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Promoter Variant-Dependent mRNA Expression of the *MEF2A* in *Longissimus Dorsi* Muscle in Cattle

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The myocyte enhancer factor 2A (*MEF2A*) gene encodes a member of the myocyte enhancer factor 2 (MEF2) protein family that is involved in vertebrate skeletal, cardiac, and smooth muscle development and differentiation during myogenesis. According to recent studies, *MEF2* genes might be major regulators of postnatal skeletal muscle growth; thus, they are considered to be important, novel candidates for muscle development and body growth in farm animals. The aim of the present study was to search for polymorphisms in the bovine *MEF2A* gene and analyze their effect on the *MEF2A* mRNA expression level in the *longissimus dorsi* muscle of Polish Holstein-Fresian cattle. In total, 4094 bp of the whole coding sequence and the promoter region of *MEF2A* were re-sequenced in 30 animals, resulting in the detection of 6 novel variants as well as one previously reported SNP. Three linked mutations in the promoter region (*-780T/G*, *g.-768T/G*, and *g.-222A/G*) and only two genotypes were identified in two Polish breeds (TTA/TTA and TTA/GGG). Three SNPs in the coding region [*g.1599G/A (421aa)*, g.*1626G/A (429aa)*, and *g.1641G/A (434aa)*] appeared to be silent substitutions and segregated as two intragene haplotypes: GGG and AAA. Expression analysis showed that the mutations in the promoter region are highly associated with the *MEF2A* mRNA level in the *longissimus dorsi* muscle of bulls carrying two different genotypes. The higher *MEF2A* mRNA level was estimated in the muscle of bulls carrying the TTA/TTA ($p < 0.01$) genotype as compared with those with TTA/GGG. The results obtained suggest that the nucleotide sequence mutation in *MEF2A* might be useful marker for body growth traits in cattle.

Introduction

The myocyte enhancer factor 2 (MEF2) family of transcription factors has been shown to play a crucial role in the activation of muscle-specific gene transcription in skeletal, cardiac, and smooth muscle cells. The products of four *MEF2* genes—*MEF2A*, *-B*, *-C*, and *-D*, that are known in vertebrates—bind as homo- and heterodimers to the A/T-rich DNA consensus sequence associated with many muscle-specific genes (Black *et al.*, 1998). Among others, the genes encoding muscle creatinine kinase; skeletal α-actin; the α-myosin heavy chain; cardiac troponins T, C, and I; dystrophin; desmin; and Ca^{2+}-ATPase have binding sites in their promoters for these TFs (Molkentin and Markham, 1993). All of the mammalian *MEF2* gene family shares the MADS-box and MEF2 domains, which mediate DNA binding and the dimerization of MEF2 monomers. MEF2 factors play an important role in several differentiation and developmental processes. For example, *MEF2C* is reported to be essential for bone development (Arnold *et al.*, 2007) and neurogenesis (Heidenreich and Linseman, 2004), while *MEF2A* is associated with cardiovascular development (Edmondson *et al.*, 1994; Black and Olson, 2004). During myogenesis in skeletal muscle cells, *MEF2C* is expressed within the somite myotome beginning at about 9.0 days postcoitus and *MEF2A* and *-D* are expressed immediately thereafter (Edmondson *et al.*, 1994). After birth, *MEF2A*, *-B*, and *-D* transcripts are expressed ubiquitously, while *MEF2C* transcripts are restricted to skeletal muscle, the brain, and spleen. Several studies suggested that MEF2 might be regulated at the transcriptional, posttranscriptional, and translational levels (Breitbart *et al.*, 1993; Black *et al.*, 1998). In addition, *MEF2A* appears to be controlled at the level of translation; *MEF2A* transcripts are expressed in a wide array of tissues, while MEF2A protein appears to be considerably more restricted, and is abundant in skeletal muscle, heart, and brain tissues (Suzuki *et al.*, 1995). Black *et al.* (1997) revealed that the 3′-UTR acts as a posttranscriptional repressor of MEF2A protein expression during the differentiation of muscle cells. Despite extensive literature describing the *MEF2A* mutations as a major cause of the risk of developing disease in humans, little is known about the significant associations between *MEF2A* gene polymorphism and gene expression, growth, and muscle development in farm animals.

