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Associations between bovine beta-lactoglobulin polymorphism within coding and regulatory sequences and milk performance traits

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Abstract. The bovine beta-lactoglobulin (LGB) gene is considered a potential quantitative trait locus in dairy cattle breeding. In Black-and-White dairy cattle the LGB gene has two predominant alleles A and B. This can result in three possible genotypes AA, AB and BB. Moreover, within the promoter of the gene several point mutations were found. A herd of one hundred and twenty-four Black-and-White cows were genotyped for two loci: locus LGB (exon IV, alleles A and B) and locus LGB-R (SSCP polymorphism within a fragment of LGB promoter: SSCP patterns R2, R3, R1, R9). In our sample 13 AA, 58 AB and 53 BB LGB cows and 66 R2, 16 R3, 40 R1 and 2 R9 LGB-R cows were identified. A statistical analysis revealed significant associations between LGB, LGB-R genotypes as well as intragenic haplotypes LGB/LGB-R and milk protein content during the first complete lactation. Cows with AA LGB genotype, R3 LGB-R SSCP pattern and AA/R3 haplotypes had the highest protein content. These results support the hypothesis that sequence variation within the promoter of the LGB gene is probably one of the factors responsible for differences in milk protein content.

Key words: bovine, beta-lactoglobulin, milk protein content, promoter, SSCP.

Introduction

Bovine beta-lactoglobulin gene (LGB) is located on chromosome 11q28 (HAYES, PETIT 1993, EGGEN, FRIES 1995). The complete genomic sequence of the bovine LGB gene was characterized by ALEXANDER et al. (1993). Among the eight protein variants (A, B, C, D, E, F, G, W) (BELL et al. 1970, 1981, BRAUNITZER et al.1973, BRIGNON, RIBADEAU-DUMAS 1973, CONTI et al. 1988, GODOVAC-ZIMMERMANN et al. 1990), A and B are predominant in Black-and-White

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dairy cattle. The A and B variants differ by two amino acid substitutions. In variant A Asp (GAT) at position 64 is changed into Gly (GGT) and in variant B Val (GTC) at position 118, is changed into Ala (GCC) (ALEXANDER et al. 1993).

The review of literature shows that LGB genotypes are associated with different milk performance traits (NG-KWAI-HANG et al. 1990, MAO et al. 1992, HILL 1993) and some technological properties of milk (JAKOB, PUHAN 1992, WALAWSKI et al. 1994). The differential expression of A and B alleles in LGB AB heterozygous cows observed by several authors (McLEAN et al. 1984, GRAML et al. 1989, FORD et al. 1993, KIM et al. 1996, NG-KWAI-HANG, KIM 1996) suggested that mutations responsible for quantitative variation of milk protein may be located also within regulatory elements of the LGB gene (promoter).

Apart from the protein variants coded within exons III and IV of LGB gene, numerous point mutations within the 5' flanking region of LGB gene were identified (ALEXANDER et al. 1993, WAGNER et al. 1994). One of them, transversion G to C in position – 435, has been shown to change binding affinity of transcription factor AP-2 to LGB promoter (LUM et al. 1997).

In this paper we attempt to combine both polymorphisms, in exon IV (LGB) and within the fragment of LGB promoter (LGB-R) to recognize the importance of both polymorphisms by looking for the associations between LGB genotypes, LGB-R genotypes as well as intragenic LGB/LGB-R haplotypes and milk performance traits in Polish Black-and-White cattle.

Materials and methods

Altogether, one hundred and twenty-four Black and White cows held in one herd were included in the analysis. Approximately 4 ml of blood were taken from each animal. Genomic DNA was isolated from leukocytes by the MasterPure DNA Purification Kit (Epicentre).

Two PCR products were obtained: a 208 bp fragment of LGB promoter (designated LGB-R) and a 240 bp fragment of LGB exon IV (designated LGB). PCR amplifications of 208 and 240 bp LGB gene fragments and SSCP genotyping were previously described in detail (KAMIŃSKI, ZABOLEWICZ 1997, 1998).

Data on milk performance traits collected during the first complete 305-day lactations, and included: milk yield (kg), fat content (%), fat yield (kg), protein yield (kg) and protein content (%). These data were extracted from breeding documentation and provided by the owner of the animals.

