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THE DNA-BINDING CAPACITY OF GENETIC VARIANTS OF THE BOVINE STAT5A TRANSCRIPTION FACTOR

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Abstract: The STATs are a family of transcription factors. STAT5A, previously known as MGF, transduces prolactin signals to the milk protein genes. Here, we describe the detection of nucleotide sequence polymorphism in exon 16 of the bovine STAT5A gene, coding for the SH2 domain. SSCP was found in a 281-bp PCR amplified gene fragment, lying between positions 12,525 and 12,806, and encompassing parts of intron 15 and exon 16 of the bovine STAT5A gene (GenBank AJ 237937). Three SSCP patterns (genotypes) were identified in a group of 108 animals of different cattle breeds. The DNA sequencing showed that they differed by a CCT deletion at position from 12,549 in intron 15, and a T→C substitution at position 12,743 in exon 16. The latter mutation changes an amino acid sequence in the STAT5A protein – a Val/Ala substitution at position 686. Since T→C substitution creates a new *MspI* site, genetic variants in the bovine STAT5A gene can be distinguished with RFLP analysis. The frequency of alleles T and C varied between the different cattle breeds studied; the CC genotype was the least frequent and the frequency of alleles T and C was 0.842 and 0.158, respectively. Proteins were extracted from the cell nuclei of liver

Abbreviations used: STATs - signal transducers and activators of transcription; MGF - mammary gland factor; SSCP - single-strand conformation polymorphism; PCR - polymerase chain reaction; Val - valine; Ala - alanine; EMSA - electrophoretic mobility shift assay; GH - growth hormone; PRLR - prolactin receptor; JAK - Janus tyrosine kinase; bp - base pairs; Kb - kilobases; dNTP - deoxynucleoside 5'-triphosphate; TBE buffer - TRIS-borate-EDTA buffer; RFLP - restriction fragment length polymorphism; EDTA - ethylenediaminetetraacetic acid; A - deoxyadenosine; C - deoxycytosine; G - deoxyguanosine; T - thymidine; SD - standard deviation; dCTP - deoxycytosinetriphosphate; GR - glucocorticoid receptor; QTLs - quantitative trait *loci*.

tissues derived from bulls of different STAT5A genotypes and subjected to EMSA in order to study if the amino acid substitution might change the DNA-binding capacity of STAT5A transcription factor. Statistically significant ($p < 0.05$) differences in nuclear protein binding to DNA were observed between genotypes TT and CC; nuclear proteins derived from CC animals always showed less DNA protein complexing than those of TT animals. EMSA competition experiments confirmed that STAT5 transcription factors take part in the formation of the DNA-protein complexes.

Key Words: STAT5A, Bovine, DNA Polymorphism, SSCP, RFLP, EMSA

INTRODUCTION

The STATs, a family of transcription factors, mediate the actions of a variety of peptide hormones and cytokines [5, 8, 16]. The DNA-binding activity of STAT5 was first identified in the mammary gland, and it was thus first named MGF [21]. STAT5 is a key intracellular mediator of prolactin signalling, and can activate the transcription of milk protein genes in response to prolactin [21]. STAT5 is also known as the major mediator of GH action on target genes [2]. This protein is likely to play an important role in establishing or maintaining lactation in the mammary gland. Some key components of this signal transduction pathway have recently been identified. The PRLR has no intrinsic tyrosine kinase activity, and belongs to the class I cytokine receptor family. It was recently demonstrated that signal transduction of these cytokine receptors is mediated by members of the JAK family [19]. Upon ligand binding to the receptor, the latent cytoplasmic STAT factors are activated through phosphorylation of a distinct tyrosine residue [19, 20]. STAT5 exists in two isoforms – A and B – which differ by a few amino acids in the carboxylic end of the protein molecule; separate genes code both of them. In cattle, the STAT5A and STAT5B genes are located close to each other (within 40 Kb) at chromosome 19 [12, 18]. The STAT5 factors interact and functionally synergize with the receptors for glucocorticoid hormones and insulin [4, 13]. Only in a few cases was nucleotide sequence polymorphism detected in the bovine STAT5A gene. McCracken *et al.* [11] found TG repeats of different lengths within the gene in intron 12. Antoniou *et al.* [1] found two SSCP variants of the gene fragment that encodes the SH2 domain in bovine STAT5A protein. Two members of our team, Flisikowski and Zwierzchowski [7], observed 6 different SSCP patterns in exon 7 of the bovine STAT5A gene. It is necessary to detect additional polymorphisms to help in the investigation of the role of STAT5A variation in the milk production traits.

