25 μl reaction mixture containing 20 ng plasmid (T-MSTNpro), 1×Taq reaction buffer, 5 nmol dNTPs, 20 pmol of each primer, and 0.25 units Taq DNA polymerase (Tiangen). 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°C and 1 min at 72°C), and a final 10 min extension at 72°C in a Biometra® T-gradient thermocycler. The sense primer and anti-sense primer were respectively 5'-ATCAAGCTTAGACCTTACCCCAAATCC-3' and 5'-CGCGGATCCGGTTTTAAAATCAATACA-3'. The underlined sequences indicated the introduced digestion sites.

Site-directed mutations in the individual E1 to E8, GRE, PRE, MEF2 and MTBF motifs or in the combinational E-box motifs (E3 + E5 and E3 + E5 + E7) were introduced by PCR. The PCR mixture and conditions were same as described above except for the primers. The primers or short fragments with suitable digestion sites were designed for generating some fragments with mutational motif (X) (Table 1, 2). Briefly, the targeted motif sequence was replaced with a suitable digestion site and the two fragments flanked the targeted motif were amplified respectively. The left-fragment flanked E8 was artificially synthesized because its length was less than 50 bp. The left-fragment flanked MTBF was ignored because its length was less than 10 bp.

The reporter vector pEGFP-N1 (4.1 kb) without promoter was obtained by removing cytomegalovirus (CMV) promoter from pEGFP-N1 (4.7 kb) vector (Clontech). Then MSTNPro^{W}-EGFP and

Table 1. The oligonucleotides used to amplify or synthesize the sheep myostatin promoter fragments with the mutations in the individual motifs

Oligo-nucleotides	Sequence	Oligo-nucleotides	Sequence
X*M*L (s)	5' ATCAAGCTTAGACCTTACCCCAAATCC 3'	X*M*R (a-s)	5' CGCGGATCCGGTTTTAAAATCAATACA 3'
GRE*M*L (a-s)	5' CGG*CTGCAG*CCTACTTTAATATTAAAT 3'	GRE*M*R(s)	5' AGA*CTGCAG*GAATTTATTCAGGGGAAC 3'
PRE*M*L (a-s)	5' AGA*CTGCAG*ATGTTCCATTTTTCTGCT 3'	PRE*M*R (s)	5' AAT*CTGCAG*GCGTTCCTTTCCTTTTCT 3'
MEF2-1*M*L (a-s)	5' CGG*CTGCAG*TAGTTAGAAAATACAGAT 3'	MEF2-1*M*R (s)	5' CGC*CTGCAG*ATAACGAATAAAACTCTT 3'
MEF2-2*M*L (a-s)	5' ATA*CTGCAG*GGGTAACAAACATTTCCT 3'	MEF2-2*M*R (s)	5' CGC*CTGCAG*ATCTTCTAAGTCATTCTA 3'
E1*M*L (a-s)	5' CGA*CTGCAG*GCTTTTTATATTCCAACT 3'	E1*M*R (s)	5' CGC*CTGCAG*GAATACAGTATAAAAGAT 3'
E2*M*L (a-s)	5' ATA*CTGCAG*TCGTCGGGATCTGTGATT 3'	E2*M*R (s)	5' TAG*CTGCAG*TCTCATCAAAGTTGGAAT 3'
E3*M*L (a-s)	5' GCG*CTGCAG*ACTACTTCTTAAAAGGAA 3'	E3*M*R (s)	5' TAT*CTGCAG*AATCAGCTCACCCTTGAC 3'
E4*M*L (a-s)	5' CCG*CTGCAG*AAAAATTAAAAACTTTAG 3'	E4*M*R (s)	5' TAT*CTGCAG*TCACAGACAGCCTTTATT 3'
E5*M*L (a-s)	5' CGA*CTGCAG*CACTTAGATCTTATTCAT 3'	E5*M*R (s)	5' CGC*CTGCAG*TTATTGTTACTAAAGTTT 3'
E6*M*L (a-s)	5' CCG*CTGCAG*AAATAAAAAGAGTTTTAT 3'	E6*M*R (s)	5' CCG*CTGCAG*CTTACTTAAATAGTATAA 3'
E7*M*L (a-s)	5' GTA*CTGCAG*AAAATTGCATTTCAGTTT 3'	E7*M*R (s)	5' GGG*CTGCAG*AATAAAGATATTATTTAA 3'
E8*M*L	5' AGCTTAGACCTTACCCCAAATCCCTGCC AGGAGTCTGCCCTCCGGT*CTGCA* 3' 5' *G*ACCGGAGGGCAGACTCCTGGCAGGGA TTTGGGGTAAGGTCTA 3'	E8*M*R (s)	5' TAT*CTGCAG*AGAAACTTCAAAGGAAGT 3'
		MTBF*M* (s)	5' ATT*AAGCTT*TGCCAGGAGTCTGCCCTC 3'
		MTBF*M* (a-s)	5' CGCGGATCCGGTTTTAAAATCAATACA 3'

