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Nucleotide sequence polymorphisms in the promoter region of bovine growth hormone receptor gene (*GHR*) have no effect on its expression level in liver*

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The gene coding for bovine GHR consists of nine protein-coding exons and untranslated, alternative exons 1A, 1B, and 1C in its 5'-region. Distinct promoters regulate transcription from each of the alternative exons. The P1 promoter which drives growth hormone receptor expression in the liver is associated with exon 1A. Earlier the nucleotide sequence polymorphisms have been identified in the bovine *GHR* gene promoter region, including several single nucleotide polymorphisms (SNPs) and one TG repeat (microsatellite) of variable length. Using computer-aided analysis in TESS programme it has also been shown that the A/G transition at position -154 (RFLP-*Nsi*I) and the C/T transition at position -1104 (*Fnu*4HI), both located upstream the exon 1A, co-localized with putative transcription factor-binding sites. In light of this the authors decided to study possible effects of these polymorphisms on *GHR* gene expression in cattle of different *GHR* genotypes, using Real-time PCR. Interestingly, no difference was found in *GHR* mRNA accumulation in liver between young Black-and-White (BW) bulls carrying (+/+), (+/-) or (-/-) genotypes at RFLP-*Nsi*I site, (+/-) or (+/+) genotypes at RFLP-*Fnu*4HI site, and $TG_{17/17}$ or $TG_{21/21}$ alleles at TG_n microsatellite, located within the P1 promoter of the bovine *GHR* gene.

KEY WORDS: cattle / gene expression / growth hormone / liver / polymorphism

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Growth hormone is the main regulator of postnatal growth and metabolism in mammals, stimulating anabolic processes such as cell proliferation, skeletal growth and protein synthesis, by modulating the expression of many genes [Burton *et al*. 1994]. Growth hormone (GH) activity depends on the GH receptor (GHR). The GHR mediates biological actions of growth hormone on target cells by transducing the GH signal across the cell membrane and inducing transcription of many genes, including *IGF1* [Rotwein *et al*. 1994, Argetsinger and Carter-Su 1996]. Therefore, genes encoding GH and GHR are viewed as promising candidate markers for selection purposes in cattle [Parmentier *et al*. 1999]. The gene coding for bovine GHR consists of nine exons (numbered 2 to 10) in the translated part, and of a long 5'-noncoding region that includes several alternative untranslated exons, of which only exons 1A, 1B, and 1C have been studied in detail [Jiang and Lucy 2001]. Distinct promoters regulate transcription from each of the alternative exons. The P1 promoter, which drives GHR expression in the liver, is associated with exon 1A [Jiang *et al*. 1999]. The liver expression of the GHR gene is regulated by various hormones, including GH and steroids, and by the level of nutrition [Schwartzbauer and Menon, 1998].

Several polymorphic sequences have been identified in the bovine *GHR* gene [Falaki *et al*. 1996, Moisio *et al*. 1998, Aggrey *et al*. 1999, Hale *et al*. 2000]. A DGGE polymorphism was reported in the promoter P1 upstream of a transcription starting site of exon 1A [Ge *et al*. 1999], next identified as an A/G transition at position -154, recognized as RFLP with *Nsi*I endonuclease [Ge *et al*. 2003]. In the earlier authors' study a novel RFLP was found in the bovine *GHR* gene 5'-region at *Fnu*4HI/*Tse*I site [Maj *et al*. 2005b]. The C/T transition was determined by sequencing at position -1104. Two alleles and three genotypes were identified within the analysed dairy and beef cattle. The RFLP-*Fnu*4HI site [Maj *et al*. 2005b] and RFLP sites for restriction endonucleases *Alu*I and *Acc*I previously identified by Aggrey *et al*. [1999], were shown to be located within the 1,206 bp LINE-1 element.

Earlier, the authors of the present paper reported the associations occurring between *GHR* RFLP-*Nsi*I and certain production traits of cattle, *i.e*. milk yield and composition, growth performance and carcass traits [Maj *et al*. 2004bc, 2005a]. Better performance for most beef production traits was shown in BW bulls of (+/+) genotype at the *Nsi*I site, while cows of the RFLP-*Nsi*I (-/-) and (+/-) genotypes yielded more milk with higher content of fat, protein, and lactose than those of the +/+ genotype. Moreover, in Angus cattle an association of RFLP-*Nsi*I *GHR* polymorphism was found with the IGF-1 blood level [Ge *et al*. 2003]; in the (+/+) individuals the mean IGF-1 concentration of blood was higher than in (-/-) animals.

