Associations of a Polymorphic AP-2 Binding Site in the 5'-Flanking Region of the Bovine β -Lactoglobulin Gene with Milk Proteins

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ABSTRACT

Studies on a polymorphic position (R10) in an Activator-Protein-2 (AP-2) binding site of the bovine β -Lactoglobulin (β -Lg) gene promoter region and quantitative traits of individual milk proteins were based on material from 79 German Holstein Friesian (HF) and 61 Simmental (Sm) cows. At least four milk samples per cow were analyzed with alkaline Urea-PAGE in combination with densitometry for quantification of individual milk proteins. The two alleles of the R10 single nucleotide polymorphism (SNP) carry either G or C in position -435 bp of the β -Lg promoter region. G- and C-alleles were found in Sm with nearly equal frequencies, while in HF the C-allele frequency was higher (0.73) than that of the G-allele. In both breeds, the R10 G-homozygotes had higher ($P < 0.001$) amounts of β -Lg secreted per day and proportion of β -Lg in milk protein compared with the C-homozygotes. A similar association was found for α -lactalbumin, whereas the relative proportions and daily secreted amounts of caseins (α_{S1} , β , κ) showed lower values in β -Lg R10 G-homozygotes. A positive association ($P < 0.001$) of R10 CC with milk yield has also been observed and indicates a close proximity of the β -Lg locus to a candidate gene for this trait. The association between the SNP in the AP-2 binding site of the β -Lg gene and its gene product can be explained as the result of differences in protein binding activity, and, therefore, allele specific differences in gene expression. Thus, our study clearly links a DNA polymorphism of molecular function very closely with in vivo expression parameters of the same locus.

(Key words: Activator Protein-2, β -Lactoglobulin, polymorphism, milk protein, milk yield)

Abbreviation key: AP-2 = Activator protein 2, β -Lg = β -Lactoglobulin, HF = German Holstein Friesian, RFLP = restriction fragment length polymorphism, Sm = Simmental, SNP = single nucleotide polymorphism.

INTRODUCTION

β -Lactoglobulin (β -Lg) is the major whey protein in the milk of ruminants and several nonruminant species (for review see Perez and Calvo, 1995). Polymorphisms have been found not only in the open reading frame of the β -Lg encoding gene which result in protein variants (Aschaffenburg and Drewry, 1955; 1957; Braunitzer et al., 1973; Bell et al., 1981; Eigel et al., 1984; Godovac-Zimmerman et al., 1990; 1996), but also in noncoding areas such as the 5'-flanking region (Wagner et al., 1994).

Associations between β -Lg protein variants and milk protein yield and composition have been observed by several authors (e.g. McLean et al., 1984; Lunden et al., 1997) but their molecular basis remains unclear. Correlations between single strand conformation polymorphisms in regulatory sequences of the β -Lg gene and milk performance traits have been reported but the effects could not be assigned to specific point mutations (Kaminski and Zabolewicz, 2000).

Ehrmann et al. (1997a), who found close associations between polymorphisms in the 5'-flanking region and proportion in milk protein as well as amount of β -Lg in bovine milk, suggested that differences in these parameters are due to different allelic influences on gene expression. Their study, however, included cows from several breeds and trait evaluation was in many cases based on one observation per cow only. Furthermore, the polymorphic sites were not analyzed for regulatory significance. Therefore, our study is focused on a single nucleotide polymorphism (SNP) in an Activator Protein 2 (AP-2) binding site within the 5'-flanking region of the β -Lg gene. Pronounced differences in protein binding capacity of the variants of this locus have been observed (Lum et al., 1997; Kock, 1998), thus allowing for a causal interpretation of associations between the gene variants and quantitative characteristics of the gene product. In addition to that, our experiments are based on large herds and at least four observations per cow, so genetic effects could be analyzed with high accuracy.

MATERIALS AND METHODS

Animals

Samples and data were collected between April 1997 and March 1999 from 79 cows of a German Holstein

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Table 1. Structure of herds.

a) Sm					b) HF				
Offspring per Parent	Sires	Σ Offspring (Sires)	Dams	Σ Offspring (Dams)	Offspring per Parent	Sires	Σ Offspring (Sires)	Dams	Σ Offspring (Dams)
1	30	30	51	51	1	32	32	59	59
2	5	10	5	10	2	6	12	7	14
3	1	3	—	—	3	5	15	2	6
4	1	4	—	—	4	2	8	—	—
14	1 ¹	14	—	—	5	1	5	—	—
Total	38	61	56	61	7	1	7	—	—
					Total	47	79	68	79

¹Sire (Genotype GG) with 14 daughters which were excluded in a second stage of analysis of variance in order to examine possible pedigree effects.

Friesian (**HF**) herd and 61 cows of a Simmental (**Sm**) herd, both from experimental stations of the University of Hohenheim. Pedigrees of the animals are summarized in Table 1.