Note: The oligonucleotides with flanking digestion sites (underlined) were designed to amplify or synthesize the sheep myostatin promoter fragments with the mutations in the individual motifs. The italics indicated the motif sequences mutated into digestion sites. "X" represented GRE (glucocorticoid response element), PRE (progesterone response element), MEF2 (myocyte enhancer factor 2, MEF2-1 to MEF2-2) and E (E-box, E1 to E8). MTBF is an abbreviation of muscle-specific Mt binding site. *M*L (*M*R) indicated the left (right) myostatin promoter fragments flanked the mutational-motif. "s" or "a-s" indicated the sense or anti-sense primers.

Table 2. The oligonucleotides used to amplify the sheep myostatin promoter fragments with the mutations in the combinational E-box motifs

Oligo-nucleotides	Sequence	Oligo-nucleotides	Sequence
E3*M*R (s)	5' AAT*GGTACC*AATCAGCTCACCCTTGAC 3'	E5*M*L (a-s)	5' GCA*CTGCAG*CACTTAGATCTTATTCAT 3'
E3*M*R (a-s)	5' CGCGGATCCGGTTTTAAAATCAATACA 3'	E5E7*M* (s)	5' CGC*GAATTC*GAATAAAGATATTATTTA 3'
E3E5*M* (s)	5' CGC*CTGCAG*TTATTGTTACTAAAGTTT 3'	E5E7*M* (a-s)	5' GCA*CTGCAG*CACTTAGATCTTATTCAT 3'
E3E5*M* (a-s)	5' CGC*GGTACC*ACTACTTCTTAAAAGGAA 3'	E7*M*L(s)	5' ACTAAGCTTAGACCTTACCCCAAATCC 3'
E5*M*L (s)	5' ACTAAGCTTAGACCTTACCCCAAATCC 3'	E7*M*L (a-s)	5' CGG*GAATTC*AAAATTGCATTTCAGTTT 3'

Note: The oligonucleotides with flanking digestion sites (underlined) were designed to amplify the sheep myostatin promoter fragments with the mutations in the combinational E-box motifs (E). The italics indicated the E-box sequences mutated into digestion sites. E*M*L (E*M*R) indicated the left (right) myostatin promoter fragment flanked the mutational-E. E3E5*M* (E5E7*M*) indicated the myostatin promoter fragments during two mutational E-boxes. "s" or "a-s" indicated the sense or anti-sense primers.

MSTNPro^{XM}-EGFP (including MSTNPro^{E1M}-EGFP, MSTNPro^{E2M}-EGFP, MSTNPro^{E3M}-EGFP, MSTNPro^{E4M}-EGFP, MSTNPro^{E5M}-EGFP, MSTNPro^{E6M}-EGFP, MSTNPro^{E7M}-EGFP, MSTNPro^{E8M}-EGFP, MSTNPro^{E35M}-EGFP, MSTNPro^{E357M}-EGFP, MSTNPro^{GREM}-EGFP, MSTNPro^{PREM}-EGFP, MSTNPro$^{MEF2\text{-}1M}$-EGFP, MSTNPro$^{MEF2\text{-}2M}$-EGFP and MSTNPro^{MTBFM}-EGFP) vectors, with same lengths and sequences except for the mutational target-motifs, were constructed by inserting the 1.2 kb wild-type myostatin promoter fragment (MSTNPro^{W}) or inserting successively two (for mutations in the individual motifs) or more (for mutations in the combinational motifs) relational myostatin promoter fragments flanked the mutational target-motifs into the corresponding digest sites of the reporter vector pEGFP-N1(4.1kb). The mutational sites were verified by sequencing with ABI automated DNA sequencer.

