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RFLP-*Tsp*RI polymorphism within exon 1 of the bovine estrogen receptor-α (*ER*α) gene*

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Estrogens regulate cellular activity by interacting with specifying intracellular receptor proteins. Due to the functions played in reproduction, development of the mammary gland, growth and differentiation of cells, estrogen receptors and their genes are considered candidates for the markers of production and functional traits in farm animals, including cattle. The detection is reported of a single-strand conformation polymorphism (SNP) within the coding region of the bovine estrogen receptor *ER*α gene – the A→C transversion at position 503 in exon 1 – recognizable by RFLP with *Tsp*RI restriction endonuclease. This mutation does not change the amino acid sequence of ERα **protein; both triplets – CCA and CCC – code for proline. This is the first ever report on the SNP polymorphism in the bovine ER protein-coding sequence.**

KEY WORDS: **cattle / DNA sequencing / *ER*α / gene polymorphism / PCR-RFLP**

Estrogen hormones are fundamental in the regulation of female sexual differentiation and reproduction. Estrogens regulate cellular activity by interacting with specifying intracellular receptor proteins. Estrogen receptors (ER), similarly as other nuclear receptors, are transcription factors, which after binding of a proper ligand (17β-estradiol, estron or estriol) are capable of regulating transcription of target genes. Known are two isoforms of the estrogen receptor – ERα and ERβ. In the human genome each of

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them is encoded by a separate gene, localized on chromosome 6 and 14, respectively. ER and its transcripts were found in many tissues, but the major expression sites in the reproductive organs in females are uterus, vagina and ovary, and in males – testes, epididymis, and prostate [Enmark *et al*. 1997]. High expression of ER was also found in other organs and tissues such as liver, lungs, mammary gland, kidneys, pituitary, gut, brain, and stomach. In some tissues differential expression of ERα and ERβ was found [Pfaffl *et al*. 2001].

In humans, ERα protein is coded by 8 exons, but in the 5' region of the *ER* genes additional exons are located that do not code for protein, but were shown to code for transcripts of different length with different 5'-UTR (untranslated region) – Kos *et al*. [2001]. The mRNA variants are created by an alternative splicing of the primary transcripts. Functions of the different ER transcripts are not known, but in some cases, tissue- or developmental stage-specific expression has been reported [Grandien *et al*. 1997].

The sequence and the structure of *ER* genes of humans, mouse, and rat is known and available in databases (e.g. GenBank). In addition, known is a partial sequence of the coding regions (cds) and of the 5' region of the gene coding for ERα in sheep and pigs, as well as the sequence of the exons 1, 5-7 of the bovine *ER*α gene. Recently, the whole sequence of the bovine *ER*α mRNA appeared in the GenBank (AY538775, Nishimura N. and Tetsuka M. 2004, unpublished).

So far, studies on the *ER* gene polymorphism in farm animals are limited. Rothschild *et al*. [1996] regarding the function of steroid hormones and their receptors in reproduction, proposed *ER* gene as a candidate marker for prolificacy in pigs. They identified nucleotide sequence polymorphism both in coding and non-coding regions of the porcine *ER*α gene. One of these mutations was found to be significantly associated with the mean number of piglets born per litter in PIC Meishan pigs [Rothschild *et al*. 1996]. The polymorphic site was located in an intron (not shown which one) and was recognized by RFLP method with *Pvu*II restriction nuclease. Other authors [e.g. Kmieć *et al*. 2002] further confirmed this relationship. In their study, allele B in a sow's genotype appeared significantly associated with the litter size in Polish Landrace pigs. However, Korwin-Kossakowska *et al*. [2000], in their studies on Złotnicka Spotted × Polish Landrace crosses failed to find any effect of *ER* genotype on the litter size.

The aim of present study was a search for a polymorphism in the coding region of the bovine estrogen receptor-α gene – in exon 1 that codes for the transactivating domain of the ER protein.

Material and methods

Animals and DNA isolation

The DNA samples were isolated from young bulls or cows of the following breeds: Polish Friesian (n=16), Charolaise (n=17), Hereford (n=13), and Simmental (n=11), maintained at the Institute Farm, Jastrzębiec, or from Polish Red cows (n=13) from

the Polish Academy of Sciences Research Station, Popielno. Approximately 10 ml blood was withdrawn from each animal to test tubes containing K_2EDTA by authorized veterinarian. DNA was isolated from leukocytes [Kanai *et al*. 1994]. All experimental procedures involving animals were approved by the Local Ethics Commission (permission No. 40/2003).

