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Polymorphism in the 5’flanking region of the myostatin gene affects myostatin and TGF-β_1 expression in bovine skeletal muscle*

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ABSTRACT

Myostatin (GDF-8) is a key protein responsible for skeletal muscle growth and development so mutations in the *mstn* gene can have major economic and breeding consequences. The aim of the present study was to investigate polymorphism in the 5’flanking region of the *mstn* gene and its possible influence on the myostatin level in skeletal muscles of Polish Black-and-White bulls. The relation between expression of myostatin and another member of the TGF-β superfamily, TGF-β_1, was also examined. We uncovered polymorphism in the 5’flanking region of the *mstn* gene: G/C substitution at position -7828 (relative to translation start codon ATG). The most frequent genotype was GC (43.6%), followed by genotypes CC (34.2%) and GG (22.2%). The concentration of the active form of myostatin (26 kDa) in *M. semitendinosus* of homozygotes (CC) was the lowest, whereas the expression of this cytokine in heterozygotes was the highest. The changes in *mstn* expression did not, however, influence average carcass traits (weight of valuable cuts and weight of lean in valuable cuts). The pattern of differences in the TGF-β_1 concentration corresponded to that observed for myostatin.

KEY WORDS: cattle, myostatin, gene expression, polymorphism, skeletal muscle, TGF-β_1

INTRODUCTION

Myostatin (GDF-8) is a key protein responsible for skeletal muscle growth and development. As a negative regulator it inhibits proliferation and terminal

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differentiation of myogenic cells, thus limiting the final number of muscle cells at birth.

McPherron and Lee (1997) identified the gene encoding myostatin in the mouse. Knock-out of the GDF-8 gene resulted in increased growth, mainly due to increased muscle mass. Individual muscles of these mice were even 2-3-fold heavier than in control animals. Increased muscle growth was a result of both hyperplasia and hypertrophy and was not influenced by the animal's age or sex. In the same year these authors showed that the double-muscle phenotype in Belgian Blue cattle is a result of a deletion in the *mstn* gene (McPherron and Lee, 1997). Double-muscled animals had, on average, a 20% higher skeletal muscle mass when compared with normal cattle. This observation was confirmed by two other research groups (Grobet et al., 1997; Kambadur et al., 1997), but in subsequent studies some cattle breeds were identified in which the presence of this mutation was not always accompanied by the double-muscle phenotype (Dunner et al., 2003). The mutation mentioned above, nt821(del11) in exon 3, is a deletion of 11 nucleotides resulting in a shift of the reading frame and appearance of the stop codon, UGA. The product of this gene is a biologically inactive, 273 amino acid peptide. This mutation was also identified in other cattle breeds: Blonde d'Aquitaine, Limousine, Parthenaise, Asturiana, Rubia Gallega. It was also identified in Polish Red cattle, however, the double-muscle phenotype was never found in this breed (Klauzińska et al., 2000). In this case, the frequency of the mutation was relatively low-it was present in 15 of the 315 studied animals and always in heterozygotes.

Since the nt821(del11) mutation may have important economic and breeding consequences, other possible mutations in the *mstn* gene that could also have a major impact on meat production in cattle were investigated. At present, eight other mutations in exons of the *mstn* gene are known, among which five are destructive mutations resulting in extreme forms of muscle hypertrophy and the remaining three are missense mutations (Grobet et al., 1998; Dunner et al., 2003; Marchitelli et al., 2003).

Dunner et al. (2003) suggest that muscle hypertrophy in cattle resulting from the nt821(del11) mutation originated in Northern Europe and spread in a few steps among dairy cattle all over Europe. Other mutated variants of the *mstn* gene, however, come from single mutations that arose in intensively selected cattle breeds and were transmitted to other breeds.