Cell culture and transfection. C2C12 myoblasts (MB) (ATCC) were routinely cultured in DMEM-F12 (GIBCO) supplemented with 10% FBS (TBD), 100U/ml penicillin and 100 μg/ml streptomycin (GIBCO). The negative control vector pEGFP-N1 (4.1 kb), positive control vector pEGFP-N1 (4.7 kb) and test vectors MSTNpro$^{W(M)}$-EGFP were transfected into C2C12 myoblasts cultured in 12-well plates using lipofectamine (Invitrogen). Briefly, cells were washed and 300 μl fresh DMEM-F12 (serum-free, penicillin-free and streptomycin-free) was added before the DNA-lipofectamine mixture was added. Plasmids and lipofectamines were first diluted respectively with 100 μl DMEM-F12 (serum-free, penicillin-free and streptomycin-free) and incubated for 5 min at room temperature. Then the diluted plasmids and lipofectamines were mixed and incubated for 20 min at room temperature before the mixtures were introduced to the cells. After culturing for 4 h at 37°C (5% CO_2), the transfected cells were added with 500 μl DMEM-F12 containing 20% FBS and were cultured for a further 36 h till the fluorescence analysis was done. At this stage, the various treatment solutions such as dexamethasone, progesterone and RU-486 were added. To control for variations in transfection efficiency, the transfections were performed in duplicate each time and each experiment was performed for six times.

Fluorescence assay and statistical analysis. After transfected and cultured for 40 h, the C2C12 myoblasts were collected and diluted and were analyzed for the relative fluorescence strength of EGFP in a fluorescence spectrophotometer (HITACHI F-4500). The duplicates of the same treatment at each time were mixed before the relative fluorescent strength of EGFP was determined. Data for six replicates, which were test groups (transfected with vectors) minus blank groups (transfected without any vector), were analyzed by the multiple comparison procedure of SAS (Version 6.12) statistical software. $P < 0.01$ and $P < 0.05$ were taken as the level of statistical significance.

Results

Effects of E-box motifs on the transcriptional regulation activity of the sheep myostatin promoter. The higher fluorescence activity in the positive group transfected with pEGFP-N1 (4.7 kb) vector containing CMV promoter suggested the better transfection efficiency (Fig. 2B). The fluorescence was hardly observed in the negative control group transfected with the pEGFP-N1 (4.1 kb) vector without promoter (Fig. 2A) but the 1.2 kb sheep myostatin promoter could activate the transcription and expression of EGFP in C2C12 (Fig. 2C).

Multiple (eight) E-boxes within the 1.2 kb sheep myostatin promoter (Du *et al*., 2005) suggested the importance of E-boxes for the transcription and expression of the myostatin gene. The mutational analysis of the individual E-box motifs showed that E3, E4, E5 and E7, particularly E3, E5 and E7, had significant effects on the activity of the sheep myostatin promoter ($P < 0.01$) (Fig. 3). The combinational mutations of E3 + E5 (E35M) or E3 + E5 + E7 (E357M) did not further decrease the activity of the sheep myostatin promoter compared to the individual mutation of E3, E5 or E7 ($P > 0.05$) (Fig. 4).

Effects of MEF2 and MTBF motifs on the transcriptional regulation activity of the sheep myostatin promoter. The mutational analysis showed that both the MTBF motif and one of the MEF2 motifs (MEF2-1, positioned in the near upstream of the third TATA-box) had obvious positive effects on the sheep myostatin promoter activity ($P < 0.01$), but another MEF2 motif (MEF2-2) found by MatInspecter had no anticipated influence ($P > 0.05$) (Fig. 5).