According to the earlier authors' study, the RFLP-*Fnu*4HI affected neither the milk yield and composition [Maj *et al*. 2004b] nor the meat production traits [Maj *et al*. 2005a] in BW cattle. However, Charolaise, Limousine, Aberdeen Angus and Hereford bulls of the (+/+) genotype and with the allele (+) at the RFLP-*Fnu*4HI site appeared superior for most growth performance and meat production traits [Maj *et al*. 2004c].

Lucy *et al*. [1998] reported the polymorphism in length of the TG-repeat (microsatellite) of the bovine *GHR* gene P1 promoter, located 86 bp upstream from the start site of exon 1A. They found that a 11-TG-repeat allele commonly occurred in *Bos indicus* cattle while alleles with 16 to 20 consecutive TGs were most common in *Bos taurus* breeds. However, the short 11-TG-repeat allele was found at low frequency also in European cattle, e.g. in Aberdeen Angus. An association was reported between TG-repeat microsatellite marker and growth rate in Angus steers [Hale *et al*. 2000]. The authors' earlier data [Maj *et al*. 2004a] showed that in Polish BW bulls the TG-repeat polymorphism was significantly associated with daily gain, live body weight, cold carcass weight, and weight of lean and fat in valuable cuts. The longest 320-bp allele, with 21 TG repeats, was superior for most carcass traits, but if the growth rate was considered, the 320-bp allele homozygotes proved inferior to others.

Since the three mutations – A/G transition at position -154 (RFLP-*Nsi*I), C/T transition at position -1104 (RFLP-*Fnu*4HI), and variable TG_n repeat – are located in the regulatory sequences for the *GHR* gene, upstream to the alternative exon 1A, it could be supposed that they might influence the gene expression in the liver. Therefore, it was decided to identify the possible effects of these mutations on liver GHR mRNA accumulation in animals with different *GHR* genotypes, using Real-time PCR.

Material and methods

Search for putative transcription factor binding sites

Computer-aided analysis was performed of the of 5'- flanking region of the bovine *GHR* gene sequence for the presence of putative transcription factor (TF) binding sites and their possible co-localization within the three polymorphic sites studied: (1) RFLP-*Nsi*I, (2) RFLP-*Fnu*4HI, and (3) variable TG_n microsatellite. Analysis was performed using the TESS programme (J.Schug and Ch.G. Overton – http://www.cbil.upenn.edu/tess), the TRANSFAC database (http://www.biobase-international.com/pages/index.php?id=transfacdatabases), and Hibio DNAsis (HITACHI) programme package.

Animals

The study was performed on 153 Black-and-White (BW) bulls with more than 80% Holstein-Friesian blood. From birth to slaughter (day 348 of life) the animals were housed on local farm and fed twice daily the ration formulated according to their age and growth rate (corn silage, concentrates and hay). About 10 ml blood samples were withdrawn from each animal on K_2EDTA and genomic DNA was isolated from leukocytes according to Kanai *et al*. [1994]. Genotyping for RFLP-*Nsi*I, RFLP-*Fnu*4HI, and variable TG_n microsatellite was performed as described by Maj *et al*. [2004ab, 2005b]. Below given are symbols used for marking the SNP alleles.

Within the RFLP-*Fnu*4HI: (-) or (+) for the non-cut or cut allele, respectively, with nucleotide T or C at position -1104, and

Within the RFLP-*Nsi*I: (-) or (+) for the non-cut or cut allele, respectively, with nucleotide A or G at position -154.

All procedures concerning the animals were approved by the Local Ethics Commission (permission No 67/2001).

Tissue and RNA isolation

Post-slaughter liver samples obtained from young BW bulls of known genotypes were immediately frozen to -80°C until required for analyses. Five animals were used of each genotype group. Total RNA was isolated from liver tissue samples (middle part of the small lobe) as described by Chomczynski and Sacchi [1987] using TRIsol® Reagent (INVITROGEN™ Life Technologies) and treated with DNAseI (SIGMA) prior to reverse transcription (RT), to prevent DNA contamination.

Reverse transcription (RT)

The RT reaction was performed for 1 h at 42°C in 40 µl containing 2 µg RNA, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.3 mM dNTP mix, 40 U of RNasin, 0.5-µg oligo $d(T)_{15}$ primer and 200 U of M-MLV reverse transcriptase, all from PROMEGA (Madison, WI, USA).

