ORIGINAL ARTICLE

Polymorphism in genes of growth hormone receptor (GHR) and insulin-like growth factor-1 (IGF1) and its association with both the IGF1 expression in liver and its level in blood in Polish Holstein-Friesian cattle

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Submitted: 2008-09-05 Accepted: 2008-09-18 Published online: 2008-12-29

Key words: **ghr gene; IGF-1;polymorphism; gene expression; blood level; cattle**

Neuroendocrinol Lett 2008; **29**(6):981–989 **PMID:** 19112400 NEL290608A11

Abstract

BACKGROUND: Polymorphisms in the bovine *ghr* and *igf1* genes. *Ghr* and *igf1* genes have been associated with milk and meat production of cattle. However, the molecular and physiological mechanisms underlying such associations are unknown. The objective of this study was to examine the effects of polymorphisms in 5'-regions of the bovine *ghr* and *igf1* genes on the *igf1* gene expression in the liver and on the level of IGF1 in blood of Polish Holstein-Friesian cattle.

METHODS: Individual and combined effects of single nucleotide polymorphisms (SNPs) in the 5'noncoding regions of the bovine *igf1* and *ghr* genes on the IGF1 level in blood and *igf1* gene expression in liver were examined. One SNP in the *igf1* gene and four SNPs in the *ghr* gene were analyzed. IGF1 level in blood was measured by radioimmunoassay (RIA) in 211 heifers and bulls of Polish Holstein-Friesian cattle (of Black-and-White type).

The *igf1* gene expression was measured in livers of bulls carrying different *igf1* and *ghr* genotypes (from three to nine animals per genotype) using real-time reverse transcription-PCR methods with the *gapdh* gene as a reference.

RESULTS: We showed that C/T transition in the promoter region of the *igf1* gene influences the gene expression; relative *igf1* expression was higher for animals with the CC genotype than for those with the TT and CT genotypes. TESS analysis showed that C/T transition in the *igf1* gene co-localizes with the NF1 transcription factor binding site. Also, the *ghr* genotype appeared to significantly influence the *igf1* gene expression in the liver, and we found the highest expression for the genotypes: RFLP-*Alu*I (AT), RFLP-*Fnu*4HI(CC), and RFLP-*Nsi*I(GA), and for the combined *ghr* genotype: *Alu*I(AT)/ *Fnu*4HI(CC)/*Nsi*I(GA). We discovered a significant association between the *igf1* genotype and the IGF1 blood level. The

highest IGF1 content in blood serum was found in CC genotype animals (1024 ng/ml) *vs* 698 ng/m and 859 ng/min in the TT and CT *igf1* genotypes, respectively. Moreover, we noticed significant differences between *ghr* genotypes. The highest blood levels of IGF1 were for the animals carrying the *ghr* genotypes: RFLP-*Alu*I(AA), RFLP-*Fnu*4HI(CC), and RFLP-*Nsi*I(AG). *Ghr* haplotypes also significantly affected the IGF1 blood level. Animals of the combined *ghr* genotypes *Alu*I(AA)/*Acc*I(CC)/*Fnu*4HI(CC)/*Nsi*I(AG) and *Alu*I(AA)/AccI(CT)/Fnu4HI (CC)/ NsiI(AG) had a higher IGF1 concentration in blood than other genotype carriers.

CONCLUSIONS: The present results indicate that the effects of polymorphism in the *igf1* and *ghr* genes on cattle milk or meat production traits could be at least partially mediated through their effects on the *igf1* gene expression in the liver and the IGF1 level in blood.

INTRODUCTION

The genes of growth hormone (GH), GH receptor (GHR), and insulin-like growth factor 1 (IGF1) are viewed as promising candidate markers for selection purposes in farm animals, including cattle (Parmentier *et al.* 1999). Several genetic polymorphisms have been found in the bovine *ghr* and *igf1* genes. *Ghr* and *igf1* gene polymorphisms have been associated with milk synthesis and meat production of cattle. However, the molecular and physiological mechanisms underlying such associations are unknown.

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 (Burton *et al*. 1994; Etherton and Bauman 1998; Etherton 2004). Therefore, there is great interest in using growth hormone to improve production traits in farm animals. GH actions on target cells are mediated through the GH receptor (GHR), which is a transducer of GH signals within the cell inducing transcription of target genes, including *igf1* (Rotwein *et al*. 1994; Argetsinger and Carter-Su 1996).

Several genetic polymorphisms were found in the bovine *ghr* gene. It was reported that these polymorphisms may influence the function of the cattle organism in view of its productivity properties including milk yield and composition, growth performance and carcass properties (Aggrey *et al*. 1999; Falaki *et al*. 1996; Blott *et al*. 2003; Maj *et al*. 2004a). Moreover, it was found that the A/G polymorphism at a *Nsi*I site in the 5' region of the bovine *ghr* gene is associated with the level of IGF1 in the blood (Ge *et al*. 2003). One nucleotide sequence polymorphism was found in the bovine *igf1* gene and was reported to affect animal growth rate and meat performance modification (Ge *et al*. 2001; Li *et al.* 2004; Siadkowska *et al.* 2006). The SSCP in the 5'-flanking region of the *igf1* gene in Angus cattle was described by Ge *et al.* (1997). This polymorphism was subsequently identified as T/C transition, also recognizable as RFLP-*Sna*BI (Ge *et al*. 2001). A significant association of the RFLP-*Sna*BI *igf1* genotype was found with the growth rate in Angus cattle (Ge *et al*. 1997) and with milk composition traits in HF cows (Siadkowska *et al*. 2006). However, the molecular and physiological mechanisms involved in this very complex action remain unsolved and need further structural and functional multidisciplinary research.

In order to explain certain important genetic and physiological relationships in the physiology of cattle, the objective of this study was to examine the effects of polymorphisms in 5'-regions of the bovine *ghr* and *igf1* genes on the *igf1* gene expression in the liver and on the level of IGF1 in blood of young Polish Holstein-Friesian cattle.