PCR conditions

Based on sequence available from GenBank (AY641986) and using the Primer3 software available from Internet (www.genome.wi.mit.edu) the following PCR primers were designed:

ER1 F: 5'-ACCGCCCGCAGCTCAAG-3'

ER2 R: 5'-TGTCGCCCTTCCTGCACCCGC-3'

With these primers a 294-bp PCR-fragment encompassing a part of exon 1 of the bovine *ER*α gene, was amplified from nucleotides 232 to 526.

The polymerase chain reactions (PCR) were performed using a PCR-mix with: 0.5 µl of primers ER1 and ER2, each at concentration of 10 pmol/ml, 1 U Taq polymerase (QIAGEN), 1 µl Taq polymerase buffer, 0.8 µl dNTPs each in concentration of 2.5 mM, ca 100 ng of genomic DNA, and H_2O up to 10 µl. The PCR reactions were carried out in a MJ TETRAD thermocycler. The following PCR protocol was used: 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C – 36 cycles. The yield and specificity of PCR products were evaluated after electrophoresis in 4% LMP agarose gel (GIBCO BRL), with ethidium bromide.

SSCP analysis

The single-strand conformation polymorphism (SSCP) analysis was carried out with the use of Hoefer SE 600 electrophoresis apparatus (PHARMACIA). A thermostatically controlled refrigerated circulator Multitemp III (PHARMACIA) was used to maintain the constant temperature (12°C) of the gel. The 10% polyacrylamide gel was prepared with a 1 × TBE buffer and electrophoresis was run under the following conditions: (1) Initial (without samples) – 120 V, 50 mA, 8 W, 2 h; (2) At 80 V, 40 mA, 5 W, 16 h. Ten µl of PCR product was 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 gels were stained using the Silver Staining System (KUCHARCZYK T.E. Inc.).

DNA sequencing

PCR products of the *ER*a gene showing different SSCP patterns (genotypes), after purification with QIAquick® PCR Purification Kit (QIAGEN), were automatically sequenced in an ABJ377 sequencer (APPLIED BIOSYSTEMS, USA). The sequencing was done at the Polish Academy of Sciences Institute of Biochemistry and Biophysics, Warsaw. The sequence was analysed using the Sequence Analyser 2.01 programme.

RFLP analysis

Ten µl of mixture containing 294-bp PCR products were digested with 10 U of *Tsp*RI restriction endonuclease. The PCR products and restriction fragments were subjected to electrophoresis in 4% agarose/ethidium bromide gel. Gels was visualised and documented by the Molecular Imager System FX (BioRad).

Results and discussion

A PCR-SSCP method was used to identify a polymorphism in the coding region in exon 1 of the bovine *ER*α gene. The 294-bp PCR product, encompassing part the exon 1 was denatured and then subjected to polyacrylamide gel electrophoresis to find the sequence variation. The number of bands and their position in the gel clearly showed the occurrence of DNA sequence variation (Photo 1). Within the analysed population of cattle, three different highly reproducible SSCP patterns were observed. The DNA samples representing SSCP patterns 1 and 2 (putatively homozygotes) were sequenced. A nucleotide substitution, the A→C transversion was identified at nucleotide 503, upstream the putative transcription start site of the exon 1 (Photo 1). The SNP was located within the proline CCA codon. However, this mutation does not change the amino acid sequence of ERa protein, since both triplets – CCA and CCC – code for proline.