Milk Samples

At least four milk samples per cow were collected in intervals of eight weeks during lactation. Fat was removed after centrifugation (10 min at 4550 × g and 4°C), and the nonfat samples were stored at -80°C.

Quantification of Milk Proteins

Milk proteins were separated by vertical Urea-PAGE (8 M Urea, 8.4% Acrylamide; see Ehrmann et al., 1997b). The gels were then stained (staining buffer: 8% w/v TCA, 26.7% v/v Methanol, 9.3% v/v Acetic Acid, 0.033% w/v CBB R250) overnight with agitation. After standardized destaining (29% v/v Methanol, 5% v/v Acetic Acid; 4 h with agitation), milk protein fractions (α -s1-Casein, β -Casein, κ -Casein, α -Lactalbumin, β -Lactoglobulin) were quantified using a densitometer (ATH Elscript 400, Hirschmann, Germany) together with software of the manufacturer.

Typing of Polymorphic Milk Proteins

Isoelectric focusing (see Ehrmann et al., 1997b) was used for identification of β -Lg protein variants A and B. Polyacrylamide gels (4.65% Acrylamide) were pre-focused for 350 Vh (25 min) and after sample application focused for 3500 Vh (95 min). Instrument settings were: 2000 V, 20 mA, 30 W. This was followed by 500 Vh with 20 mA and 30 W. The anode buffer was 1 M NaOH and the cathode buffer was 1 M H₃PO₄.

Blood Samples and DNA-Isolation

In order to be able to trace sampling errors, two blood samples per animal were taken. Genomic DNA was pre-

pared using the NucleoSpin Blood Quick Pure kit (Macherey Nagel, Düren, Germany) according to the instructions of the manufacturer.

Genotyping of DNA Variants

A fragment of the bovine β -Lg 5'-flanking region between -795 bp and +58 bp was amplified from genomic DNA by PCR.

Forward primer: GGGGGTATCAGCAACACACCCAGC ACCAG

Reverse primer: GGGAAGCTTCAAGCAGGAGGCACT TCATG.

The amplicon was digested with Fnu4HI (New England Biolabs, Frankfurt, Germany) and subsequently submitted to agarose gel analysis for the restriction fragment length polymorphism (**RFLP**) determination of the R10 site (SNP at -435 bp, defined by Wagner et al., 1994). Genotype nomenclature refers to the respective nucleotide in position -435. Fragment lengths were 294/256/145 bp for C-homozygotes, 294/172/145 bp for G-homozygotes and 294/256/172/145 bp for heterozygotes.

Statistical Analysis of Associations Between Genotypes and Trait Values

The GLM-Procedure of SAS, version 6.12, was applied within breed, using the following model:

$$Y_{ijklmn} = \mu + MFL_i + A_{ij} + L_k + LS_l + S_m + b_1(C_{ijklmn} - \bar{C}) + b_2(C_{ijklmn}^2 - \bar{C}^2) + e_{ijklmn}$$

where

Y_{ijklmn} = trait value

μ = population mean

MFL_i = fixed effect of marker genotype

A_{ij} = fixed effect of animal j within genotype i

L_k = fixed effect of lactation number

Figure 1. Associations between R10 genotypes and proportion (%) of the β -Lg fraction in milk protein as well as daily secreted amount (g/day) of β -Lg in Sm (white bars) and HF (grey bars). Significant differences ($P < 0.01$; t-Test) between genotypes are indicated by different lower-case letters.

LS_1 = fixed effect of lactation stage
 S_m = fixed effect of season (trimester)
 b_1, b_2 = regression coefficients on cell number, linear and squared
 C_{ijklmn}, C_{ijklmn}^2 = cell number and cell number squared
 $\bar{C}, \bar{C}^2$ = mean of cell number and mean of cell number squared
 e_{ijklmn} = residual effect

RESULTS AND DISCUSSION

The R10 locus (Wagner et al., 1994) lies in a site for which AP-2 binding activity has been shown (Lum et al., 1997) and for which we could verify allele specific quantitative differences in nuclear protein binding (Kock, 1998). In keeping with the fact that a Guanine in position -435 results in complete homology with the AP-2 consensus sequence (CCCAGGGC), whereas the occurrence of Cytosine causes a decrease in homology, both studies yielded a marked superiority of the G-variant in protein binding. Accordingly, an increased β -Lg gene activity in animals carrying R10-G alleles can be assumed. This is mirrored by our results concerning the associations of the R10-G variant with the amount of β -Lg secreted per day as well as the proportion of β -Lg in milk protein as shown in Figure 1. Concerning both parameters, there was a significant superiority of G-homozygotes compared with C-homozygotes in either breed. For GG-cows, higher β -Lg proportions in milk protein were observed than for heterozygous animals,