MATERIALS AND METHODS

Animals

Young bulls and heifers (211 in total) of Polish Holstein-Friesian (the Black-and-White type) were used in the experiment. IGF1 concentration was measured in blood samples taken from 105 heifers and 106 bulls, at the age of 335 (±8) days. Liver tissue for the study of *igf1* gene expression was taken from 54 young (15 months) HF bulls, slaughtered at the local abattoir. All animals originated from 29 Holstein sires, and progeny groups ranged from 1 to 28 (an average of 7.4 calves per sire). The animals were born at different farms but housed in one experimental station at IGAB Jastrzębiec, starting from the age of 4 months. The animals had access twice daily to diets, which were formulated according to age under a standardized feeding regimen (Polish Norms 1993). Water was freely available. The average gain calculated over the period from birth to 11 months of age was 692 ± 90 g/d (mean ± SD) in heifers and 887 ± 99 g/d in bulls. The animals were kept in individual tie-stalls.

All procedures involving the animals were performed in accordance with the Guiding Principles for the Care and Use of Research Animals and were approved by the Local Ethics Commission (permission No. 3/2005).

Determination of ghr and igf1 polymorphism

Approximately 10 mL of blood was withdrawn by an authorized veterinarian from each animal and placed into test tubes containing K_2EDTA. DNA was isolated from the blood by the method of Kanai *et al.* (1994). The following single nucleotide polymorphisms were genotyped in the *ghr* gene 5'-region: A/T transversion at pos. -1177 (RFPL-*Alu*I; Aggrey *et al.* 1999); C/T transition at pos. -887 (RFPL-*Acc*I; Aggrey *et al.* 1999); C/T transition at pos. -1104 (RFPL-*Fnu*4HI; Maj *et al.* 2005); A/G transition at pos. -154 (RFPL-*Nsi*I; Ge *et al.* 2003). The RFLPs at *Alu*I, *Acc*I, *Fnu4*HI and *Nsi*I sites were estimated as previously described (Maj *et al.* 2004b). De-

tection of the *igf1* gene C/T transition (RFLP-*SnaB*I) at position -472 (Ge *et al.* 2001) was performed as previously described (Siadkowska *et al.* 2006). Positions of all mutations are given relative to the initiation of the transcription site.

The polymerase chain reactions (PCR) were carried out in MJ Research PTC-225 Thermal Cycler. The PCR-amplified DNA fragments were digested at 37°C for 3 hours with 5 units of a respective restriction endonuclease (New England BioLabs, USA). The digestion products were separated on 2% agarose (GIBCO-BRL, England) gels in 1 × TRIS-borate-EDTA (TBE) buffer. The gels were stained with ethidium bromide and visualised and scanned in FX Molecular Imager (Bio-Rad).

Igf1 gene expression in liver

Samples of liver tissue were taken from young 15-month-old Friesian bulls of the known *ghr* and *igf1* genotypes, immediately after slaughter at the local abattoir. Samples were flash-frozen in liquid nitrogen and then stored at –80 °C until used. Total RNA from frozen tissue samples were isolated using TRIzol reagent (Invitrogen, Carlsbad, USA) according to manufacturer's protocol. The RNA samples were treated with RNase-free DNase (Promega corp., Madison, USA) to remove any possible contaminating genomic DNA and dissolved in diethylpyrocarbonate-treated water. Quantity and quality of total RNA was estimated by native agarose gel electrophoresis with ethidum bromide and with the Nanodrop apparatus (USA). Constant amounts of 2 μg of RNA were reverse transcribed to cDNA with 200 units of M-MLV Reverse Transcriptase (Promega corp., Madison, USA) according to the manufacturer's instructions.

The IGF1 mRNA levels were measured using real-time PCR techniques. Samples without cDNA were used as a negative control. The following primers were used in RT-PCR reactions (Pfaffl *et al.* 2002): IGF1-F – 5'-TCGCATCTCTTCTATCTGGCCCTGT-3'; IGF1-R – 5'-GCAGTACATCTCCAGCCTCCTCAGA-3'. These primers amplified a 240-bp-long DNA fragment which spanned two exons (3, 4) in the highly conserved IGF1 region and included all known alternatively spliced mRNA variants (Pfaffl *et al.* 2002). As a reference the "housekeeping" *gapdh* gene was used, amplified with the following primers: GAPDH-F – 5' – GCGATACTCACTCTTCTACCTTCGA-3'; GAPDH-R – 5'-TCGTACCAGGAAATGAGCTTGAC-3'. PCR amplification was performed on 7500 ABI PRISM apparatus (Applied Biosystems, Foster City, USA) using 96-well optical plates with SYBR Green technique. A PCR mix (25 μl) was prepared to give the indicated end concentration: 11.1 μl water, 0.2 μl forward primer (10 μM), 0.2 μl reverse primer (10 μM), 1 μl cDNA and 12.5 μl SYBR GREEN PCR Master Mix (Applied Biosystems). The following amplification procedure was used: 10 min of denaturation at 95 °C, 40 cycles of 4-segment amplification with 15 s at 95 °C for denaturation, 30 s at 58 °C for annealing, 40 s at 72 °C for elongation. Emulation was selected and a dissociation stage was added to ensure that the desired amplicon was detected. The *igf1* gene expression was quantified in terms of *igf1* cDNA copies using LightCycler software 3.5 based on the Second Derivative Maximum Method (Roche Diagnostics; Switzerland). 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 (-, 5x, 25x, 125x, 625x) were primed separately (in different PCR tubes) for *igf1* and *gapdh* as a reference gene. Relative mRNA expression for the *igf1* gene was calculated using the formula:

$$R = (1+E_{(IGF1)} - CT_{(IGF1)})/(1+E_{(GAPDH)} - CT_{(GAPDH)}).$$

Hormone assay

IGF1 concentrations were determined in duplicates of acid-ethanol plasma extracts according to the RIA method of Breier *et al.* (2004)] using sheep anti-IGF1 antibody (Y32; against human N-Met-IGF1). Human recombinant IGF1 (Sigma, St. Louis, Mo, USA) was used for [^{125}I] labeling as well as for a reference standard. The validity of the assay was verified by dilution test (with or without standard added) resulting in slope 1.02 ± 0.05, and recovery above 80%. The detection limit of the assay was 2.5 ng/tube, and the intra- and interassay CV were 8 and 12%, respectively.