Comparison of the restriction maps of both *ERa* gene variants revealed that A→C substitution created a restriction site for *Tsp*RI endonuclease, thus enabling PCR-RFLP

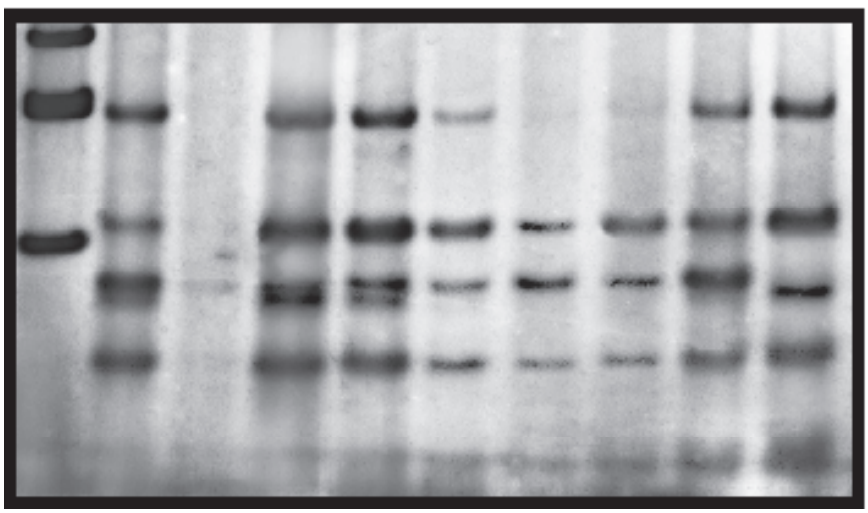

Photo 1. Polyacrylamide (8%) gel electrophoresis showing three PCR-SSCP patterns of the bovine estrogen receptor-α (*ER*α) gene. M – 26-501-bp DNA marker (*Msp*I digest of pUC19). AC, CC, AA – different SSCP patterns.

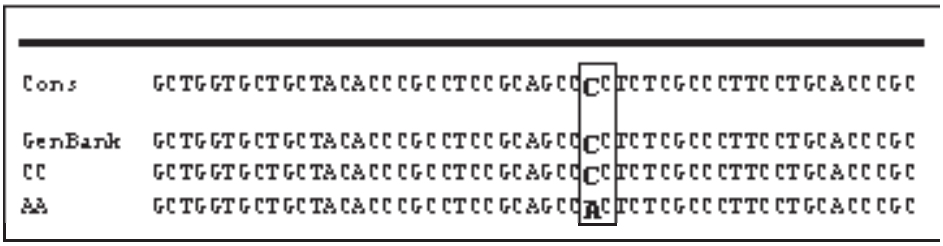

Fig. 1. Polymorphic variants in the analysed fragment of the bovine *ERα* gene. Sequence of the 53-bp fragment of the bovine *ERα* gene showing A→C transversion at position 503 relative to transcription start site of the exon 1. Sequenced A and C variants were compared to the GenBank mRNA sequence (AY538775). Cons – the consensus sequence.

analysis of the gene polymorphism to be performed. The following restriction fragments resulting from digestion of the 294 bp PCR product with *Tsp*RI endonuclease were found: the uncut 294-bp amplicon for genotype *CC*, 277-bp and 17-bp bands for genotype AA, and 294-bp, 277-bp, and 17-bp for heterozygotes AC (Photo 2). Although the shortest 17-bp fragment was not visible on the gels, the band patterns enabled clearly distinction between genotypes.

Using the PCR-RFLP method genotyped were 70 individuals representing different cattle breeds (Tab. 1). All three genotypes – *AA, AC*, and *CC* – were identified in the

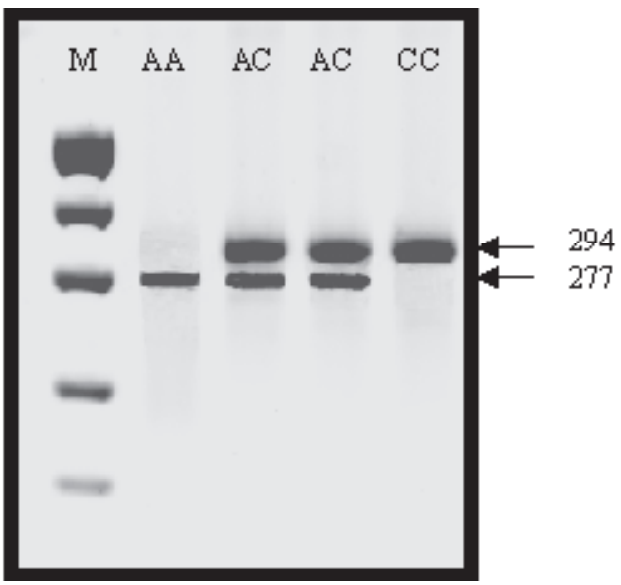

Photo 2. The RFLP-*Tsp*RI genotyping of the bovine *ERα* gene. Agarose (4%) gel showing *ERα* genotypes after digestion of the 294-bp PCR product with *Tsp*RI restriction endonuclease. Genotypes (*AA, CC, AC*) are shown at the top of each line. M – 26-501-bp marker (*Msp*I digest of pUC19).