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In the present study, genetic variations in the promoter and coding regions of the *MEF2A* gene were investigated in 200 bulls representing four cattle breeds using multi-temperature single strand chain polymorphism (MSSCP) and DNA sequencing methods. In addition, the promoter variant effect on *MEF2A* mRNA expression was evaluated in the *longissimus dorsi* muscle of Polish Holstein-Fresian (HF) bulls.

Materials and Methods

Blood, tissue sampling, DNA and RNA isolation, and cDNA preparation

The blood samples were obtained from 200 unrelated bulls of four cattle breeds including Polish HF ($n=130$), Polish Red (PR, $n=30$), Hereford (HER, $n=20$), and Limousine (LIM, $n=20$). DNA was extracted from blood using Wizard® Genomic DNA Purification Kit (Promega) and stored at −20°C. *Longissimus dorsi* muscle samples for quantitative polymerase chain reaction (qPCR) expression analysis in promoter region were taken from 20 Polish HF bulls of known *MEF2A* genotypes. Animals were maintained at the Polish Academy of Science Experimental Farm, Jastrzębiec, and kept under the same housing and feeding conditions. The bulls were housed in a tie-stall and fed silage, hay, and concentrate *ad libitum* with constant access to water. After 24 h fattening, all bulls were slaughtered at the age of 12 months, with body weight about 370 kg. All tissues were harvested and immediately frozen in liquid nitrogen and then stored at −80°C. Total RNA was extracted from tissues using a Qiagen RNeasy® Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions, afterward a DNase treatment was performed. The quality and quantity of RNA was examined by spectrophotometric NanoDrop measurements and by 2% agarose gel electrophoresis. Reverse transcriptions were performed on 1 μg of total RNA using Transcription First Strand cDNA Synthesis Kit with oligo primers (Roche), according to manufacturer's instructions. The resulting cDNA was stored at −20°C until use. All procedures carried out on animals were approved by the Local Ethics Commission, permission No. 29/2007.

Identification and analysis of the polymorphism

The genomic organization of the *MEF2A* was performed using the ScanGen (http://genes.mit.edu/GENSCAN.html) and Apollo sequence annotation editor (http://apollo.berkeleybop.org/current/install.html). Searching for putative binding sites for transcription factors was performed by using TESS software (www.cbil.upenn.edu/cgi-bin/tess/tess). Fourteen overlapping DNA fragments flanking all exons as well as the promoter region were PCR amplified and used for polymorphism search with the MSSCP technique. PCR primers are shown in Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/dna. The PCR was performed according to standard manufacturer's instruction (Qiagen). PCR products were then separated on acrylamide gel and applying MSSCP electrophoresis in Pointer System (Kucharczyk) with 40 W constant powers for 70 min. The electrophoresis temperatures were as follows: 35°C, 15°C, and 5°C for 350 Vh. The gels were silver stained for 30 min using the Silver Stain Kit (Kucharczyk) and documented by Molecular Imager System FX (BioRad).

PCR products representing different MSSCP patterns were sequenced using a 3130xl Genetic Analyzer (Applied Biosystems Applera). Sequence alignments and identification of SNPs were performed using Clustal.W (www.ebi.ac.uk/tools/msa/clustalW2) and Chromas Lite v2.01 programs (www.technelysium,com.au/chromas). Three SNPs, the *g.-780T/G*, *-768T/G*, and *g.1641G/A*, were genotyped using *XmnI*, *RsaI*, and *BslI* restriction enzymes (Biolabs) respectively, and the *g.-222A/G* was screened using MSSCP method. Genotype and allele frequencies and deviation from the Hardy–Weinberg equilibrium were calculated using POPGENE V3.1 software (www.ualberta.ca/∼fyech).