Statistical analysis

The effects of *igf1* genotypes and single or combined *ghr* genotypes on the blood serum IGF1 concentration were analyzed by the least-squares method as applied in the general linear model (GLM) procedure of SAS (1991/2001) according to the following statistical model:

$$y_{ijkl} = \mu + G_i + SEX_j + YS_k + \beta(BW_{ijkl} - BW) + e_{ijkl}$$

where: y_{ijkl} – IGF1 concentration; μ - overall mean; G_i - the fixed effect of the *ghr* genotype (i = 1, 2, 3), or combined genotype (i = 1.....18); SEX_j – the fixed effect of sex (1, 2); YS_k – the fixed effect of season-year at blood sampling (k = 1...9); $\beta(BW_{ijkl} - BW)$ – overall regression on body weight at 11 mo of age; e_{ijkl} – random residual effect. The differences in the *igf1* gene expression between genotypes were tested by Tukey's, Benferrony's, Newman-Keul's, and Dunnet's Multiple comparision tests. The most "restrictive" results were taken for data interpretation. The data were processed by using the GLM procedure of SAS program (SAS/STAT User's Guide, 1989).

Table 1. The effects of the *igf1* genotypes on IGF1 concentration in cattle blood. Overall least-squares means (LSM) and standard error (SE) of blood serum IGF1 concentration (ng/ml) across the *igf1* C/T genotypes.

***Igf1* genotype**	**Blood serum IGF1 concentration, ng/ml**	
	LSM	**SE**
TT (n=50)	698[a]	164
CT (n=95)	859	145
CC (n=61)	1024[a]	155

[aa] Values marked with the same letter are significantly different at $P \leq 0.05$

Table 2. The effect of the *ghr* genotypes on IGF1 concentration in cattle blood. Overall least-squares means (LSM) and standard error (SE) of blood serum IGF1 concentration (ng/ml) across the *ghr* RFLP-*Alu*I, *-Acc*I, *-Fnu*4HI, *and -Nsi*I genotypes.

***Ghr* genotype**	**Blood serum IGF1 concentration, ng/ml**	
	LSM	**SE**
RFLP-*Alu*I		
AA (n=85)	1100[a]	67
AT (n=101)	950[a]	62
TT (n=23)	1045	124
RFLP-*Acc*I		
CC (n=141)	1042	56
CT (n=65)	1056	82
RFLP-*Fnu*4HI		
CC (n=159)	1078[b]	51
CT (n=47)	866[b]	91
RFLP-*Nsi*I		
+/+ (n=25)	1035[c]	138
AG (n=88)	1095[d]	75
AA (n=98)	856[cd]	67

[a,b,c] Values marked with the same letter are significantly different at $P \leq 0.05$.

RESULTS

Association of ghr and igf1 polymorphisms with IGF1 blood levels

IGF1 concentrations were determined in blood samples collected from animals at their peripubertal period when the highest IGF1 level occurs. The detected level of IGF1 was on average 868 ng/ml for heifers and 1088 ng/ml for bulls. The significantly greater values in males than in females are typical for non-primate species (Gatford *et al*. 1998). Although there are a few reports on IGF1 blood levels analyzed at 11 months of age in larger cattle groups, the obtained numerical values correspond well to those reported by Röpke *et al*. (1994). Those authors also observed that intensive feeding (also used in the present study) caused further elevation of IGF1 circulatory levels in both sexes. The calculated LSM was 1020 ng/ml (SE = 63) for heifers and 1063 ng/ml (SE = 66) for bulls. Since the difference in LSM between bulls and heifers was not significant, associations between the *ghr* genotypes and IGF1 levels were calculated jointly for both sexes, but the sex was included into the model (statistical model formula; Materials and methods section).

An association was estimated of the C/T polymorphism in 5'-region of the *igf* gene with IGF1 blood level in HF cattle (Table 1). The average IGF1 concentration in the CC genotype animals (1024 ng/ml) was by about 50% higher than that in TT genotypes (698 ng/ml); the difference was significant at $P \leq 0.05$. In CT heterozygotes the IGF1 level (859 ng/ml) was an average between both homozygotes. The IGF1 blood levels for different *ghr* genotypes are given in Table 2. A significant association was found for RFLP-*Alu*I ($P \leq 0.05$). Animals with the AA genotype had on average a 15% higher IGF1 concentration in blood (1100 ng/ml) than those with the AT genotype (950 ng/ml). Animals with the *ghr* genotype CC at RFLP-*Fnu4*HI had on average a 25% higher level of IGF1 (1078 ng/ml) than those with the CT genotype (866 ng/ml). Only three animals with the homozygous TT genotype were found and consequently the results concerning this genotype are not shown in the results and tables. The *ghr* GA genotype at RFLP-*Nsi*I was associated with a significantly higher ($P \leq 0.05$) level of IGF1 in blood (1095 ng/ml) than that of the AA genotype (856 ng/ml). No significant associations were found for the RFLP at the *Acc*I site.

The SNPs analyzed are located close to each other (within 1023 bp) in the 5'-region of the *ghr* gene and thus they might be in linkage disequilibrium. Since an independent analysis of each SNP might not provide independent results, the associations were calculated between combined *ghr* genotypes and IGF1 levels in blood. Out of 81 (3^4) theoretically possible combinations of four individual genotypes, only 30 were identified in the sample of animals, with 18 represented by four or more individuals. Thus, more than half of the possible genotype combinations were missing. This suggests the existence of predominating intra-genic haplotypes within the bovine GHR locus.