breeds studied. The frequency of the allele A varied from 0.42 in Polish Red cattle to 0.63 in Simmentals. Preliminary results presented showed that occurrence of different genotypes and alleles might not differ between breeds.

Due to the functions that estrogens play in the female reproduction and in development of the mammary gland, estrogen receptors and their genes are considered candidates for the markers of production and functional traits in farm animals, including cattle. In the

Table 1. Occurrence of the A→C genotypes and alleles of the exon 1 of the *ERα* receptor gene identified by *Tsp*RI RFLP in a sample of dairy and beef cattle

Breed	Number of animals			Allele frequency		Total number of animals
	genotype *AA*	genotype *CC*	genotype *AC*	*A*	*C*	
Polish Friesian	5	3	8	0.56	0.44	16
Charolaise	7	5	5	0,56	0.44	17
Hereford	3	4	6	0,46	0.54	13
Simmental	5	2	4	0.63	0,37	11
Polish Red	3	5	5	0.42	0.58	13

earlier study [Szreder and Zwierzchowski 2004], sequenced was the 2853-bp fragment of the 5'-region of the bovine *ERα* gene. The sequence was deposited in the GenBank database under accession no AY340597. Using this sequence, we identified a polymorphism within 5' region of the bovine *ERα* gene – the A→G transition, recognizable by RFLP technique with *Bgl*I restrictase, located upstream to the exon C. However, no polymorphisms in the coding sequences of the bovine *ERα* gene were reported, so far. Detection of additional polymorphisms is necessary to help elucidate the effect of *ER* variation on the production traits in cattle.

In the present study, using SSCP, RFLP, and sequencing methods, an attempt was made to find out a polymorphism in the coding region of the bovine *ERα* gene in exon 1 that codes for the transactivating domain of the estrogen receptor protein. The A→C transversion was found at position 503 within the proline codon CCA. The mutation does not change the amino acid sequence of ERa protein as both triplets – CCA and CCC – code for proline. Although silent, this mutation has a potential for a genetic marker for production and functional traits in cattle. Such silent mutations might be linked to other, causative mutations located in the vicinity. In summary, the SNP polymorphism has been identified in the exon 1 of the bovine *ERα* gene. This is the first ever report on the polymorphism in the protein-coding sequence of the bovine *ER* receptor gene.

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Polimorfizm RFLP/*Tsp*RI w eksonie 1 genu receptora estrogenu α (*ER*α) bydła

Streszczenie

Estrogeny pełnią ważną rolę w rozrodzie samic, a także regulują wzrost i różnicowanie komórek i całego organizmu. W działaniu estrogenów na komórkę pośredniczą specyficzne wewnątrzkomórkowe receptory. Receptor estrogenu (ER) jest białkowym czynnikiem transkrypcyjnym, które wiążąc się z DNA reguluje ekspresję genów. U ssaków występują dwie izoformy receptora estrogenów – ERα i ERβ. Ze względu na liczne funkcje estrogenów, ich receptory i kodujące je geny są kandydatami na markery genetyczne cech produkcyjnych i funkcjonalnych u zwierząt gospodarskich. Stosując metody SSCP, RFLP i sekwencjonowania, zidentyfikowano polimorfizm typu SNP (*single nucleotide polymorphism*) w rejonie kodującym genu *ER*α bydła – transwersję A→C w pozycji 503 w eksonie 1. Mutacja rozpoznawana jest metodą RFLP z użyciem endonukleazy *Tsp*RI. Ta mutacja nie zmienia sekwencji aminokwasów w białku ERα; oba tryplety – CCA i CCC kodują prolinę. Jest to pierwszy opisany polimorfizm genu *ER*α bydła w rejonie kodującym białko.