The statistical analysis included: statistical characteristics of the experimental material ($\overline{x}$ = arithmetic mean, s = standard deviation), and testing the significance of differences between the genotypic means for single traits using the F-test and Duncan's test incorporated in the software package STATISTICA for Windows (STATSOFT 1995) for the following factors: LGB genotype (AA, AB, BB), LGB-R genotype (R1, R2, R3 and R9) and haplotype LGB/LGB-R (AA/R3,

AB/R2, BB/R2, BB/R1). Three possible LGB/LGB-R combinations: AA/R1, AA/R9 were not identified within the group investigated. Five groups of intragenic haplotypes (AA/R2, AB/R3, AB/R1, AB/R9, BB/R3) were excluded from the individual statistical analysis (too low number of animals) and combined in one group designated "Other haplotypes".

Table 1. Means and standard deviations (in parentheses) of LGB, LGB-R genotypes or LGB/LGB-R haplotypes for milk performance traits

Genotypes / Haplotypes	Milk performance traits				
	milk (kg)	fat (kg)	fat (%)	protein (kg)	protein (%)
LGB					
AA n = 13	6726	290.6	4.32	236.1	3.51^{A}
	860	41.9	0.37	30.5	0.24
AB n = 58	6455	275.6	4.27	215.0	3.33^{B}
	1243	60.7	0.30	47.8	0.21
BB n = 53	6409	273.7	4.27	213.4	3.33^{B}
	1009	50.4	0.39	39.1	0.26
LGB-R					
R2 n = 66	6429	272.6	4.24	213.4^{a}	3.32^{B}
	1212	58.5	0.30	46.8	0.24
R3 n = 16	6772	297.3	4.39	239.7^{b}	3.54^{A}
	873	45.6	0.40	31.6	0.23
R1 n = 40	6334	270.5	4.27	210.3^{a}	3.32^{B}
	992	48.4	0.39	37.2	0.22
R9 n = 2	7757	351.4	4.53	265.3	3.42
	205	29.0	0.50	15.6	0.29
LGB/LGB-R					
AA/R3 n = 12	6799	290.3^{ab}	4.27^{A}	235.9^{ab}	3.47^{AB}
	856	43.7	0.35	31.8	0.22
AB/R2 n = 54	6417	271.4^{a}	4.23^{A}	212.4^{ab}	3.31^{B}
	1262	60.6	0.26	48.4	0.20
BB/R2 n = 11	6539	277.38^{a}	4.24^{A}	215.8^{ab}	3.30^{B}
	1028	52.8	0.43	42.2	0.35
BB/R1 n = 39	6335	269.9^{a}	4.26^{A}	209.7^{a}	3.31^{B}
	1005	48.7	0.39	37.6	0.22
Other haplotypes n = 8	6804	320.5^{b}	4.71^{B}	248.3^{b}	3.65^{A}
	956	42.2	0.32	27.6	0.22

n = number of animals recorded. Significant differences are marked by capital letters ($P \leq 0.01$) or by small letters ($P < 0.05$).
The group "Other haplotypes" included: AA/R2 (n=1), AB/R3 (n=1), AB/R1 (n=1), AB/R9 (n=2), BB/R3 (n=3).

Results

Each of the 124 cows included in the analysis was genotyped for both loci: LGB and LGB-R. We identified 13 AA, 58 AB and 53 BB cows for LGB and 66 R2, 16 R3, 40 R1 and 2 R9 cows for LGB-R. A new LGB-R SSCP pattern, designated R9, was detected. In comparison with LGB-R SSCP patterns already described (KAMIŃSKI, ZABOLEWICZ 1998), R9 has four bands: a, b, c and f. Among the 124 cows analysed we found only two cows with the R9 SSCP pattern. Four further R9 SSCP patterns were identified in a population of 140 A.I. bulls (unpublished data).

Complete results of statistical analysis are presented in Table 1. Statistical analysis revealed significant associations between individual LGB and LGB-R genotypes as well as intragenic haplotypes LGB/LGB-R for milk protein content, fat content, milk protein yield and milk fat yield. The milk from cows with genotype LGB AA has a significantly ($P < 0.01$) higher protein content (= 3.51) than milk from cows with genotypes AB ($\bar{x} = 3.33$) and BB ($\bar{x} = 3.33$). The LGB-R cows with SSCP pattern R3 produced milk with a significantly ($P < 0.01$) higher protein content ($\bar{x} = 3.54$) than cows with SSCP patterns R2 ($\bar{x} = 3.32$) and R1 ($\bar{x} = 3.32$). SSCP pattern R3 is also associated with a higher protein yield, but at the lower level of significance ($P < 0.05$).