Real-time analysis

Ten Polish HF bulls from each genotype group—TTA/TTA and TTA/GGG—were used to measure the effect of SNPs in the promoter region on *MEF2A* mRNA expression level in the *longissimus dorsi* muscle. Real-time PCR amplification was done in triplicate using an SYBR Green detection and the Roche Light Cycler 2.0 system (Roche). Real-time PCR primers were designed to anneal to adjacent exons or exon–exon junctions (Supplementary Table S1). Raw results were normalized relative to the geometric mean of mRNA detected from three reference genes *SF3AI*, *EEFIA2*, and *TBP* genes. The amplification efficiencies of the genes were determined using Light Cycler 3.5 Software (Simpson *et al.*, 2000). The results were calculated using the mathematical formula for relative mRNA quantification in real-time PCR given by Pfaffl (2001). The statistical analysis between the *MEF2A* genotypes and mRNA expression level was performed with the Duncan's test.

Results

In total, 4094 bp were re-sequenced in 30 animals, resulting in the detection of 6 novel variants in the bovine *MEF2A*, specifically, three SNPs (*g.-780T/G*, *g.-768T/G*, and *g.-222A/G*) in the promoter (Supplementary Fig. S1), 1 InDel (*g.167747C/-)* in the intron 9, and 2 synonymous SNPs *g.1599G/A* (421aa) and g.*1626G/A* (429aa) in the exon 11 (Supplementary Fig. S2). Moreover, we detected a previously described SNP by Chen *et al.* (2010) in the exon 11, namely the synonymous *g.1641G/A* (434aa) substitution. The nucleotide sequences and polymorphism information for bovine *MEF2A* have been deposited in the *GenBank* database under accession No. GU211003, JN944535, and JN944536. The distribution of genotypes and allele frequencies was studied in 200 animals representing four cattle breeds (Supplementary Table S2). The distribution of genotypes showed that variations in the promoter region segregated as two intragene haplotypes TTA and GGG in the animals studied. Genotyping performed for *g.-780T/G*, *g.-768T/G*, and *g.-222A/G* SNPs revealed two genotypes for each locus in the HF and PR breeds, only. All studied HER and LIM animals showed variant TTA. No GGG/GGG genotype was found in all cattle breeds tested. The frequencies of the haplotype TTA and GGG for HF and PR were 0.829, 0.108 and 0.950, 0.050, respectively.

Three SNPs within exon 11 segregated as two haplotypes GGG and AAA, with higher AAA haplotype frequencies in

all examined breeds (Supplementary Table S2). All genotypes for the tested SNPs distributed according to the Hardy–Weinberg equilibrium. The *g.167747C/-* InDel polymorphism in the intron 9 was not genotyped.

In silico analysis of the promoter SNPs using TESS software revealed that allele T at the *g.-780T/G* SNP creates putative binding sites for *HiNF-A*, *SGF-1*, and *POU1F1a* transcription factors, which are abrogated by the G allele, respectively. The SNP *g.-768T/G* was located within the potential sites for *c-Ets-2*, *PU.1*, and *H-2RIIBP*, which are abrogated or introduced by the T allele, respectively. While the *g.-222A/G* SNP changes the binding sites for *IHF* transcription factors (Supplementary Fig. S3).

The *in silico* transcription factor binding site analysis is in the line with the real-time PCR results that showed a genotype-dependent *MEF2A* mRNA expression level ($p < 0.01$) in the *longissimus dorsi* muscle. The mRNA expression of *MEF2A* was higher in animals carrying homozygous TTA/TTA genotype, than in those with heterozygous TTA/GGG variant (Fig. 1).

Discussion

Members of the MEF2 family of transcription factors are upregulated during skeletal muscle differentiation and cooperate with the MyoD family of myogenic basic helix-loop-helix (bHLH) transcription factors to control the expression of muscle-specific genes (Molkentin and Markham, 1993; Black *et al*., 1998).