Some of the combined genotypes "haplotypes" appeared to have statistically significant effects on the IGF1 levels. Animals of the combined *ghr* genotypes: **(1)** - *Alu*I(AA)/*Acc*I(CC)/ *Fnu*4HI(CC)/*Nsi*I(AG) and **(5)** - *Alu*I(AA)/*Acc*I(CT)/*Fnu*4HI(CC)/*Nsi*I(AG) had a higher IGF1 concentration in blood (1434 ng/ml and 1450 ng/ml, respectively) than those with most of the other genotypes (difference significant at $P \leq 0.01$), while the combined *ghr* genotype (**14**) - *Alu*I(AT)/*Acc*I(CT)/*Fnu*4HI(CT)/*Nsi*I(AG) was associated with the extremely low IGF1 concentration in blood serum (547 ng/ml).

Table 3. The effect of combined genotypes (haplotypes) in the *ghr* gene 5′ region on IGF1 concentration in cattle blood. Overall least-squares means (LSM) and standard error (SE) of blood serum IGF1 concentration (ng/ml) across the combined *ghr* genotypes.

Combined *ghr* genotype "haplotype"		n	IGF1 concentration (ng/ml)	
			LSM	SE
(1)	*Alu*I(AA)/*Acc*I(CC)/*Fnu*4HI(CC)/*Nsi*I(AG)	11	1434^{aA}	178
(2)	*Alu*I(AA)/*Acc*I(CC)/*Fnu*4HI(CC)/*Nsi*I(AA)	24	981^{b}	121
(3)	*Alu*I(AA)/*Acc*I(CC)/*Fnu*4HI(CT)/*Nsi*I(AG)	4	1083	294
(4)	*Alu*I(AA)/*Acc*I(CC)/*Fnu*4HI(CT)/*Nsi*I(AA)	7	1010	226
(5)	*Alu*I(AA)/*Acc*I(CT)/*Fnu*4HI(CC)/*Nsi*I(AG)	8	1447^{A}	206
(6)	*Alu*I(AA)/*Acc*I(CT)/*Fnu*4HI(CC)/*Nsi*I(AA)	11	1166	183
(7)	*Alu*I(AA)/*Acc*I(CT)/*Fnu*4HI(CT)/*Nsi*I(AG)	4	847	302
(8)	*Alu*I(AA)/*Acc*I(CT)/*Fnu*4HI(CT)/*Nsi*I(AA)	7	1003	229
(9)	*Alu*I(AT)/*Acc*I(CC)/*Fnu*4HI(CC)/*Nsi*I(GG)	8	1132	210
(10)	*Alu*I(AT)/*Acc*I(CC)/*Fnu*4HI(CC)/*Nsi*I(AG)	34	1005^{b}	108
(11)	*Alu*I(AT)/*Acc*I(CC)/*Fnu*4HI(CC)/*Nsi*I(AA)	22	954^{b}	131
(12)	*Alu*I(AT)/*Acc*I(CC)/*Fnu*4HI(CT)/*Nsi*I(AG)	4	927	300
(13)	*Alu*I(AT)/*Acc*I(CT)/*Fnu*4HI(CC)/*Nsi*I(AG)	10	1202	187
(14)	*Alu*I(AT)/*Acc*I(CT)/*Fnu*4HI(CT)/*Nsi*I(AG)	7	547^{B}	222
(15)	*Alu*I(AT)/*Acc*I(CT)/*Fnu*4HI(CT)/*Nsi*I(AA)	7	886^{b}	225
(16)	*Alu*I(TT)/*Acc*I(CC)/*Fnu*4HI(CC)/*Nsi*I(GG)	6	1456	241
(17)	*Alu*I(TT)/*Acc*I(CC)/*Fnu*4HI(CC)/*Nsi*I(AG)	4	1175	299
(18)	*Alu*I(TT)/*Acc*I(CC)/*Fnu*4HI(CC)/*Nsi*I(AA)	9	924^{b}	199

Values marked with different letters are significantly different - ^{a,b} at $P \leq 0.05$; and $^{A,B....}$ - at $P \leq 0.01$.

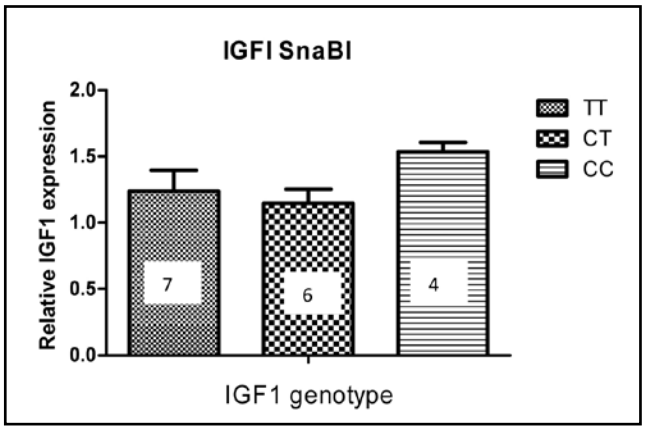

Fig. 1. The effect of the C/T transition (RFLP-*Snab*I) in the *igf1* gene 5′-region on the gene expression in bovine liver. Accumulation of the IGF1 mRNA in livers of animals with different *igf1* genotypes was measured with real-time PCR and expressed in arbitrary units. The data were referenced to the corresponding values of the *gapdh* gene. Values represent the average (mean ± S.D) of triplicate reactions with RNA derived from four to seven animals of each genotype (actual numbers of animals are shown in the figure).