Real-time PCR

The following PCR primers were used [Pfaffl *et al*. 2002] to amplify a 138-bp fragment of the bovine *GHR* cDNA spanning exons 8 and 9:

forward: 5'- CCAGTTTCCATGGTTCTTAATTAT-3' and
reverse: 5'-TTCCTTTAATCTTTGGAACTGG-3'.

Prior to Real-time PCR the ordinary PCR amplification was done and the specificity of the desired *GHR* and β-actin amplification products was documented with agarose (SIGMA-ALDRICH, Germany) gel electrophoresis and additionally with melting curve analysis.

A mastermix of the following components was prepared to give the indicated end concentrations: 6.4 µl water, 1.2 µl $MgCl_2$ (4 mM), 0.2 µl forward primer (0.4 µM), 0.2 µl reverse primer (0.4 µM) and 1.0 µl LightCycler Fast Start DNA Master SYBR Green I® (ROCHE DIAGNOSTICS, Germany). Glass capillaries were filled with 9 µl of the mastermix and 1 µl containing 30 ng of reverse transcribed RNA was added as the PCR template. As an internal control β-actin was used in each reaction. The capillaries were closed, centrifuged and placed into the rotor of the LightCycler PCR apparatus (ROCHE). The following amplification programme was applied: 10 min of denaturation at 95°C, 45 cycles of four-segment amplification with 10 s at 95°C for denaturation, 15 s at 61°C for annealing, 20 s at 72°C for elongation, and 4 s at 82°C for a single fluorescence measurement above the melting temperature of possible primer-dimers. The fourth segment eliminated a nonspecific fluorescence signal and

ensured accurate quantification of the desired product. Subsequently, a melting step was performed consisting of 2 s at 95°C, 5 s at 60°C and slow heating with a rate of 0.1°C per s up to 95°C, with continuous fluorescence measurement, and finally followed by cooling down to 35°C.

To create standards for calculating the amplification efficiency ($E=10^{-(1/b)}-1$; b = regression coefficient) during Real-time PCR, five dilutions of bovine liver cDNA (no dilution; 5×; 25×; 125×, 625×) were primed separately (in different PCR tubes) for *GHR* and β-actin as a reference gene. Relative mRNA expression for the *GHR* gene was calculated using the formula:

$$AU = (1 + E_{(GHR)}) - CT_{(GHR)}/(1 + E_{(\beta\text{-actin})}) - CT_{(\beta\text{-actin})}$$

The significance of differences between the expression levels of *GHR* genotypes was estimated with the Duncan's test.

Results and discussion

Transcription-factor binding sites

A computer-aided analysis was performed of the 5'-noncoding region of the bovine *GHR* gene using the TESS programme and TRANSFAC database. Two fragments were analysed, both located upstream the exon 1A of the gene – from nucleotide (nt) -177 to nt -58 and from nt -1137 to nt -1078. The presence was shown of several putative TF-binding sites 100% identical with the respective sequence cosensuses: NF-1, GR (from -1137 to -1078 fragment – Fig. 1-A), and C/EBP, HNF-3, GR, GATA1 (from -177 to

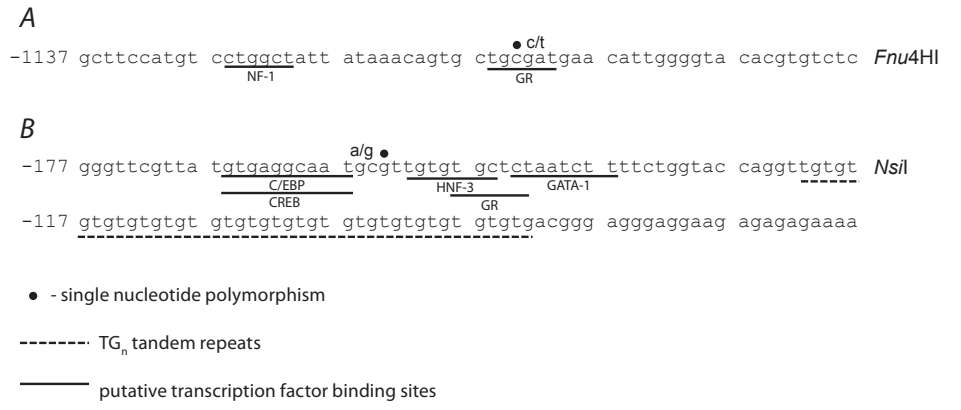

Fig. 1. Computer-aided search for putative transcription factor binding sites in two fragments of 5'-noncoding region of the bovine *GHR* gene, located within the promoter region for the exon 1A, using the TESS programme and TRANSFAC database. Also shown are the studied single nucleotide polymorphisms in *GHR* gene 5'- region: RFLP-*Fnu*4HI, RFLP-*Nsi*I and TG-repeat. **A –** from -1137 to -1078 gene fragment, with the C/T transition at position -1104 (RFLP-*Fnu*4HI). **B –** from -177 to -58 gene fragment, with the A/G transition at position -154 (RFLP-*Nsi*I), and with the TG_n repeat of variable length.