Recently, a number of published studies have clearly shown that MEF2 factors are involved in the postnatal regulation of skeletal muscle development, growth, and homeostasis (Knapp *et al.*, 2005; Hennebry *et al.*, 2009). Zhao *et al.* (2011) confirmed that muscle regulatory factor (MRF) and *MEF2* families are critical for the phenotypic differences between two pig breeds and proposed a novel model myogenesis. According to these authors, *MyoD* and *MEF2A* control the balance between intermuscular adiopogenesis and myogenesis by regulating the CCAAT/enhancer-binding protein (CEBP) family, while *MEF2C* and *Myf5* are important during the whole myogenesis process and *MEF2D* affects muscle growth and maturation. The expression level of MEF2 family genes is downregulated after birth but increases during skeletal muscle regeneration (Parker *et al.*, 2003), while Musaro *et al.* (1995) showed that *MEF2C* was expressed at lower levels in newborn and young mice but increased conspicuously in adults and remained high in senile mice. The increased expression of the *MEF2C* in adult mice was associated with increased slow myosin isotype, indicating the possible role of *MEF2C* in the induction of the myogenic pattern specific for type I fibers in mature muscles (Senna *et al.*, 2004; Hennebry *et al.*, 2009).

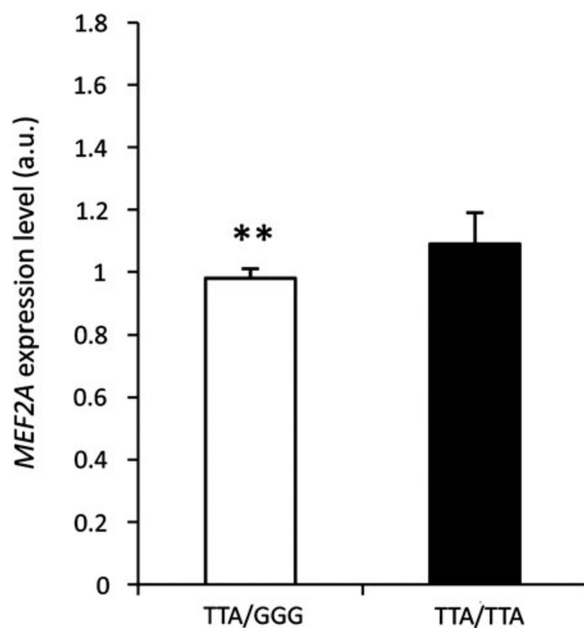

FIG. 1. The effect of SNPs at position *-780T/G*, *-768T/G*, and *-222A/G* in the promoter region on the *MEF2A* expression level in *longissimus dorsi* muscle; $^{**}p < 0.01$.

Several studies have established that MEF2 factors govern the intricate process of cardiogenesis by regulating cardiac-specific gene expression (Black *et al.*, 1998). In addition, MEF2 transcription factors are involved critically in the regulation of inducible gene expression during myocardial cell hypertrophy, inasmuch as the MEF2-binding site within the *MLC2* promoter is required during PE-mediated and ET-1–mediated hypertrophy (Zhu *et al.*, 1991) and MEF2 DNA-binding activity is increased in the hearts of rats subjected to pressure or volume overloads (Molkentin and Markham, 1993).

Several SNPs identified in the human *MEF2A* gene are associated with hypertrophic cardiomyopathy and coronary artery disease (CAD). Several missense mutations of the *MEF2A* gene were identified in CAD patients, which reduced the transcriptional activation activity of MEF2A and could increase the risk of suffering a myocardial infarction (Bhagavatula *et al.*, 2004; Weng *et al.*, 2005; González *et al.*, 2007; Elhawari *et al.*, 2010). Coto *et al.* (2009) reported that mutation CAG-repeat in exon 12 of the human *MEF2A* gene could be a risk factor for left ventricular hypertrophy.