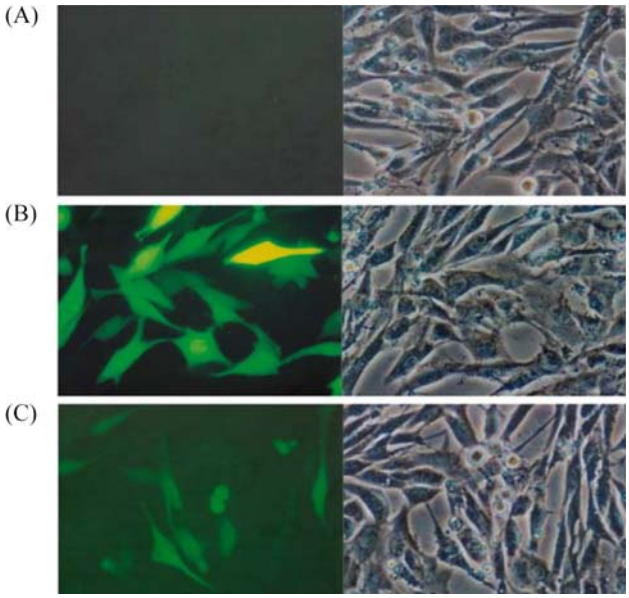

Fig. 2. The 1.2 kb sheep myostatin promoter activate the transcription and expression of EGFP in C2C12 myoblasts (10 × 20) (C). (A) and (B) indicated the negative control group transfected with pEGFP-N1(4.1 kb) vector without promoter and the positive group transfected with pEGFP-N1(4.7 kb) with CMV promoter, respectively.

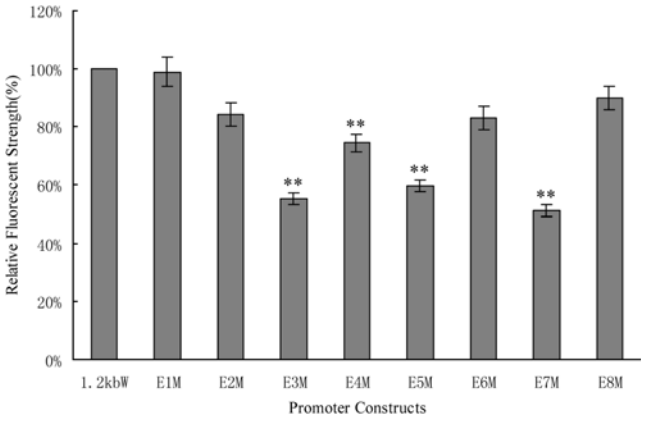

Fig. 3. Effects of single E-box mutation on the transcriptional regulation activity of sheep myostatin promoter in C2C12 myoblasts. 1.2 kbW indicates that the EGFP expression vector with the 1.2 kb wild-type myostatin promoter fragment. E1M to E8M indicates respectively that the EGFP expression vectors with the 1.2 kb mutational-type myostatin promoter fragments containing the individual E-box (E1 to E8) mutations. Bars indicate means ± standard deviations for six replicates. ** indicates there were significant differences compared to the 1.2 kb wild-type (1.2 kbW) sheep myostatin promoter ($P < 0.01$).

Effects of GRE and PRE motifs on the transcriptional regulation activity of the sheep myostatin promoter. The mutational analysis showed that both GRE and PRE motifs had obvious positive effects on the sheep myostatin promoter activity ($P < 0.01$) (Fig. 5). This suggested that glucocorticoid

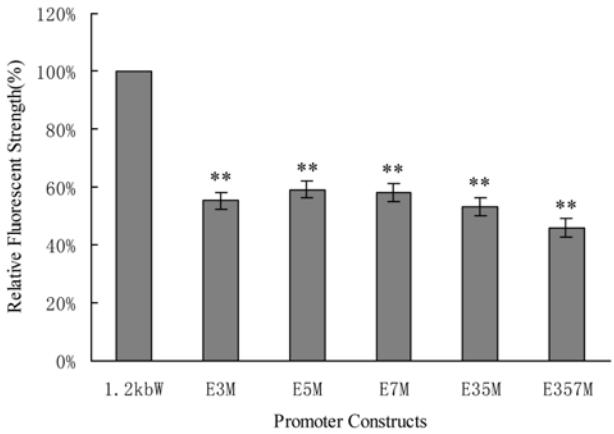

Fig. 4. Effects of combinational E-boxes (E) mutation on the transcriptional regulation activity of sheep myostatin promoter in C2C12 myoblasts. 1.2 kbW indicated that the EGFP expression vector with the 1.2 kb wild-type myostatin promoter fragment. E3M, E5M and E7M indicated respectively that the EGFP expression vectors with the 1.2 kb mutational-type myostatin promoter fragments containing the individual E-box (E3, E5 or E7) mutations. E35M and E357M indicated respectively that the EGFP expression vectors with the 1.2 kb mutational-type myostatin promoter fragments containing the combinational mutations of more E-boxes (E3M + E5M and E3M + E5M + E7M). Bars indicate means ± standard deviations for six replicates. ** indicates that there were significant differences compared to the 1.2 kb wild-type (1.2 kbW) sheep myostatin promoter ($P < 0.01$).