-58 bp fragment – Fig. 1-B). The GR (glucocorticoid receptor) binding site (½GRE – glucocorticoid response element) perfectly co-localized with the C/T transition at position -1104 (RFLP-*Fnu*4HI). Another two TF-binding sites were localized close to RFLP-*Nsi*I; putative binding sites for C/EBP and HNF-3 were removed by only 3 nt and 2 nt, respectively, from the A/G transition at position -154.

GHR genotypes

One-hundred-and-fifty-three young Black-and-White (BW) bulls were genotyped for *GHR* RFLP-*Nsi*I, RFLP-*Fnu*4HI, and variable TG_n microsatellite. For the A/G transition at position -154 (RFLP-*Nsi*I) three genotypes were found: AA (-/-), AG (-/+), and GG (+/+). For the C/T transition at position -1104 (RFLP-*Fnu*4HI) identified were only two genotypes – CT (+/-) and CC (+/+). Seven genotypes were detected of the *GHR* gene TG_n microsatellite, including four homozygotes – $TG_{17/17}$, $TG_{18/18}$, $TG_{19/19}$, and $TG_{21/21}$.

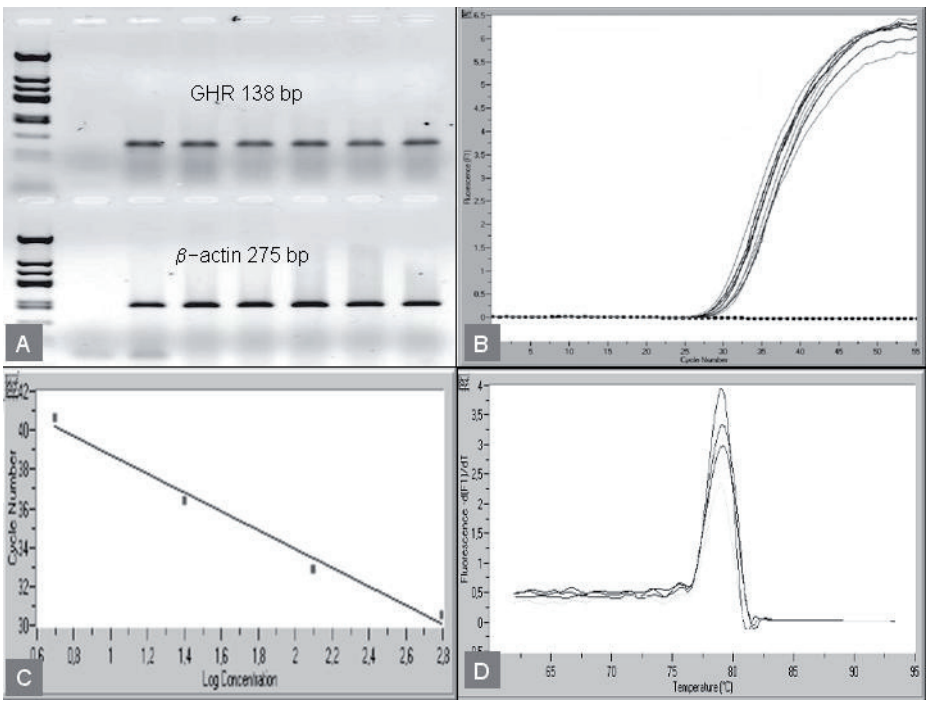

Fig. 2. Characteristics of the RT-PCR reaction done to measure expression of the *GHR* gene in the bovine liver. A – PCR amplification with primers specific for bovine *GHR* and bovine β–actin genes done using bovine cDNA as a template; results documented with agarose gel electrophoresis. B – quantification of *GHR* gene expression in Light Cycler PCR apparatus (Roche Diagnostics; Switzerland); amplification plots of the GHR gene in livers of bulls of different Fnu4HI genotype. C – a representative standard curve; the calculated slope value – -4.830. D – representative melting curves of the *GHR* gene RT-PCR products.