Effect of igf1 and ghr polymorphism on igf1 gene expression in liver

The *igf1* gene expression was measured in livers of bulls carrying different *igf1* and *ghr* genotypes using real-time-PCR methods with the *gapdh* gene as a reference (Fig. 1). Statistically significant differences were found. Higher relative IGF1 expression was shown for animals with the *igf1* CC genotype at RFLP-*Snab*I than for those with the TT and CT genotypes (difference non-significant, but approaching significance at $P \leq 0.05$). In the bovine *igf1* gene the C/T transition is located at position -472 relative to the transcription start site (512 bp upstream from the ATG codon; according to the GenBank sequence AF210383), in the gene region where many *cis* regulatory sequences are located. Therefore, we searched for putative transcription factor binding sites (TFBS) in the bovine *igf1* gene fragment, between nucleotides –670 and –310 (Fig. 2). The TESS program and TRANSFAC database were used. Several putative TFBS were identified adjacent to the SNP under study, including the NF-1 binding site that co-localized with the C/T substitution. Only the allele with T at position

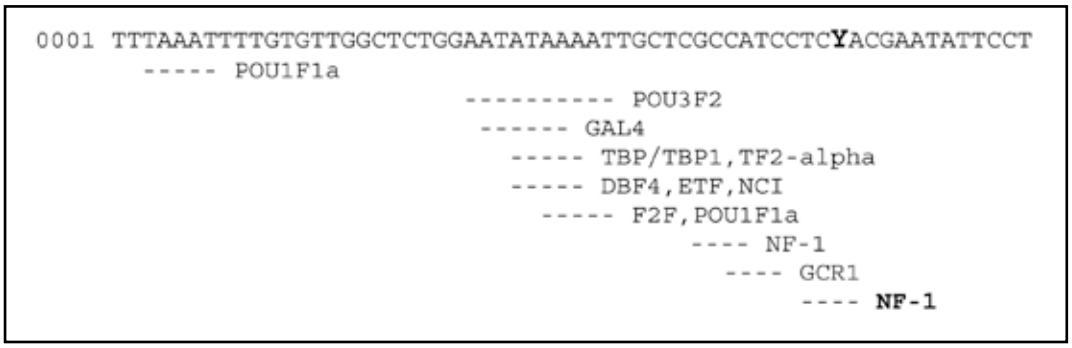

Fig. 2. Computer-aided search for putative transcription factor binding sites (TFBS) in the bovine *igf1* gene 5'-region, encompassing the C/T transition at position -472. The TESS program and TRANSFAC database were used.

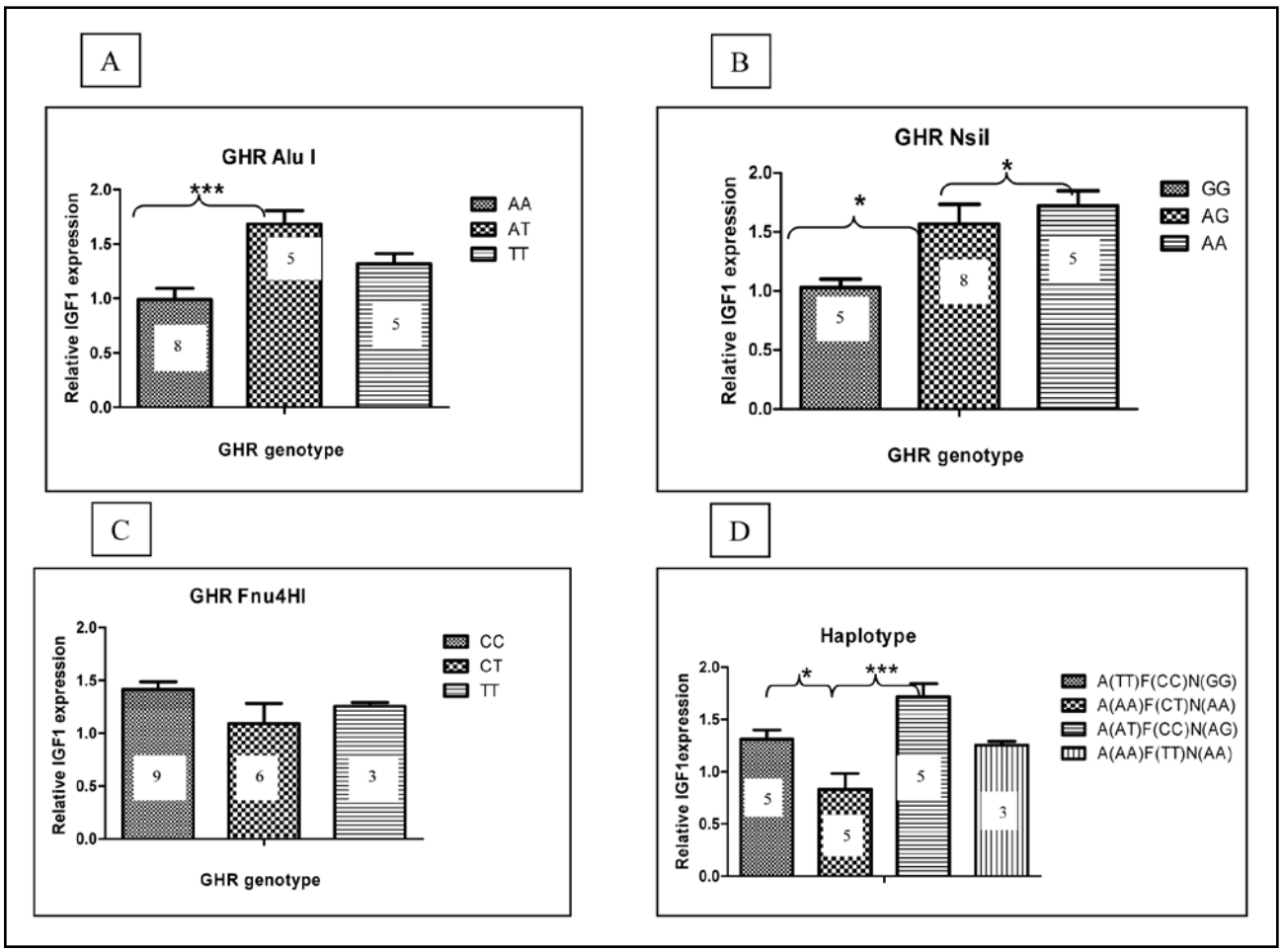

Fig. 3. The effect of single nucleotide polymorphisms (SNPs) in the *ghr* gene 5'-region on *igf1* gene expression in bovine liver. Accumulation of the *igf1* mRNA in livers of animals with different *ghr* genotypes was measured with real-time PCR and expressed in arbitrary units. The data were referenced to the corresponding values of the *gapdh* gene. Values represent the average (mean ± S.D) of triplicate reactions with RNA derived from three to nine animals of each genotype.; actual numbers of animals are shown in the figure). **(A)** A/T transversion at pos. -1177 (RFPL-*Alu*I); **(B)** C/T transition at pos. -1104 (RFPL-*Fnu*4HI); **(C)** A/G transition at pos. -154 (RFPL-*Nsi*I); (D) combined RFLP- *Alu*I/-*Fnu*4HI/-*Nsi*I genotypes.