Despite enormous progress in functional genomics, the skeletal muscle phenotype can not always be associated with polymorphism in the coding region of the *mstn* gene. Therefore, polymorphism in the 5'flanking region of this gene is also of interest. A mutation in this region can increase or decrease *mstn* gene expression in skeletal muscles, affecting muscle growth and development. In

contrast to gene structure, however, the regulatory region turned out to be highly conservative and only several mutations having a very low frequency were found. Jeanplong et al. (2000) described mutation BTAFJ in the 5'flanking region of the *mstn* gene, which is probably a region acting as a transcription silencer. Using SSCP, RFLP and sequencing methods, Klauzińska (2002) failed to find mutations in the 1269-bp gene fragment (GenBank, AJ438578.1) immediately before the transcription initiation site. There were no mutations in 255 cattle from two breeds, Polish Red and Holstein. Crisa et al. (2003) described a polymorphism in the 5'flanking region of the bovine *mstn* gene transitions T/A in pos. -371 and G/C at pos. -805 (with relation to codon ATG), however, the influence of this mutation on *mstn* expression in skeletal muscles was not studied.

In the present study we describe a new polymorphism in the 5'flanking region of the *mstn* gene and its influence on the myostatin level in skeletal muscles of Polish Black-and-White bulls. The relation between expression of myostatin and another member of the TGF-β superfamily, TGF-β_1, was also examined.

MATERIAL AND METHODS

Animals and sampling

One hundred and seventeen young Black-and-White bulls, with more than 75% share of HF blood, were examined. They were born in herds yielding 4500-5000 kg milk and were the progeny of 24 AI sires. The number of half-sibs varied from 3 to 9. Only 6 sires were represented in all genotype groups: CC, CG, and GG. The numbers of sires per genotype group were as follows: myostatin genotype CC: progeny of 16 sires; genotype CG: progeny of 13 sires; genotype GG: progeny of 16 sires.

The bulls were housed in tie-stalls, and fed silage, hay and concentrate *ad libitum* up to the age of 15 months. All bulls were then slaughtered after 24 h fasting. At slaughter, the live body weights for CC homozygotes, CG heterozygotes and GG homozygotes were 338.9 ± 43.2 kg; 324.8 ± 57.1 kg, and 324.5±52.1 kg, respectively, and did not differ significantly between genotypes. The carcasses were chilled for 24 h at 4°C. Valuable cuts (round, shoulder, tenderloin, best ribs + fore ribs) were obtained from the right carcass-sides and dissected into lean, fat and bone.

The association of carcass traits with the genotypes was evaluated using regressions on body weight at slaughter. The differences between genotypes were assessed by Duncan's test.

Approximately 10-ml blood samples were collected from each animal on K_2EDTA, and genomic DNA was isolated from the leukocytes (Kanai et al.,

1994). Samples of *M. semitendinosus* were taken immediately after slaughtering and frozen in a dry ice-acetone mixture for Western blot analysis. All of the procedures carried out with the use of animals were approved by the Local Ethics Commission, permit No. 67/2001.

PCR conditions

Based on the sequence available from GenBank (AF348479) and using Primer3 software available from the Internet (www.genome.wi.mit.edu), the following PCR primers were designed:

BATFJ2-F - 5’-CACATTTTGCAGCTTTCCTG-3’
BATFJ2-R - 5’-GCCAATGTTTGTCAGCTCAA-3’

With these primers, a 219-bp DNA fragment, encompassing a distal 5’flanking region of the *mstn* gene, was amplified. The polymerase chain reactions were performed using a PCR-mix with: primers BATFJ2-F and BATFJ2-R, each at a final concentration of 3 pmol, 5 µl REDTaq ReadyMix PCR REACTION Mix (SIGMA), 1 µl (about 100 ng) of genomic DNA and H_2O up to 10 µl. The following PCR protocol was used: 1 min at 94ºC, 1 min at 62ºC and 1 min at 72ºC - 32 cycles; 72ºC - 7 min. The yield and specificity of the PCR reactions were evaluated by electrophoresis of the products in 2% agarose gels (Gibco) with ethidium bromide.