The representative results demonstrating the specificity of the *GHR* and β-actin RT-PCR-amplification products, as documented with agarose gel electrophoresis, are shown in Figure 2-A. Additionally, shown are the representative amplification plots (Fig. 2-B), standard curve (Fig. 2-C), and the results of melting curve analysis (Fig. 2-D). These results confirmed the high specificity of the RT-PCR products and good yield of the PCR reaction.

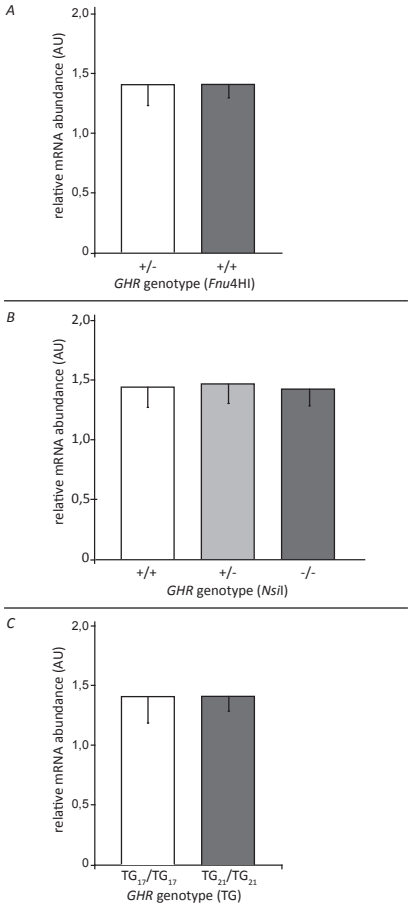

Fig. 3. Accumulation of the *GHR* mRNA in livers of animals with different *GHR* genotypes (expressed in arbitrary units – AU). The data are referred to the corresponding values of the β-actin gene and represent means (±SD) for Real-time PCR experiments with RNA derived from five animals of each genotype. **A** – C/T *GHR* genotypes at position -1104 (RFLP-*Fnu*4HI). No significant difference in *GHR* expression level was identified with the Duncan's test between RFLP-*Fnu*4HI (+/-) and RFLP-*Fnu*4HI (+/+) genotypes. **B** – A/G *GHR* genotypes at position -154 (RFLP-*Nsi*I). No significant difference in expression level was identified between RFLP-*Nsi*I (+/+), (+/-), and (-/-) genotypes. **C** – TG_n tandem repeat of variable length in *GHR* gene promoter P1. No significant difference in the *GHR* mRNA level was identified between $TG_{17/17}$ and $TG_{21/21}$ genotypes.

The possible effect of the RFLP-*Fnu*4HI at the *GHR* gene promoter region was studied using liver samples derived from (+/-) and (+/+) genotype animals, since no materials were available from bulls carrying the (-/-) genotype. No significant difference between bulls of different genotypes was identified (P>0.1). As shown in Figure 3-A, the animals carrying the (+/+) and (+/-) genotype accumulated the same amount of *GHR* mRNA in their livers.

Five animals were used from each genotype group: (+/+), (+/-) and (-/-), to search for possible association between RFLP-*Nsi*I genotype and *GHR* gene expression at mRNA level in the bovine liver. As shown in Figure 3-B no significance of difference was confirmed (P>0.1) in the level of *GHR* mRNA between livers of bulls of different RFLP-*Nsi*I genotypes.

Liver samples derived from BW bulls extremely differing in length of the TG_n microsatellite located at position -86 within the *GHR* gene promoter P1 were used to study the possible effect of this polymorphism on *GHR* gene expression in the bovine liver. As shown in Figure 3-C, no difference was identified in the *GHR* mRNA level between bulls carrying homozygous $TG_{17/17}$ and $TG_{21/21}$ genotypes (P>0.1).