In this report, we describe our study, the aim of which was to search for a new genetic polymorphism in the bovine STAT5A gene and its effect on the STAT5A protein function. For the first time, the genotype/function relationship was detected; statistically significant differences in nuclear protein binding to DNA were observed between STAT5A genetic variants.

MATERIALS AND METHODS

Animals and DNA isolation

Young bulls of different breeds – Friesian, Charolais, Limousin, Aberdeen Angus, Hereford and Simmental – were included in the study. Approximately 10-ml blood samples were collected from each animal on K₂EDTA, and genomic DNA was isolated from the leukocytes [9]. Liver samples were excised immediately after death from animals routinely slaughtered at the local abattoir. The liver samples were frozen at -25°C and stored at -80°C until use. All the procedures were approved by the Local Ethical Commission, permission No 67/2001.

PCR conditions

Based on the sequence available from GenBank (AJ 237937) and using the Primer3 software available from the Internet (www.genome.wi.mit.edu), the following PCR primers were designed:

STAT 1: 5'-AGCCCTACAGCTCCAATCCT-3'

STAT 2: 5'-GGGTGTACCCGCTGCTTAG-3'.

With these primers, a 281-bp PCR fragment, encompassing parts of intron 15 and exon 16 of the STAT5A gene, was amplified. The polymerase chain reactions were performed using a PCR-mix with: primers STAT1 and STAT2, each at a final concentration of 2 pmol/μl, 1 U Taq polymerase (Sigma), 1 μl Taq polymerase buffer, dNTPs of 2.0 mM/μl, ca 100 ng of genomic DNA, and H₂O up to 10 μl. The following PCR protocol was used: 1 min at 94°C, 1 min at 61°C, and 1 min at 72°C – 34 cycles. The yield and specificity of the PCR reactions were evaluated by electrophoresis of the products in 2% agarose gels (Gibco) with ethidium bromide.

SSCP analysis

SSCP analyses were carried out using a Hoepfer SE 600 electrophoresis apparatus (Pharmacia). A thermostatically controlled refrigerated water circulator was used to maintain the gel at a constant temperature of 12°C. The 8% polyacrylamide gel was prepared with a 1 x TBE buffer. Initial electrophoresis (without samples) was run for 2 h at 120 V, 50 mA, 8 W. Ten-μl samples of PCR products were mixed with 10 μl of denaturation buffer (formamide, 0.25% bromophenol blue, 0.5 M EDTA), denatured for 5 min at 94°C, rapidly chilled on ice, and then loaded onto the gel. The electrophoresis was run at 80 V, 40 mA, 5 W, for approx. 16 h. The gels were stained using the Silver Staining System (Kucharczyk, Poland).

DNA sequencing

PCR products of different genotypes in the STAT5A 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- μ l aliquots with 10 U of *Msp*I restriction nuclease (BioLabs, New England, USA) for 3 hours at 37°C. The restriction fragments were subjected to electrophoresis in 2% agarose/ethidium bromide gels (Gibco, BRL, England) in 1 x TBE buffer (0.09 M Tris-boric acid, 0.002 M EDTA). The gels were examined under UV light and documented in a FX Phosphoimager apparatus (Bio-Rad).