-472 has the NF-1 binding site 100% identical with the consensus sequence for this TF.

The *ghr* genotypes also appeared to influence the *igf1* expression levels in bovine liver (Fig. 3 a,b,c). The highest relative IGF1 mRNA levels were detected in livers of young bulls carrying RFLP-*Alu*I genotype AT (significantly different from AA, at $P \leq 0.001$), RFLP-*Fnu*4HI genotype CC (non-significant), and RFLP-*Ns*iI genotype AA (significantly different from AG and GG, at $P \leq 0.05$). The effects of combined *ghr* genotypes were also shown (Fig. 3 d). The highest IGF1 mRNA level was found in the case of the "haplotype" with AT genotype at RFLP-*Alu*I, CC genotype at RFLP-*Fnu*4HI, and AG genotype at RFLP-*Ns*iI, while the lowest – for the

"haplotype" with AA/CC/GG genotypes at the respective RFPLs. The differences between "haplotypes" were significant at $P \leq 0.05$ or $P \leq 0.001$.

DISCUSSION

A number of studies have shown associations of *ghr* gene polymorphisms with milk and meat production traits of cattle (Lee *et al*. 1996; Aggrey *et al*. 1999; Blott *et al*. 2003). In our studies, we have shown that genotypes at the *ghr* 5'-noncoding region significantly influenced several dairy traits in Polish BW cattle (Maj *et al*. 2004a). Cows of the RFLP-*Nsi*I genotypes AA and AG produced more milk with a higher content of most milk components (fat, protein, and lactose) than did cows with the GG genotype. Also the RFLP-*Alu*I AA genotype appeared favorable for most of the traits. The heterozygous genotype CT at RFLP-*Acc*I appeared superior with respect to several milk yield and composition parameters - percentage of total solids, fat, protein, and lactose. Also, the combined *ghr* genotypes were shown to influence some dairy traits. It was proven that single base substitution in the *ghr* gene influenced the receptor density in bovine liver (Maj *et al*. 2007).

The genetic variants at the 5'-noncoding region of the bovine *ghr* gene also had a marked effect on beef production traits (Maj *et al*. 2004b). The T allele at the polymorphic *Alu*I site appeared favorable for several meat production-related traits, including average daily gain, weight of carcass, and weight of lean in selected cuts. The animals with the *ghr* genotype CC for the RFLP-*Acc*I seemed better for feed consumption, while those with the genotype CC and allele C at the polymorphic *Fnu*4HI site appeared superior for most meat-production traits studied. A better performance for most traits was shown for bulls of the AA or AG genotypes at the *Nsi*I site.

GH receptor mediates the action of GH in the liver on the synthesis of the insulin-like growth factors, mainly IGF1, which in turn is a regulator of postnatal growth and of numerous metabolic processes in animals and humans (Burton *et al.* 1994; Jones and Clemmons 1995; Philips *et al*. 1998). The *ghr* gene mutations naturally occurring in humans or knockout of the *ghr* gene in mice resulted in reduction of the blood IGF1 concentration (Rosenfeld *et al.* 1994; Zhou *et al*. 1997). So, it was rational to consider associations of nucleotide sequence polymorphisms of the *ghr* gene with blood IGF1 levels in cattle. In the present study such associations were found for SNPs located at *Alu*I, *Fnu*4HI, and *Nsi*I sites.

Nucleotide substitutions located in 5'-regions (promoters) of different genes were shown to influence expression in cattle of different genes: casein in the mammary gland (Szymanowska *et al*. 2004), signal transducer and activator of transcription 5A (STAT5A) in the liver (Flisikowski *et al*. 2004), and leptin in the fat tissue (Adamowicz *et al*. 2006). The altered gene expression may then affect animals' physiology, e.g. influencing hormone levels or biochemical pathways. An association was previously found between the *ghr* gene RFLP-*Nsi*I and blood IGF1 level by Ge *et al*. (2003) in Angus cattle; the animals with genotype GG had a higher IGF1 level as compared to animals with genotype AA. Thus, our present results agree with the previous report. However, studies by Ge *et al*. (2003) failed to show any significant association between other *ghr* gene polymorphisms located in the 5'-region (RFLP-*Alu*I, -*Acc*I, -*StuI*) with serum IGF1 concentration or growth traits in Angus cattle. In their other study Ge *et al*. (2001) showed an association of the *igf1* gene polymorphism (RFLP-*SnaB*I) with IGF1 blood levels. In the post-weaning period (days 28 – 56) the Angus calves with the CC genotype had lower serum IGF1 concentration in serum and higher body weight than those with the TT genotype. Moreover, the allele with nucleotide (nt) T at position -472 appeared significantly more often than that with nt C in a group of animals selected for the high IGF1 in blood. These results are somewhat in contrast to ours, since we have shown a 47% higher IGF1 blood level in young HF cattle (at 10 months of age) carrying the *igf1* genotype CC as compared with the TT genotype. This discrepancy might result from the different age of the animals used in experiments and different breed.