All animals were also divided into five further groups depending on the intragenic haplotype LGB/LGB-R. The milk from cows belonging to group "Other haplotypes" and haplotype AA/R3 shows the highest protein content, respectively $\bar{x} = 3.65$ and $\bar{x} = 3.47$. The same haplotype groups are also outstanding in milk protein yield, $\bar{x} = 246.4$ kg and $\bar{x} = 236.2$ kg for "Other haplotypes" and AA/R3, respectively. Cows having rare haplotypes ("Other haplotypes") showed a higher fat content ($\bar{x} = 4.71$) than the remaining groups (from $\bar{x} = 4.23$ to $\bar{x} = 4.27$). The same group of cows was also outstanding in fat yield but at the lower level of significance ($P < 0.05$).

Discussion

So far, the polymorphism within exons III and IV of the bovine LGB gene has been considered as strongly affecting many milk performance traits. MAO et al. (1992) in a relatively large population of 11,015 Holstein cows showed that the B allele is favourable for fat content and protein yield. NG-KWAI-HANG et al. (1990) found in a population of approximately 8000 cows that the replacement of allele B by allele A at the LGB locus is followed by an increase in protein content by 0.05, 0.07 and 0.08%, for the first, second, and third lactation, respectively. Favourable chromosome substitution effects (effects attributed not only to a certain marker but also to unknown genes linked to the marker and located on the same chromosome) on transmitting abilities for fat content, protein yield, and the gain of protein and fat were observed for allele A (COWAN et al. 1992). BOVENHUIS and

WELLER (1994) found that BB and AB genotypes are negatively associated with milk yield and positively with fat content. LUNDEN et al. (1997) reported a positive additive effect of the LGB B allele on casein content and on the ratio of casein to total protein.

The achievements of molecular biology clearly indicate that the quantitative variance of gene expression in most of eucaryotic genes is coded not in exons but rather within 5'regulatory regions of the genes (LEWIN 1997, PAPAVASSILIOU 1997). WAGNER et al. (1994) sequenced up to –795 bp the 5' flanking region of LGB gene (EMBL GeneBank acc. no X6313). The entire 5' flanking region contains experimentally confirmed or computationally deduced common as well as specific DNA motifs engaged in the regulation of LGB gene transcription (GROENEN and van der POEL 1994, WAGNER et al. 1994, MALEWSKI 1998). Also, numerous point mutations within the 5' flanking region of LGB gene were identified (ALEXANDER et al. 1993, WAGNER et al. 1994).

In this study we chose the fragment of LGB promoter to find the relationships between the sequence variation within this fragment and variation in milk performance traits, especially in milk protein content. This is a LGB promoter fragment of 208 bp, between –501 to –293 according to the sequence published by ALEXANDER et al. (1993). It was chosen for three reasons: (1) this subregion is the most conservative fragment in the entire 5' part of the LGB gene (95% homology with ovine beta-lactoglobulin gene); (2) five point mutations are located within the 208 bp PCR product (WAGNER et al. 1994), (3) one of these mutations, named R10 (S) in position – 435, was shown to affect the binding affinity of transcription factor AP-2 (LUM et al. 1997). If G appears in position – 435, AP-2 recognition sequence has 100% homology to AP-2 consensus sequence (CCCCAGGC) and if C occurs in this position, the AP-2 site decreases its homology to AP-2 consensus sequence. It can be concluded that the higher homology of AP-2 may result in a formation of a more efficient transcriptional complex, as it increases the yield of transcription, the yield of LGB protein synthesis and finally the total protein content of milk. Similar assumptions were proved experimentally for bovine beta-casein; in vitro studies showed that mutations within the binding site of the mammary-gland-specific nuclear factor (MGF) strongly affect the transcriptional activity of the beta-casein gene (SCHMITT-NEY et al. 1991).