In the current study, six novel SNPs of the *MEF2A* gene were identified, suggesting that the bovine *MEF2A* gene is lowly polymorphic and evolutionarily conserved. In addition, three SNPs in the promoter region appeared to be breed specific for the HF and PR cattle breeds. Recently, Zhou *et al.* (2010) described three SNPs in 5′-UTR, exon 4, and intron of the chicken *MEF2A* gene. In our previous study, we found in the bovine Mef2C gene two substitutions and two *InDel*s upstream from the putative transcription start of exon 1 that were deposited in the *GenBank* database (Juszczuk-Kubiak *et al.*, 2011).

In the current study, the promoter variants are associated with the *MEF2A* mRNA expression level in the *longissimus dorsi* muscle in 12-month-old HF bulls. The SNPs in the *MEF2A* promoter region are localized within the binding sites for transcription factors, namely *HiNF-A*, *SGF-1*, *c-Ets-2*, *PU.1*, *H-2RIIBP*, *IHF*, and *POU1F1a*. Salvatori *et al.* (2002) reported that the *GHRHR* promoter is regulated by POU1F1 and that promoter mutations impairing POU1F1 binding can reduce the expression of *GHRHR*. In addition, POU1F1 regulates GH secretion in the pituitary gland and plays crucial roles in the development and growth of mammals (Gil-Puig *et al.*, 2005). This suggests that the transcription factor might also be involved in the *cis*-regulation of the *MEF2A* transcription in muscles. Similar effects have been noted previously in other bovine genes where mutations localized in the promoter region changed the affinity of transcription factors

to the promoter sequence and acted as *cis*-regulators in the expression of the target gene (Flisikowski *et al.*, 2004; Adamowicz *et al.*, 2006). Additionally, these SNPs might be in linkage disequilibrium with the variation in other regions of the gene with functional or structural significance. So far, the potential effect of any polymorphisms in the regulatory region of the *MEF2A* gene on its expression in the muscles of cattle has been not reported in any available study.

It is known that MEF2 proteins act as major transducers of Ca^{2+} signaling events and that these events have a central role in the hypertrophic growth and remodeling of adult skeletal muscle in response to mechanical loads (Olson and Williams, 2000). Additionally, increased *MEF2* expression is induced by exercise in response to acute endurance exercise, which is correlated with the increased proportion of oxidative fibers without promoting fast to slow fiber transformation (Vissing *et al.*, 2008). It is also possible that *MEF2A* promoter variants could have a potential effect on *MEF2A* transcription levels in human muscles and might influence the homeostasis of muscles and their performance during exercise. Therefore, it is possible that postnatal skeletal muscle growth depends more on Ca^{2+} signaling and MEF2 proteins than on myogenic bHLH factors (Knapp *et al.*, 2005).

Two SNPs, *g.1599G/A* (P421P) and *g.1626G/A* (P429P) in exon 11, could not change amino acids but might have caused codon alternations, which probably affected translation efficiency and the efficiency of cotranslational protein folding (Kimchi-Sarfaty *et al.*, 2007). Additionally, proline residue confers unique structural constraints on peptide chains and markedly influences the speed of protein folding (Wedemeyer *et al.*, 2002).

The bovine *MEF2A* gene is localized on BTA21 and contains 11 exons, encoding for 492 amino acids, and has a genomic structure similar to that in humans and pigs (Wu *et al.*, 2011), and recent studies have shown that polymorphisms in the *MEF2A* gene are associated with body weight in chicken and cattle (Chen *et al.*, 2010; Zhou *et al.*, 2010). Chromosome 21 harbors 2 QTLs associated with birth weight. Davis *et al.* (1998) and Casas *et al.* (2003) detected a QTL for birth weight in the centromeric region (located at 4 centimorgans) of this chromosome, but Kim *et al.* (2003) found a QTL for birth weight at 62 centimorgans from the beginning of the linkage group. These implied that *MEF2A* might have an important role in postnatal muscle development, and is considered to be a potential molecular marker for carcass quality traits in farm animals. This information on genotype-dependent *MEF2A* mRNA expression can be utilized for future marker selection in association studies of carcass traits in cattle and for more detail in *in vitro* functional analyses.

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Disclosure Statement

No competing financial interests exist.

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