Electrophoretic mobility shift assays (EMSA)

Liver tissues of Charolaise and Polish Black-and-White (Friesian) bulls of different genotypes in the STAT5A gene were used as a source of nuclear proteins. DNA-binding assays were performed essentially as previously described [10]. The oligonucleotide STAT probe 5'-GGGAGATTCTAGGAATTCAATCC-3' (of the sequence given by Santa Cruz Biotechnologies, Inc.) containing the STAT binding site (underlined) was synthesised in TIB MOL-BIOL (Poznań, Poland) and labelled with [α -³²P]dCTP (ICN). For EMSA competition experiments, the unlabeled STAT probe was used. Competitor oligonucleotides were included at two different concentrations – 100- or 50-fold molar excess of the labelled probe. The specificity of protein binding to DNA was also tested using a mutated STAT probe – 5'-GGGAGATTTT**AGGT**ATTCATCC-3' labelled with [α -³²P]dCTP, in which the STAT-binding site was altered by two point mutations (shown in bold). The EMSA gels were dried in a BioRad GEL DRYER 543 apparatus and subjected to autoradiography with Kodak Screen. The autoradiograms were scanned with Bio-Rad Molecular Imager FX and densitometry was done with Quantity One program (Bio-Rad).

Statistics

The significance of differences in the nuclear protein binding to STAT5 was estimated with the Duncan's test.

RESULTS AND DISCUSSION

We used a PCR-SSCP method to identify a new polymorphism in intron 15 and exon 16 of the bovine STAT5A gene. According to the theory of the SSCP method [14], a DNA polymorphism is detected when the electrophoresis mobility of DNA bands is reproducibly different. First we obtained a specific PCR product of the desirable 281-bp size. Then the PCR product was subjected to SSCP analysis to find the sequence variation. The number of bands and their position in the gel very clearly showed the occurrence of DNA sequence variation (Fig. 1).

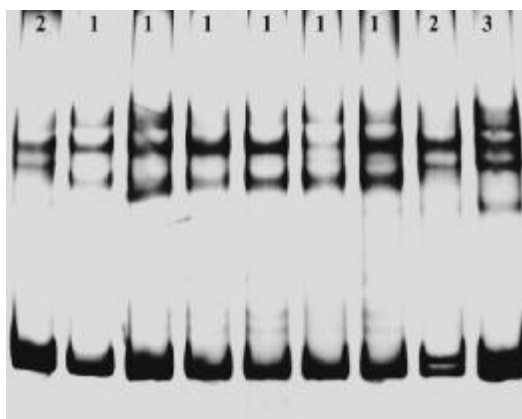


Fig. 1. SSCP polymorphism within intron 15/exon 16 of the bovine STAT5A gene. A polyacrylamide gel electrophoresis of the 281-bp gene fragment amplified from DNA of 10 animals. The PCR products were denatured, resolved in non-denaturing 8% polyacrylamide gels, and then silver stained. Three different SSCP patterns 1, 2, 3 (genotypes) were observed.

Within the analysed population of bulls, three SSCP patterns (gene variants) were observed, differing in the number and position of bands. The individual SSCP patterns were highly reproducible. Then we aimed to precisely identify the nucleotide mutations underlying the SSCP polymorphism. Different SSCP variants of the 281-bp PCR fragment were sequenced. The sequence of SSCP variant 1 appeared identical to the relevant fragment of the bovine STAT5A gene available in the GenBank database AJ 237937 (variant T; Fig. 2).