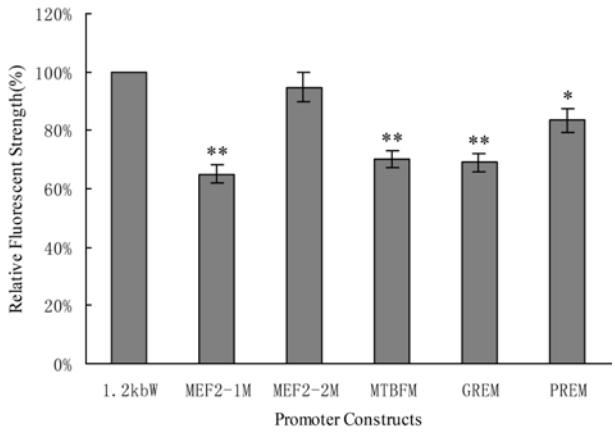

Fig. 5. Effects of single MEF2, MTBF, GRE or PRE motif mutation on the transcriptional regulation activity of sheep myostatin promoter in C2C12 myoblasts. 1.2 kbW indicated that the EGFP expression vector with the 1.2 kb wild-type myostatin promoter fragment. The MEF2-1M, MEF2-2M, MTBFM, GREM and PREM indicated respectively that the EGFP expression vectors with the 1.2 kb mutational-type myostatin promoter fragments containing the individual mutation of MEF2 (myocyte enhancer factor 2, MEF2-1 to MEF2-2), MTBF (muscle-specific Mt binding site), GRE (glucocorticoid response element) or PRE (progesterone response element) motifs. Bars indicate means ± standard deviations for six replicates. ** or * indicates that there were significant differences compared to the 1.2kb wild-type (1.2 kbW) sheep myostatin promoter ($P < 0.01$ or $P < 0.05$).

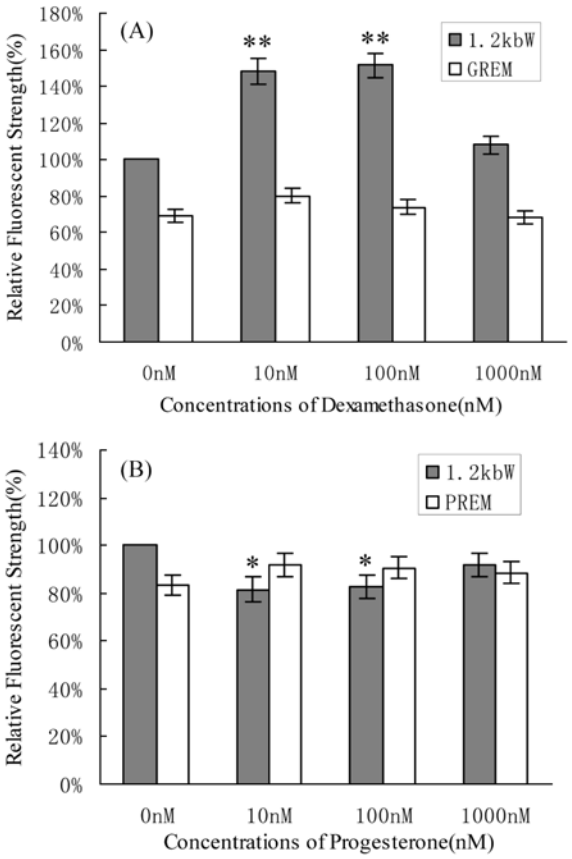

Fig. 6. Effects of dexamethasone (A) or progesterone (B) on the transcriptional regulation activity of sheep myostatin promoter (MSTNpro) in C2C12 myoblasts. 10, 100 and 1000 nM dose of dexamethasone or progesterone were added to C_2C_{12} myoblasts transfected with the wild-type vector MSTNpro^{W}-EGFP (filled bars) or GRE (PRE)-mutational vectors (open bars) MSTNpro^{GREM}-EGFP (MSTNpro^{PREM}-EGFP). Bars indicate means ± standard deviations for six replicates. ** or * indicates that there were significant differences ($P < 0.01$ or $P < 0.05$, respectively) compared to C2C12 myoblasts transfected with the corresponding vector but without adding dexamethasone or progesterone.