PCR-HD analysis

Polymerase chain reaction-heteroduplex (PCR-HD) analysis was carried out using a Hoepfer SE 600 electrophoresis apparatus (Pharmacia) in 8% polyacrylamide gels with added 10% glycerol. The gels were maintained at a constant temperature (20ºC) with the use of a Multitemp III circulator. The gels were pre-electrophoresed (without samples) for 2 h at 120 V, 50 mA. Ten µl of PCR product were mixed with 10 µl of denaturation buffer (%: ethylene glycol 20, formamide 30, dextran blue 0.5), denatured for 5 min at 94ºC, incubated for 60 min at 65ºC, rapidly chilled on ice, and loaded onto the gels. The electrophoresis was run at 120 V, 60 mA, 8 W, for 18 h. The gels were stained using the Silver Staining System (Kucharczyk).

DNA sequencing

PCR products of different genotypes in the *mstn* gene were purified with a QIAquick® PCR Purification Kit (QIAGEN), and automatically sequenced in an ABJ377 sequencer (Applied Biosystems, USA). The sequence was analysed using the Sequence Analyser 2.01 program.

RFLP analysis

PCR products were digested in 10-µl aliquots with 5 U of *HpaI* restriction nuclease (Sigma-Aldrich, USA) for 3 h at 37ºC. The restriction fragments were subjected to electrophoresis in 2% agarose/ethidium bromide gels (Gibco, BRL, England) in 1 × TBE buffer (0.09 M Tris-boric acid, 0.002 M EDTA). The gels were examined under UV light and documented in a FX Molecular Imager apparatus (Bio-Rad).

Western blot analysis

The specificity of the monoclonal rat anti-myostatin and polyclonal chicken anti-TGF-β1 antibodies (R&D Systems, Inc., Minneapolis, USA) was evaluated in preliminary trials and they were found to be superior in comparison with other commercial antibodies.

The protein for Western blot analysis was isolated using Tissue Lysis Buffer (Sigma Aldrich) according to the producer's protocol. The lysates were mixed 1:2 (v/v) with Laemmli sample buffer (BioRad) containing 2.5% 2-mercaptoethanol and boiled for 3 min. Samples containing identical quantities of proteins were subjected to SDS-PAGE (12% gel) together with a Kaleidoscope Marker (BioRad). Electrophoresis was run for 17 h at 45 V using Mini Protean II ™ cell (BioRad). After electrophoresis the separated proteins were electroblotted on a nitrocellulose membrane (Amersham Pharmacia Biotech) for 195 min at 75V using the Mini Protean II ™ cell. The membranes were blocked overnight with a 5% w/v solution of non-fat powdered milk in TBST (pH 7.5). The following day the membranes were rinsed three times for 10 min in TBST, at room temperature, and then incubated for 1 h at room temperature with the primary antibodies diluted 1:500. The membranes were then rinsed four times for 10 min in TBST and incubated with diluted 1:2500 horseradish peroxidase-conjugated goat anti-rat IgG and goat anti-chicken IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for another 1 h at room temperature. Finally, the membranes were rinsed three times for 10 min in TBST, and labeled proteins were visualized using the ECL Western blotting detection reagent (Hercules, CA, USA) on a high performance chemiluminescence Hyperfilm ECL (Amersham Pharmacia Biotech, Little Chalfont, UK). The image on hyperfilm was then analysed with a Kodak Edas System and the integrated optical density (IOD) was measured.

Statistical evaluation

The results were statistically evaluated by ANOVA and Tukey's multiple range test using Prism version 2.00 software (GraphPad Software, San Diego, CA).

