RFLP-\textit{Tsp}RI polymorphism within exon 1 of the bovine estrogen receptor-\( \alpha \) (\textit{ER}\( \alpha \)) 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 \textit{ER}\( \alpha \) gene – the A\( \to \)C transversion at position 503 in exon 1 – recognizable by RFLP with \textit{Tsp}RI restriction endonuclease. This mutation does not change the amino acid sequence of \textit{ER}\( \alpha \) protein; both triplets – CCA and CCC – code for proline. This is the first ever report on the SNP polymorphism in the bovine \textit{ER}\( \alpha \) protein-coding sequence.

KEY WORDS: cattle / DNA sequencing / \textit{ER}\( \alpha \) / 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 (17\( \beta \)-estradiol, estron or estriol) are capable of regulating transcription of target genes. Known are two isoforms of the estrogen receptor – \textit{ER}\( \alpha \) and \textit{ER}\( \beta \). 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 ERα gene. Recently, the whole sequence of the bovine ERα 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 ERα 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 PvuII restriction nuclease. Other authors [e.g. Kmiec 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 Zlotnicka 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 K₂EDTA by authorized
veterinarian. DNA was isolated from leukocytes [Kanai et al. 1994]. All experimental
procedures involving animals were approved by the Local Ethics Commission (per-
mission 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’-ACCGCCCAGCTCAAG-3’
ER2 R: 5’-TGTCGCCCTTCCCTGACCCGC-3’

With these primers a 294-bp PCR-fragment encompassing a part of exon 1 of the
bovine ERα 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 H₂O 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 ERα 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 TspRI 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 ERα 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 ERα protein, since both triplets – CCA and CCC – code for proline.

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

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*Photo 1. Polyacrylamide (8%) gel electrophoresis showing three PCR-SSCP patterns of the bovine estrogen receptor-α (ERα) gene. M – 26-501-bp DNA marker (MspI digest of pUC19). AC, CC, AA – different SSCP patterns.*
analysis of the gene polymorphism to be performed. The following restriction fragments resulting from digestion of the 294 bp PCR product with TspRI 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
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 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 BglI 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 ERα 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.

**REFERENCES**


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**Polimorfizm RFLP/TspRI w eksonie 1 genu receptora estrogenu α (ERα) 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 wiążą się z DNA regulując 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 ERα bydła – transwersję A→C w pozycji 503 w eksonie 1. Mutacja rozpoznawana jest metodą RFLP z użyciem endonukleaza TspRI. Ta mutacja nie zmienia sekwencji aminokwasów w białku ERα; oba tryplety – CCA i CCC kodują prolinę. Jest to pierwszy opisany polimorfizm genu ERα bydła w rejonie kodującym białko.