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consensus  ATCTCTCCTCTGTGGGCAATGGGCTTGTGAAATGAGCTTGGCCCTTTTCACAGCTGACCTTAACCTGTGGAAATCTGAAGCC
12550
GenBank    ATCTCTCCTCTGTGGGCAATGGGCTTGTGAAATGAGCTTGGCCCTTTTCACAGCTGACCTTAACCTGTGGAAATCTGAAGCC
Variant T  ATCTCTCCTCTGTGGGCAATGGGCTTGTGAAATGAGCTTGGCCCTTTTCACAGCTGACCTTAACCTGTGGAAATCTGAAGCC
Variant C  ATCTCTCT--GTGGGCAATGGGCTTGTGAAATGAGCTTGGCCCTTTTCACAGCTGACCTTAACCTGTGGAAATCTGAAGCC

consensus  ATTTCACACGCGGGATTTTCCTCATCCGATCCCTGGCCGACAGGTTGGGGACCTGAACTATCTCATCTACAGTGTTCCTCCG
12630
GenBank    ATTTCACACGCGGGATTTTCCTCATCCGATCCCTGGCCGACAGGTTGGGGACCTGAACTATCTCATCTACAGTGTTCCTCCG
Variant T  ATTTCACACGCGGGATTTTCCTCATCCGATCCCTGGCCGACAGGTTGGGGACCTGAACTATCTCATCTACAGTGTTCCTCCG
Variant C  ATTTCACACGCGGGATTTATCCATCCGATCCCTGGCCGACAGGTTGGGGACCTGAACTATCTCATCTACAGTGTTCCTCCG

T/C
consensus  ACCGCCCCAGGATGAGTCTTCTCCAGTACTACACTCTCTGTGCTTGGGTGGGCTGGCC
12710
GenBank    ACCGCCCCAGGATGAGTCTTCTCCAGTACTACACTCTCTGTGCTTGGGTGGGCTGGCC
Variant T  ACCGCCCCAGGATGAGTCTTCTCCAGTACTACACTCTCTGTGCTTGGGTGGGCTGGCC
Variant C  ACCGCCCCAGGATGAGTCTTCTCCAGTACTACACTCTCTGTGCTTGGGTGGGCTGGCC
    
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Fig. 2. Nucleotide sequence of the 281-bp fragment of the bovine STAT5A gene including the polymorphic RFLP-*MspI* site. The “polymorphic” C and T nucleotides are shown in bold. The deletion of the CCT trinucleotide is marked by asterisks. The sequence recognised by *MspI* nuclease is underlined.

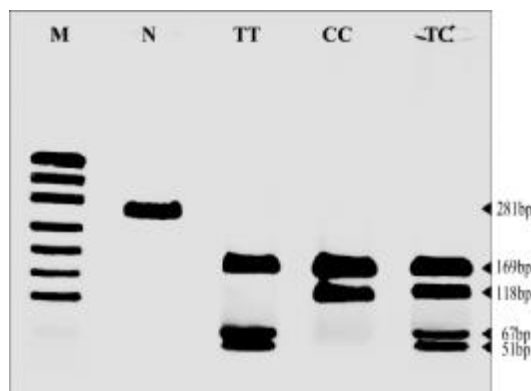


Fig. 3. RFLP-*M*s*I* polymorphism in exon 16 of the bovine *STAT5A* gene. A 2% agarose gel electrophoresis stained with Ethidium bromide. M – 11-1444 bp DNA marker (*Hae*III and *Taq*I digest of pUC19, InGen, Poland); lane N – non-cut PCR product. TT, TC, CC – *STAT5A* genotypes.

In variant 3, two mutations were identified: a deletion of trinucleotide CCT at position 12,549 in intron 15, and a T→C substitution at position 12,743 in exon 16 (variant C; Fig. 2). The latter mutation deletes the cutting site for *M*s*I* nuclease. We digested the 281-bp DNA fragment with the restriction nuclease and observed three different *STAT5A* genotypes – TT, TC, and CC (Fig. 3).

The 281-bp fragment contains two *M*s*I* restriction sites, of which only one appeared polymorphic. Digestion with the enzyme resulted in: two restriction fragments – 169 bp and 118 bp for TT homozygotes; three fragments – 169 bp, 67 bp, and 51 bp for CC homozygotes; and four fragments for TC heterozygotes. The SSCP variant 2 appeared heterozygous with respect to T→C substitution. The frequency of the different genotypes and of alleles T and C was studied in small samples from the different cattle breeds (Tab. 1).