In our study the sequence variation of this subregion was screened by the SSCP method, which allowed us to identify four SSCP patterns (R1, R2, R3 and R9). We observed that cows with SSCP pattern R3 have a significantly ($P < 0.01$) higher protein content than cows with the R2 and R1 SSCP patterns (Table 1). Cows with genotype LGB AA have a significantly ($P < 0.01$) higher protein content ($\bar{x} = 3.51$) than AB ($\bar{x} = 3.33$) and BB ($\bar{x} = 3.33$). These results are in agreement with results published by EHRMANN et al. (1997). They found a very significant association between allele A of the LGB gene and both a higher beta-lactoglobulin content and a lower casein content of milk. These results seem

to be very reliable because they show direct relationships between LGB gene polymorphism and the gene product quantity. They are consistent with earlier reports showing the associations between allele A and a higher total milk protein content (ROZZI et al. 1989, NG-KWAI-HANG et al. 1990, HILL 1993, WALAWSKI et al. 1999).

In our experiment, among the 16 cows with the R3 SSCP pattern, 12 have the LGB AA genotype. The remaining four cows have other LGB/LGB-R haplotypes. It is a question which of the two analysed polymorphisms is the cause observed differences of milk protein content. We suggest that R3 SSCP polymorphism is a more reliable marker for a high protein content in milk than A/B polymorphism in exon IV.

In our experiment we were able to identify the LGB haplotype. Haplotype is a particular combination of alleles in a defined region of chromosome, so it is a genotype in miniature (LEWIN 1997). Intragenic haplotypes limit the combination of alleles to one gene. Intragenic haplotypes for bovine beta-lactoglobulin (LGB) have been analysed by WAGNER et al. (1994). These authors identified haplotypes created by the combination of the point mutation located in bovine beta-lactoglobulin exon IV (responsible for protein variants A and B) and different point mutations located within the promoter of this gene. Among the 60 cows analysed they found 80% cows with regular haplotypes (e.g. AA pairs to BB while AB to AB, for exon IV and promoter, respectively). The remaining 20% of cows had irregular haplotypes. Results presented in this paper confirmed this irregularity. For example, we recorded twelve cows with AA/R3 genotype, but also several cows with irregular haplotypes, e.g. BB/R3 and AB/R3. Similar observations were described by GELDERMANN et al. (1996). They found two different SSCP patterns for AB and BB LGB cows. This phenomenon may be explained by linkage disequilibrium phase which could occur between mutations in the coding regions and in the regulatory sequences of the LGB gene. Linkage disequilibrium can result from reduced recombination in the region or from the founder effect, in which there has been insufficient time to reach equilibrium since one of the markers was introduced into the population. Differences in the linkage disequilibrium phase could vary across individuals, populations and breeds and might explain the confusing data on the importance of LGB gene mutations for milk performance traits reported in the literature (COWAN et al. 1992).

The small number of rare SSCP patterns, like R9 or haplotypes including R9, did not allow us to evaluate their importance for milk protein content. SSCP genotypes identified within the 208 bp PCR product are not localised point mutations; it is only clear some change in nucleotide sequence has occurred (ORITA et al. 1989). Therefore, no SSCP pattern identified in our PCR product can be referred to a specific point mutation, neither that in position – 435 nor any other. The relatively small number of animals does not allow to estimate the frequency of the genotypes and may serve only as a preliminary observations.

Probably, milk protein content is also affected by other loci mapped on chromosomes 1, 6, 9, 10 and 20 (GEORGES et al. 1995), telomeric end of chromosome 20 (ARRANZ et al. 1998) or by other genes, e.g. the growth hormone gene (LAGZIEL et al. 1999). Nevertheless, regulatory sequences of milk protein genes and other genes directly engaged in milk protein synthesis seem to be the most suitable candidates for milk protein quantitative trait loci (QTLs).

This is the first report showing the relationships between mutations within the promoter of the LGB gene and intragenic LGB haplotypes and milk performance traits.

Further studies are necessary to explain the importance of polymorphism within the LGB promoter for beta-lactoglobulin level and milk protein content. Moreover, our observations should be confirmed on a larger population of cows representing all possible genotypes and haplotypes. The possible effect of R3 or other polymorphisms on a different efficiency of transcription complex should be confirmed experimentally.

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