RESULTS

mstn gene polymorphism

Our investigations revealed the presence of a new polymorphism in the 5'flanking region of the *mstn* gene in Polish Black-and-White bulls. The 219 bp gene fragment was amplified from -7658 bp to -7877 bp. The nucleotide sequence polymorphism was initially detected using the PCR-HD (heteroduplex) technique and then precisely identified by DNA sequencing. Finally, the PCR product was digested with *Hpa*I, an enzyme recognizing the polymorphic site G/C substitution at position -7828 (relative to translation start codon ATG). Three genotypes were identified: GG, GC and CC (Figures 1 and 2). The

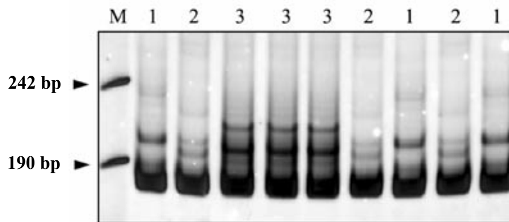


Figure 1. Polyacrylamide (8%) gel electrophoresis showing three PCR-HD (heteroduplex) patterns of the bovine *mstn* gene. M - 26-501 bp DNA marker (MspI digest of pUC19). 1, 2, 3 - different PCR-HD patterns

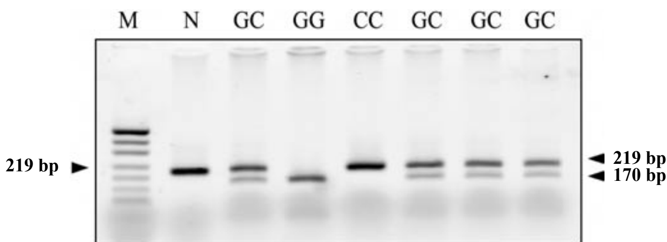


Figure 2. Agarose gel electrophoresis to detect PCR-RFLP/*Hpa*I at position -7665 in the bovine *mstn* gene 5'-flanking region; M - 11-1444 bp DNA marker (*Hae*III and *Taq*I digest of pUC19, InGen, Poland); N - the 219-bp non-cut PCR product; CC, GG, GC - myostatin genotypes, represented by 219-bp and 170-bp bands after HpaI digestion

frequency of alleles and number of *mstn* genotypes are presented in Table 1. From the total number of 117 bulls analysed, the most frequent genotype was GC, followed by genotype CC and GG (Table 1). The frequency of the C allele was slightly higher than of the G allele (0.56 vs 0.44, respectively).

Table 1. Frequencies of *mstn* genotypes and corresponding alleles in Polish Black-and-White bulls

Genotype	n	%	frequency
CC	40	34.19	C=0.56 G=0.44
GC	51	43.59	
GG	26	22.22	

Myostatin and TGF-β_1 concentration in skeletal muscle

Myostatin expression at the protein level was evaluated using the Western blot method. The average concentration of the active form of myostatin (26 kDa) in *M. semitendinosus* was the highest in heterozygotes (OD = 352300 ± 80800 [optical density ± SD]), whereas its lowest concentration was observed in CC homozygotes (OD = 131600 ± 55990) ($P \leq 0.05$) (Figure 3). Interestingly, a very