Tab. 1. The distribution of genotypes and allele frequencies of RFLP polymorphism at the *M*s*I* site in the bovine *STAT5A* gene.

Genotypes and alleles	Breed					
	Polish Friesian	Charolaise	Limousine	Aberdeen Angus	Hereford	Simmental
	Number of animals					
TT	21	12	10	8	12	9
TC	14	5	6	2	4	2
CC	2	1	-	-	-	-
	Allele frequency					
T	0.756	0.805	0.812	0.90	0.875	0.909
C	0.244	0.195	0.188	0.10	0.125	0.091

In all the breeds, the TT genotype was the most frequent. The CC genotype was found in low frequency in only two breeds – Polish Fiesian and Charolaise. The overall frequency of alleles T and C was 0.842 and 0.158, respectively.

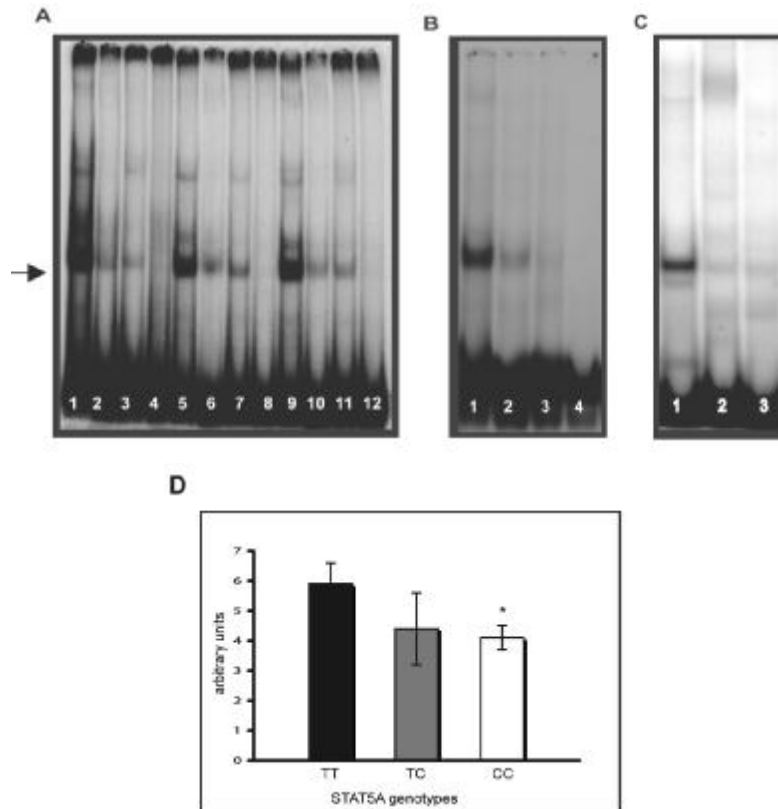


Fig. 4. The DNA-binding properties of different STAT5A genotype variants. Nuclear proteins were isolated from the livers of bulls differing by a C→T substitution at position 12,743 in exon 16 of the STAT5A gene (Val/Ala substitution at position 686 in the STAT5A protein). A. [³²P]dCTP-labeled STAT5 probe was used in EMSA. Lanes 1, 5, 9 – genotype AA; lanes 2, 6, 10 – genotype AB; lanes 3, 7, 11 – genotype BB; lanes 4, 8, 12 – control (free probe). B. Specificity of protein binding to DNA tested by competition with unlabeled STAT5A probe. EMSA were performed with a labeled STAT5 probe – lane 1 and with a 50x – lane 2 or 100x – lane 3, excess of the unlabeled probe, lane 4 – control (free probes). C. Specificity of protein binding to DNA tested with the mutated STAT probe: lane 1 – “canonical” STAT probe; lane 2 – mutated STAT probe; lane 3 – control (free mutated STAT probe). D. Quantitative differences in the binding of STAT5A variants to the STAT5 probe. The values given represent the average of six EMSA experiments with proteins derived from three animals of each genotype (mean ± SD), *difference statistically significant at p ≤ 0.05, arrow - band densitometered.