Several SNPs were found in the P1 promoter region of the bovine *GHR* gene, upstream the exon 1A. Since this promoter was shown to drive transcription of the most abundant, liver-specific *GHR* transcript in cattle, the authors of the present study hypothesized that nucleotide sequence polymorphism within this region might influence the gene expression, what could elucidate a possible mechanism of known associations between these polymorphisms and production traits in cattle. One of the polymorphisms under study, the C/T transition at position -1104 (*Fnu*4HI), perfectly co-localizes with the GR binding site, and another one – the A/G transition at position -154 (RFLP-*Nsi*I) – is very close to the putative binding sites for C/EBP and HNF-3. This made the hypothesis even more attractive. Such associations might have suggested the existence of a direct effect of the polymorphism on traits by increasing or decreasing a promoter activity and therefore concentration of the *GHR* transcripts. However, this appeared not to be the case. No effect of nucleotide substitutions or TG_n repeat polymorphism was shown in this study on *GHR* mRNA accumulation and *GHR* gene expression in bovine liver.

Results showing the effect of polymorphisms in regulatory regions on gene expression in cattle have already been reported. For the first time Lum *et al*. [1997] have shown the effect of the G/C nucleotide substitution in the bovine β-lactoglobulin gene promoter at AP2 binding site on the gene expression, later confirmed by Kuss *et al*. [2003]. Effects of SNP or InDel polymorphisms in promoter regions of bovine casein αS1 and αS2 genes on TF binding capacity, gene expression and respective protein content of milk were shown by Martin *et al*. [2002] and Szymanowska *et al*. [2004]. Similar results were reported for the other bovine genes; in the *STAT5A* gene promoter an A/G transition at position -488 affects HNF-3 binding and influences the *STAT5A* transcript and protein levels in the bovine liver [Flisikowski *et al*. 2004]. As shown by Adamowicz *et al*. [2006] the C→G transversion at position -105 in the

bovine leptin gene decreases DNA-SP-1 binding capacity and the gene expression in the adipose tissue.

Recently, the effect of the A/G transition at position -154 and of the variable TG repeat in the *GHR* gene exon 1A promoter P1 was studied by Zhou and Jiang [2005] using reporter gene *luc* constructs. No effect of promoter variants on the reporter gene expression was shown; the P1 promoter constructs bearing A or G nucleotide at position -154, and those with various number of TG repeats (TG_{11} *vs* TG_{20}), had a similar transcriptional activity. These findings were confirmed in the present study by Real-time PCR analysis performed on RNA extracted from livers of BW bulls with different *GHR* genotypes. Summarizing, the results presented here suggest that neither A/G transition at position -154 nor the length of TG microsatellite in the *GHR* P1 promoter directly affects milk and meat production traits in cattle and that the existing associations may be caused by linkage disequilibrium with nearly located alleles. Moreover, as shown by the results of the present study, another SNP located within the promoter region of bovine *GHR* gene – the C/T transition at position -1104 (RFLP-*Fnu*4HI) – also does not influence the expression level of the gene in bovine liver, despite its co-localization with the putative GR transcription factor binding site.

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Polimorfizm sekwencji nukleotydowej w rejonie promotorowym genu receptora hormonu wzrostu (*GHR*) bydła nie wpływa na ekspresję tego genu w wątrobie

S t r e s z c z e n i e

Gen kodujący receptor hormonu wzrostu bydła zbudowany jest z dziewięciu eksonów kodujących białko i trzech eksonów (1A, 1B, i 1C) nie podlegających translacji, których transkrypcja prowadzi do powstania trzech typów mRNA, różniących się długością rejonu 5'-UTR. Ekspresją transkryptu występującego w wątrobie kieruje promotor P1, położony „powyżej" eksonu 1A. W rejonie promotorowym genu *GHR* bydła występuje kilka polimorfizmów typu SNP, a także polimorficzna sekwencja mikrosatelitarna TG_n. W prezentowanej tu pracy, w wyniku analizy komputerowej w programie TESS stwierdzono, że niektóre z tych mutacji leżą w miejscu wiązania czynników transkrypcyjnych. Postanowiono zatem poszukiwać zależności między polimorfizmem sekwencji nukleotydów w rejonie promotorowym genu *GHR* bydła a ekspresją tego genu w wątrobie. Używano techniki Real-time, a jako matrycę stosowano RNA, wyizolowany z wątroby młodych buhajów cb o różnych genotypach *GHR*. Nie wykazano różnic w poziomie mRNA *GHR* w wątrobie między buhajami o genotypach (+/+), (+/-) i (-/-) w odniesieniu do tranzycji A/G w pozycji -154 (RFLP-*Nsi*I), o genotypach (+/-) i (+/+) dla tranzycji C/T w pozycji -1104 (RFLP-*Fnu*4HI) ani o genotypach $TG_{17/17}$ i $TG_{21/21}$ w odniesieniu do sekwencji mikrosatelitarnej w promotorze P1.