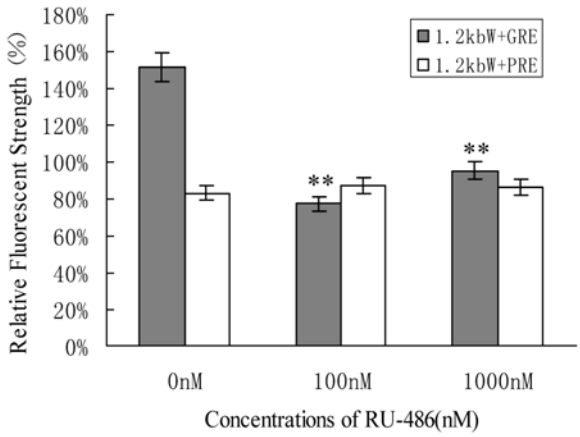

Fig. 7. Effects of RU-486 on dexamethasone-induced increases or progesterone-induced decreases of the transcriptional regulation activity of sheep myostatin promoter in C2C12 myoblasts. The C_2C_{12} myoblasts transfected with the 1.2 kb wild-type vector MSTNpro^{W}-EGFP were incubated with 100 nM dexamethasone (filled bars) or progesterone (open bars) and increasing concentrations of the glucocorticoid or progesterone antagonist RU-486 for 40 h. Bars indicate means ± standard deviations for six replicates. ** indicates significant differences ($P < 0.01$) compared to C2C12 myoblasts transfected with the same vector and added with the same hormone but without RU-486.

and progesterone might regulate the transcription of the myostatin gene. To investigate the hypothesis, we transfected the wild-type vector MSTNpro^{W}-EGFP or GRE(PRE)-mutational vector MSTNpro^{GREM}-EGFP (MSTNpro^{PREM}-EGFP) into C_2C_{12} myoblasts and added the 10, 100 and 1000 nM dose of dexamethasone or progesterone respectively to C_2C_{12} myoblasts after transfection for 4 h. The changes of the sheep myostatin promoter activity were measured after transfection for 40 h. The results showed that the concentrations of 10 nM and 100 nM dexamethasone significantly increased the activity of the wild-type myostatin promoter in sheep ($P < 0.01$) but had no obvious effect on the activity of the GRE-mutational myostatin promoter ($P > 0.05$) (Fig. 6A), and the concentrations of 10 nM and 100 nM progesterone significantly decreased the activity of the wild-type sheep myostatin promoter ($P < 0.05$) but had no obvious effect on the activity of the PRE-mutant sheep myostatin promoter ($P > 0.05$) (Fig. 6B).

To determine whether the effects of dexamethasone or progesterone on the myostatin gene transcription were mediated through a receptor-mediated pathway, we incubated C_2C_{12} myoblasts with 100 nM dexamethasone or progesterone and increasing concentrations of the glucocorticoid or progesterone antagonist RU-486 (Mifepristone). The results showed that 100 and 1000 nM RU-486 significantly inhibited the dexamethasone-induced increases in the activity of sheep myostatin promoter ($P < 0.01$) but did not have obvious effect on the progesterone-induced decreases in the activity of sheep myostatin promoter ($P > 0.05$) (Fig. 7).

Discussion

Roles of E-boxes in the myostatin gene transcription. The E-box (CANNTG) is one of the sequence motifs for the basic helix-loop-helix myogenic regulatory factors (MRFs) including the MyoD (myogenic differentiation 1), Myf5 (myogenic factor 5), 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 E-boxes with similar core sequences play roles to a different extent. Spiller *et al.* (2002) showed that out of three important E-boxes (E3, E4 and E6), E6 was crucial for the bovine myostatin promoter activity by the mutational analysis.