Computer analysis revealed that the substitution T→C at position 12,743 of the STAT5A gene changes the amino acid sequence at position 686 (Val/Ala) in the STAT5A protein. We measured the DNA-binding activity of different STAT5A genetic variants. Proteins were extracted from the cell nuclei isolated from the livers of Charolaise and Polish Black-White bulls carrying different STAT5A genotypes and then EMSA was performed. The differences were observed between all the genotypes (Fig. 4A), but only those between genotypes TT and CC were revealed as statistically significant ($p < 0.05$; Fig. 4C). In all cases, the nuclear protein derived from CC genotype animals showed a lower DNA-binding capacity than that from the other genotypes. EMSA experiments with an excess of unlabelled STAT probe and with the mutated STAT probe confirmed that STAT5 transcription factors take part in the formation of the DNA-protein complexes (Fig. 4B, C).

Very few experiments were performed dealing with the effects of gene mutations and the function of STAT proteins. In the mouse STAT5B, an artificially introduced substitution between glycine and glutamine at position 433 was shown to dramatically decrease its DNA-binding properties [3]. The glycine residue lay in the stretch of 5 amino acids that determine the DNA-binding specificity of the mouse and human STAT5 proteins. The ovine STAT5A mRNA was shown to contain a 3'-untranslated region extended by 130 nt as compared to its bovine counterpart [17]. This extended region is coded by an additional exon in the ovine STAT5A gene. The authors suggest that this difference might account for the improved translation efficiency and higher abundance of the STAT5A protein in ovine mammary tissues. Studies by Wheeler *et al.* [22] have shown that the DNA-binding properties of STAT5A were individually variable in mammary glands from lactating cows. We suggest that the genetic polymorphism of the STAT5A factor, including the RFLP-*MspI* polymorphism within exon 16, might result in such a variation in the DNA-binding properties between individuals. Although a T→C substitution at position 12,743 of the gene lies outside the DNA-binding domain of the STAT5A factor, it may change the whole protein conformation and thus influence its affinity to the relevant binding site in the target genes. Exon 16 codes for domain SH2, responsible for dimerization of the STAT5 transcription factors to the activation proteins [15]. Dimerization of the STAT molecules or their interaction with other regulatory proteins, e.g. with GR factor, was shown as a prerequisite for their DNA-binding and transactivation activities [6]. Moreover, mutations in this region might result in changes in the transactivation properties of the STAT5A protein, and thus influence the level of expression of the genes regulated by this transcription factor. However, with this approach it is impossible to discriminate between effects on the of STAT5A DNA binding activity (which could be due to differences in hormone responsiveness, STAT5A expression, changes in JAK2 kinase activation) and between changes due to the altered structure of STAT5A. Most of the research on genetic markers applied to animal breeding is focused on the analysis of mutations located within economically important structural genes and the linkage of these genes to QTLs. STAT5, originally identified in

the mammary gland of lactating animals, confers a prolactin response in mammary epithelial cells and is also known as the major mediator of growth hormone action. The STAT5A gene is a candidate gene for cattle quantitative traits. Previously, the polymorphism of a microsatellite length was described in intron 12 of the bovine STAT5A gene [11]. Also, two variants of the STAT5A gene region coding for SH2 protein domain were identified [1]. However, the effect of these polymorphisms on the production traits was not studied. The results of our study may be used in further research on the associations between different RFLP variants of the STAT5A gene and cattle performance traits. For the first time the genotype/function relationship was detected; statistically significant differences in nuclear protein binding to DNA were observed between STAT5A genetic variants.

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