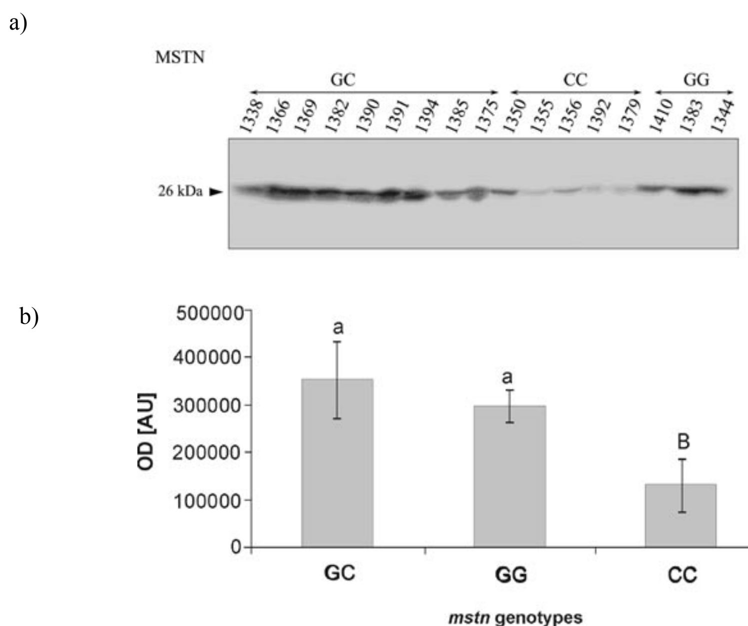
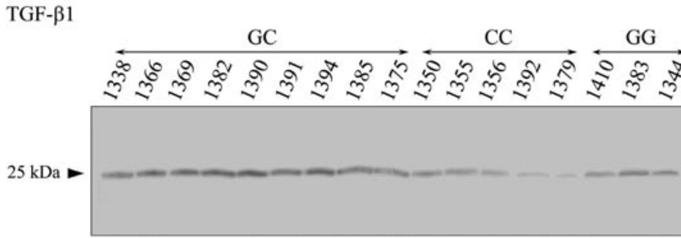


Figure 3. (a) Effect of *mstn* polymorphism on myostatin (26 kDa - active form) expression in *M. semitendinosus* of bulls analysed by Western blotting. (b) Expression of active form of MSTN (26 kDa) in *M. semitendinosus* of bulls in relation to *mstn* polymorphism. Means of optical density [OD] (± SD) denoted with a different superscript differ significantly ($P \leq 0.05$) (GG, n = 9; CC, n = 15; GC, n = 27)

similar pattern of TGF-β_1 concentrations was observed, with the highest concentration of the active form of TGF-β_1 (25 kDa) in the GC genotype (OD = 102400±17660) and the lowest concentration of this cytokine in the CC genotype (OD = 54010 ± 6833; $P \leq 0.05$) (Figure 4).

a)



b)

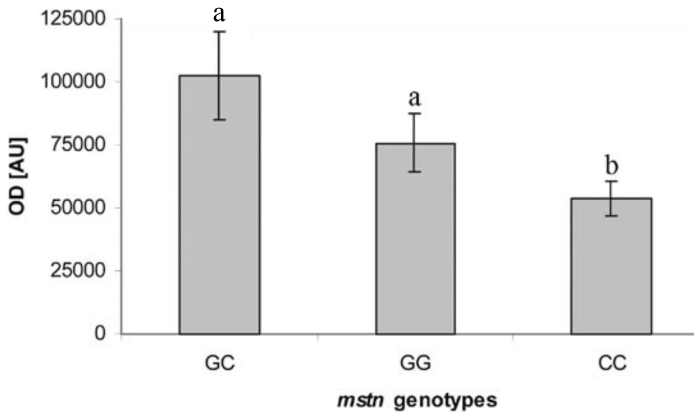


Figure 4. (a) Effect of *mstn* polymorphism on TGF-β_1 (25 kDa, active form) expression in *M. semitendinosus* of bulls analysed by Western blotting. (b) Expression of active form of TGF-β_1 (25 kDa) in *M. semitendinosus* of bulls in relation to *mstn* polymorphism. Means of optical density [OD] (± SD) denoted with a different superscript differ significantly (P≤0.05) (GG, n = 9; CC, n = 15; GC, n = 27)

Carcass traits

To evaluate the influence of *mstn* gene polymorphism and the differences in its expression on skeletal muscle phenotype, an analysis of carcass traits was conducted. A total of 117 bulls was evaluated. In animals with the CC genotype, in which the myostatin concentration was the lowest, the weight of valuable cuts in a carcass-side and the weight of lean in valuable cuts were higher than in GC and GG animals. In contrast, in GC animals, in which the myostatin concentration was the highest, the values of analysed parameters were the lowest (Table 2), however, these differences were not statistically significant.