Previously we found a significant association for the Leu/Leu genotype of the *gh* gene with the highest blood level of IGF1 in young BW bulls (Grochowska *et al*. 2001). An association of the blood IGF1 level and GHR 1A mRNA variant expression in livers of Friesian cattle was found by Radcliff *et al*. (2003).

The genes encoding growth hormone (GH), GH receptor (GHR), and insulin-like growth factor 1 (IGF1) are viewed as promising candidate markers for selection purposes in farm animals. Several genetic polymorphisms have been found in the bovine *ghr* and *igf1* genes. Associations have been reported of *ghr* and *igf1* gene polymorphism and milk and meat production traits of cattle, e.g. milk yield and composition, growth performance and carcass traits. However, the molecular and physiological mechanisms underlying such associations are not known.

As shown by Li *et al*. (2004), the *igf1* genotype CC is associated with higher body mass at weaning. No association was found by Hines *et al*. (1998) between *igf1* gene RFLP-*SnaB*I and dairy production traits in Holstein cattle. However, in our studies associations were found between RFLP-*SnaB*I and milk traits in HF cows (Siadkowska *et a*l. 2006).

In search for a physiological basis of such associations we found significant differences in the GHR B_{max} and K_d (measured with ^{125}I-GH as a ligand) between dairy and beef breeds (Grochowska *et al.* 2002). The B_{max} was greater ($P \leq 0.05$) in Polish HF bulls as compared to beef breeds, while K_d was the lowest in the

dairy HF breed ($P \leq 0.01$). Relationships were also found between the GHR ligand-binding capacity and the nucleotide sequence polymorphism in the *ghr* gene 5'-region - RFLPs at *Alu*I, *Acc*I and *Stu*I sites (Grochowska *et al.* 2002).

Numerous studies have shown that differences in hormone levels may be crucial for animal milk or meat production performance. Although no final conclusion can be drawn, our results have shown that sequence variations in 5'-regions of the candidate genes – *igf1* and *ghr* – may influence (directly or indirectly) gene expression, hormone synthesis and their levels in blood and finally animals' performance. The results of the present study suggested that the associations of the *igf1* and *ghr* gene polymorphisms with production traits of cattle could be mediated through their effects on the IGF1 level in blood.

Acknowledgements

The authors are grateful to Dr. J. Bass (Ruakura Agricultural Research Centre, New Zealand) for generous supply of IGF1 antibodies. This study was funded within Polish Ministry of Science and Higher Education grant PBZ-KBN-113/P06/2005, IGAB PAS project No S.I.2.1 and the Scientific Network of Polish Ministry of Science and Higher Education No. 20/E183/SN0013 "Scientific network of genomics, proteomics and metabolomics utilization in the control of animal growth, reproduction, health and biodiversity". The contribution of Dagmara Robakowska-Hyżorek was financially supported by a scholarship funded by the President of the Polish Academy of Sciences.