The mutation of the individual E6 could significantly decrease the bovine myostatin promoter activity (Spiller *et al*., 2002). Our results of mutational analysis in the individual E-box motifs showed that E3, E4, E5 and E7, particularly E3, E5 and E7, had important effects on the activity of the sheep myostatin promoter and E7 was most important. This crucial E6 in the bovine myostatin gene promoter was mutated in sheep but the most important E-box (E7) for the sheep myostatin gene transcription is at the same position as E5 near to E6 of the bovine myostatin promoter (Du *et al*., 2005). The combinational mutations of E3 + E4 or E3 + E4 + E6 further decreased the bovine myostatin promoter activity compared to the individual mutation of E3, E4 or E6 (Spiller *et al*., 2002). However, the combinational mutations of E3 + E5 (E35M) or E3 + E5 + E7 (E357M) had no significant difference with the individual mutation of E3, E5 or E7 in our test for the sheep myostatin promoter. These results with the sheep and bovine myostatin promoters suggested that there were both conservation and variation for the function of E-boxes across species. Of particular interest in our experiment, the close position of three important E-boxes E3, E4 and E5 in the sheep myostatin promoter suggested they might function as a cluster to better sustain the stability of DNA-protein. The E-boxes with similar core sequences played roles to a different extent, which suggested that the different sequences or motifs flanking the E-box might be a cause.

Roles of MEF2 and MTBF motifs in the myostatin gene transcription. MEF2 proteins (MEF2A, MEF2B, MEF2C and MEF2D) are required for the muscle-specific genes transcription and the myoblasts differentiation during myogenesis (Lyons *et al*., 1995; Olson *et al*., 1995; Akkila *et al*., 1997). The obvious positive effect of the MEF2-1 motif on the sheep myostatin promoter activity in our mutational analysis suggested that MEF2 was influential on the transcription and expression of the sheep myostatin gene. Like human (Ma *et al*., 2001), the influential MEF2-1 motif of the sheep myostatin promoter was positioned in the near upstream of the third TATA-box. This indicated the conservation of the MEF2 motif across species. The conservation of the MTBF motif among mammals such as sheep, goat, bovine and porcine (Du *et al*., 2005) suggested that the MTBF motif might play a role in the myostatin gene transcription. The result of our mutational analysis showed that the MTBF motif was influential on the transcriptional regulation activity of the sheep myostatin promoter, which verified the above hypothesis.

Roles of GRE and PRE motifs in the myostatin gene transcription. Glucocorticoids could induce muscle atrophy by inhibiting protein synthesis or stimulating protein breakdown in skeletal muscle (Goodlad and Clark, 1991; Hasselgren, 1999). The recent research found that the glucocorticoid-induced muscle atrophy was associated with the upregulation of the myostatin gene expression (Ma *et al*., 2003). Ma *et al.* (2001) showed that the glucocorticoid agonist dexamethasone increased the transcriptional regulation activity of the human myostatin promoter and the effects of dexamethasone on the myostatin gene transcription were mediated through a glucocorticoid receptor-mediated pathway. In our experiment, the mutation of the GRE motif resulted in a significant decrease in the sheep myostatin promoter activity, the addition of the synthetic glucocorticoid (dexamethasone) significantly increased the wild-type myostatin promoter activity but not the GRE-mutational myostatin promoter activity and the glucocorticoid antagonist RU-486 inhibited the dexamethasone-induced increase in the sheep myostatin promoter activity. Our results as evidenced by Ma's results (2001) suggested that glucocorticoid upregulating the myostatin expression might be due, at least in part, to glucocorticoid upregulating the myostatin transcription through a glucocorticoid receptor-mediated pathway.

Both the mutation of PRE motif and the supplement of progesterone could decrease markedly the sheep myostatin promoter activity as evidenced by the fact that the progesterone antagonist RU-486 did not have an effect on the progesterone-induced decrease in the sheep myostatin promoter activity. This suggested that the PRE motif found by MatInspecter might not be a progesterone response element. The decrease in the 1.2 kb sheep myostatin promoter activity by progesterone might involve in other mechanism.

In summary, our experiment suggested that some motifs be important for the transcriptional regulation of the sheep myostatin gene. More experimental methods need to be adopted to further testify the roles and mechanisms of these motifs.

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