Table 2. Overall least squares means (LSM) and standard errors (SE) of selected carcass traits across the MSTN/*Hpa*I genotypes

Trait	CC n=40		GC n=51		GG n=26	
	LSM	Se	LSM	Se	LSM	Se
Weight of valuable cuts in a carcass-side, kg	50.4	0.5	49.9	0.5	49.8	0.6
Weight of lean in valuable cuts, kg	35.6	0.5	35.2	0.5	35.3	0.6
Cold carcass dressing percentage, %	50.6	0.4	50.3	0.5	50.5	0.5

DISCUSSION

In this study we showed the presence of a polymorphism in the 5'flanking region of the *mstn* gene-a G/C substitution at position -7828 (relative to translation start codon ATG)-occurring in BW cattle (Figures 1 and 2). The polymorphic site had not been described earlier, however, Jeanplong et al. (2000) identified a BTAFJ polymorphism in the 5'flanking region of the *mstn* gene located 7.6 kb (in fact 7718) upstream from the translation start codon (ATG). Our results indicate that the concentration of the active form of myostatin in *M. semitendinosus* of homozygotes (CC) was the lowest, whereas the expression of this cytokine in heterozygotes was the highest (Figure 3). Earlier studies indicate that a high level of myostatin inhibits both proliferation and differentiation of muscle cells (Joulia et al., 2003). Mutations in the *mstn* gene, resulting in the lack of functional myostatin, lead to increased muscle mass in mice (McCroskey et al., 2003), or to the already mentioned double-muscle phenotype in cattle. If so, the bulls with the CC genotype (with the lowest myostatin concentration) should have a higher muscle mass than heterozygotes. Analysis of carcass traits in our study revealed that in CC bulls, the total weight of valuable cuts and of lean in valuable cuts was higher than in heterozygotes, however, the differences were not statistically significant. It can not be excluded that other factors, e.g., genetic, physiological and nutritional, could have interfered with the effect of myostatin on muscle growth in the examined bulls. It should be stressed that a relatively small cohort of animals was used for association studies, and the parent genotypes were not estimated. Thus, there was no possibility of studying the segregation of paternal and maternal alleles of *mstn*. Further studies using larger populations of animals with a known family structure are necessary to prove the association of the GC genotype of the *mstn* 5' region with meat production traits.

Since in our previous work we identified a direct relationship between myostatin and TGF-β_1 expression in myogenic cells *in vitro* (Budasz-Świderska et al., 2005), we also assessed the influence of *mstn* polymorphism on TGF-β_1 expression in bovine skeletal muscle. We showed that in bulls with different *mstn* genotypes,

the concentration of TGF-β_1 was also different and the pattern of changes was similar to that observed for the myostatin concentration in *M. semitendinosus* (Figure 4). These results suggest a close interdependence between expression of myostatin and TGF-β_1 (both belonging to the TGF-β superfamily of proteins and exhibiting strong structural and functional similarities) in bovine skeletal muscle. Experiments performed on C2C12 myogenic cells revealed that myostatin stimulates expression of TGF-β_1 at the protein level in both proliferating and differentiating cell cultures (Budasz-Świderska, 2005). On the other hand, TGF-β_1 upregulates myostatin expression in myogenic cells stimulated to proliferate and differentiate (Budasz-Świderska et al., 2005). The reciprocal effects of myostatin and TGF-β_1 action could be due to interactions between activators, repressors, co-activators and co-repressors of the *mstn* and *tgf-*β_1 genes.

Analysis of polymorphisms in the promoter or 5'flanking region of the *mstn* gene can give further insight into the regulatory mechanism of its expression in animals. Possible polymorphisms in binding sites for different transcriptional factors as well as the silencers present in these regions can influence these factors' activity and, indirectly, also *mstn* expression and activity. Myostatin is widely known due to its role in the double-muscle phenotype, but because of the changes occurring in the *mstn* before the ATG codon that can influence carcass quality, it can also be important in cattle breeds not carrying double muscling.

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