REFERENCES

1 Adamowicz T, Flisikowski K, Starzyński R. Świtoński M, Zwierzchowski L (2006). Mutation in the Sp1-motif on the bovine leptin gene affects its expression. *Mamm Genome* **17** (1): 77–82.
2 Aggrey SE, Yao J, Sabour MP, Lin CY, Zadworny D, Hayes JF, Kunlein U (1999). Markers within the regulatory region of the growth hormone receptor gene and their association with milk-related traits in Holsteins. *J Heredity* **90**:148–151.
3 Argetsinger LS, Carter-Su C (1996). Growth hormone signaling mechanisms: involvement of the tyrosine kinase JAK2. *Hormone Res*. **45:** 22–24.
4 Blott S, Kim JJ, Moisio S, Schmidt-Kuntzel A, Cornet A, Berzi P, Cambisano N, Ford C, Grisart B, Johnson D, Karim L, Simon P, Snell R, Spelman R, Wong J, Vilkki J, Georges M, Farnir F, Coppieters W (2003). Molecular dissection of a quantitative trait locus. A phenylalanine-to-tyrosine substitution in the trans-membrane domain of the bovine growth hormone receptor is associated with a major effect on milk yield and composition. *Genetics.* **163**: 253–266.
5 Breier BH, Gallaher BW, Gluckman PD (1991). Radioimmunoassay for insulin-like growth factor-I: solutions to some potential problems and pitfalls. *J Endocrinol.* **128:** 347–357.
6 Burton JL, McBride BW, Block E, Glimm DR, Kenelly JJ (1994). A review of bovine growth hormone. *Canadian J Anim. Sci.* **74:** 167–201.
7 Etherton TD (2004). Somatotropic function: The somatomedin hypothesis revisited. *J Anim Sci.* **82** (Suppl 13): E239–244.
8 Etherton TD, Bauman DE (1998). Biology of somatotropin in growth and lactation of domestic animals. *Physiol Rev.* **78**: 745–761.
9 Falaki M, Gengler N, Sneyers M, Prandi A, Massart S, Formigoni A, Burny A, Portetelle D, Renaville R (1996). Relationships of polymorphisms for growth hormone and growth hormone receptor genes with milk production traits for Italian Holstein-Friesian bulls. *J Dairy Sci.* **79:** 1446–1453.
10 Flisikowski K, Starzyński R, Zwierzchowski L (2004). Promoter variant-dependent expression of the STAT5A gene in bovine liver. *Biochim. Biophys. Acta – Gene Structure and Expression* **1679** (2): 195–199.
11 Gatford KL, Egan AR, Clarke IJ, Owens PC (1998). Sexual dimorphism of the somatotrophic axis. *J. Endocrinol.* **157**:373–389.
12 Ge W, Davis ME, Hines HC (1997). Two SSCP alleles detected in the 5'-flanking region of the bovine IGF-1 gene. *Anim Genet.* **28**: 155–156.
13 Ge W, Davis ME, Hines HC, Irvin KM, Simmen RC (2003). Association of single nucleotide polymorphisms in the growth hormone and growth hormone receptor genes with blood serum insulin-like growth factor I concentration and growth traits in Angus cattle. *J Anim Sci.* **81**(3): 641–648.
14 Ge W, Davis ME, Hines HC, Irvin KM, Simmen RCM (2001). Associations of a genetic marker with blood serum insulin-like growth factor-I concentration and growth traits in Angus cattle. *J Anim Sci.* **79**: 1757–1762.
15 Grochowska R, Gajewska A, Snochowski M, Zwierzchowski L (2002). Ligand-binding activity of growth hormone receptor (GH-R) in bulls of different cattle breeds with identified GH-R genotypes. *J Anim Feed Sci.* **11:** 223–236.
16 Grochowska R, Sørensen P, Zwierzchowski L, Snochowski M, Løvendah LP (2001). Genetic variation in stimulated growth hormone release and in insulin-like growth factor-I of young dairy cattle and their associations with the leucine/valine polymorphism in the GH gene. *J. Anim. Sci.* **79**: 470–476.
17 Hines HC, Ge W, Zhao Q, Davis ME (1998). Association of genetic markers in growth hormone and insulin-like growth factor I loci with lactation traits in Holsteins. *Anim Genet.* **29**(1): 69.
18 Jones JI, Clemmons DR (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev.* **16**: 3-34.
19 Kanai N, Fujii T, Saito K, Tokoyama T (1994). Rapid and simple method for preparation of genomic DNA from easily obtainable clotted blood. *J Clin Pathol.* **47:** 1043–1044.
20 Lee BK, Lin GF, Crooker BA, Murtaugh MP, Hansen LB, Chester-Jones H (1996). Association of somatotropin (bST) gene polymorphism at the 5th exon with selection for milk in Holstein cows. *Domest Anim Endocrinol.* **13** :373–381.
21 Li C, Basarab J, Snelling WM, Benkel B, Murdoch B, Hansen C, Moore SS (2004). Assessment of positional candidate genes myf5 and IGF1 for growth on bovine chromosome 5 in commercial lines of *Bos taurus*. *Anim Sci.* **82**(1): 1–7.
22 Maj A, Gajewska A, Pierzchała M, Kochman K, Zwierzchowski L (2007). Single base substitution in growth hormone receptor gene influences the receptor density in bovine liver. *Neuro Endocrinol Lett.* **28**: 401–405.
23 Maj A, Strzałkowska N, Słoniewski K, Krzyżewski J, Oprządek J, Zwierzchowski L (2004a). Single nucleotide polymorphism (SNP) in the 5'-noncoding region of the bovine growth hormone receptor gene and its association with dairy production traits in Polish Black-and-White cattle. *Czech J Anim Sci.* **49:** 419–429.
24 Maj A, Pareek CS, Klauzińska M, Zwierzchowski L (2005). Structure and polymorphism of the 5'-noncoding region of the bovine growth hormone receptor gene. *J Anim Breed Genet.* **122**: 414–417.
25 Maj A, Zwierzchowski L, Oprządek J, Oprządek A, Dymnicki E (2004b). Polymorphism in the 5'-noncoding region of the bovine growth hormone receptor gene and its association with meat production traits in cattle. *Anim Res.* **53** (Suppl 6): 503–514.
26 Parmentier I, Portetelle D, Gengler N, Prandi A, Bertozzi C, Vleurick L, Gilson R, Renaville R (1999). Candidate gene markers associated with somatotropic axis and milk selection. *Domest Anim Endocrinol*. **17**: 139–148.

27 Pfaffl MW, Georgieva TM, Georgiev IP, Ontsouka E, Hageleit M, Blum JW (2002). Real-time RT-PCR quantification of insulin-like growth factor (IGF)-1, IGF-1 receptor, IGF-2, IGF-2 receptor, insulin receptor, growth hormone receptor, IGF-binding proteins 1, 2 and 3 in the bovine species. *Domest Anim Endocrinol.* **22:** 91–102.
28 Phillips LS, Pao CI, Villafuerte BC (1998). Molecular regulation of insulin-like growth factor-I and its principal binding protein, IGFBP-3. *Prog Nucleic Acid Res Mol Biol.* **60:** 195–265.
29 Polish Norms (1993). Nutrient Requirements of Ruminants. Institute of Zootechnics, Omnitech Press, Warsaw.
30 Radcliff RP, McCormack BL, Crooker BA, Lucy MC (2003). Growth hormone (GH) binding and expression of GH receptor 1A mRNA in hepatic tissue of periparturient dairy cows. *J Dairy Sci.* **86:** (12) 3933–3940.
31 Röpke R, Schams D, Schwartz FJ, Kirchgessner M (1994). Growth-related hormones in plasma of bulls, steers and heifers given food with two different energy levels. *Anim Prod*. **59**: 367–377.
32 Rosenfeld RG, Rosenbloom AL, Guevara-Aguirre J (1994). Growth hormone (GH) insensitivity due to primary GH receptor deficiency. *Endocr Rev*. **15**(3): 369–390.
33 Rotwein P, Gronowki AM, Thomas MJ (1994). Rapid nuclear actions of growth hormone. *Hormone Res*. **42**: 170–175.
34 SAS/STAT User's Guide, Release 8 e Edition. SAS Institute Inc., Cary, NC, USA (1999–2001).
35 Siadkowska E, Zwierzchowski L, Oprządek J, Strzałkowska N, Bagnicka E, Krzyżewski J (2006). Effect of polymorphism In IGF-1 gene on production traits in Polish Holstein-Friesian cattle. *Anim Sci Pap Rep.* **24** (3): 225–237.
36 Szymanowska M, Malewski T, Zwierzchowski L (2004). Transcription factor binding to variable nucleotide sequences in 5'-flanking regions of bovine casein genes. *Int Dairy J.* **14** (2): 103–115.
37 Zhou Y, Xu BC, Maheshwari HG, He L, Reed M, Lozykowski M, Okada S, Cataldo L, Coschigamo K, Wagner TE, Baumann G, Kopchick JJ (1997). A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor-binding protein gene (the Laron mouse). *Proc Natl Acad Sci* USA. **94**: 13215–13220.