while the latter and G-homozygotes did not differ significantly as to the daily secreted amount of β -Lg. Our analyses (Tables 2 and 3) also considered α -Lactalbumin (α -La), α_{S1} -Casein (α_{S1} -Cn), β -Casein (β -Cn) and κ -Casein (κ -Cn). In case of the whey protein α -La the observed superiority of β -Lg GG-cows held true for both parameters in the Sm breed whereas in HF there was no significant difference in the amount of α -La secreted per day between cows of different R10 genotypes. In the milk of heterozygotes or G-homozygotes of both breeds the percentage of total milk protein was increased as compared to the C-homozygotes (Table 3). However, except for heterozygous Sm which showed lower values for α_{S1} -Cn than G-homozygotes, daily amounts of α_{S1} - and β -Cn as well as their proportions in milk protein were lowest in the milk of R10 G-homozygotic animals (Tables 2 and 3).

Similar associations as reported by Ehrmann et al. (1997a) for different variants in the 5'-region of the β -Lg gene might be due to the very close linkage of these loci with the R10 locus, implying the presence of distinct "intragenic haplotypes" which are stably distributed in the breeds. This interpretation could as well be applied to our results with regard to the β -Lg protein polymorphism, which also showed associations with the considered milk parameters (Data not shown).

We furthermore observed a connection between cows of different R10 genotypes and milk yield, with C-homozygotes showing the highest values in either breed. Interestingly this is more pronounced in the HF herd with

Table 2. Associations between R10 genotypes of the AP-2 binding locus in the 5'-flanking region of the β -Lg gene and amounts of protein fractions and total milk protein secreted per day.

Genotype (Frequency)			LS-Means $\pm$ SE											
a) Sm:	No. of animals	No. of milk samples	α -La [g/day]		β -Lg [g/day]		α_{S1} -Cn [g/day]		β -Cn [g/day]		κ -Cn [g/day]		Total milk protein [kg/day]	
CC (0.23)	14	69	15.55 ^a	0.56	63.58 ^a	2.13	260.76 ^a	5.76	285.22 ^a	5.81	21.93	0.82	0.65 ^a	0.01
CG (0.44)	27	131	15.68 ^a	0.41	85.62 ^b	1.54	224.27 ^b	4.17	255.87 ^b	4.20	— ²		0.61 ^b	0.01
GG (0.33)	20	95	17.26 ^b	0.44	87.93 ^b	1.66	228.76 ^b	4.49	239.67 ^c	4.53	20.80	0.64	0.59 ^b	0.01
		<i>P</i> ¹	**		***		***		***		n.s.		**	
b) HF:														
CC (0.56)	44	232	35.60 ^a	0.80	102.21 ^a	2.53	303.54 ^a	4.68	326.64 ^a	5.45	35.22 ^a	0.84	0.80 ^a	0.01
CG (0.34)	27	136	28.91 ^b	0.89	127.01 ^b	2.82	296.75 ^a	5.21	323.14 ^a	6.07	— ²		0.81 ^a	0.01
GG (0.10)	8	40	37.86 ^a	1.82	128.29 ^b	5.74	239.44 ^b	10.61	258.68 ^b	12.36	28.72 ^b	1.90	0.69 ^b	0.03
		<i>P</i> ¹	***		***		***		***		**		***	

¹****P* < 0.001, ***P* < 0.01 (F-Test); significant differences (*P* < 0.01; t-Test) between genotypes are indicated by different letters; n.s.: not significant.

²Not considered, due to low precision of trait evaluation.

25.23 $\pm$ 0.36 kg milk per day (CC) as compared to 21.41 $\pm$ 0.82 kg (GG) than in the Sm herd with 19.0 $\pm$ 0.40 kg (CC) as compared to 17.27 $\pm$ 0.31 kg (GG). In both breeds cows with the genotype GG also had lower amounts of total milk protein (kg/d), mainly caused by a decrease in the casein fraction. The frequencies of the alleles C and G were nearly equal among the Sm cows whereas the positively associated allele C had a frequency of almost 0.73 in the HF herd. This seems to be due to higher milk performance in HF than in Sm but in order to draw a definite conclusion, genotypes of sires with high and low breeding values would have to be investigated in both breeds. In view of the negative effect of R10 GG on milk yield we also suggest that a high resolution QTL analysis of the neighboring chromosomal regions of the β -Lg gene would reveal putative candidate genes for this trait.

In the Sm herd 14 cows were offspring of the same sire. In order to evaluate possible pedigree influences on our observations we excluded these offspring in a second analysis of the Sm data. The results (not shown) were almost identical with the findings in the complete material. We therefore conclude that the R10 genotype effects are much stronger than sire influences.

Cumulative contribution of the β -Lg R10 genotypes to the variance explained by the model is given in Figure 2 and showed an especially high influence on β -Lg parameters in both breeds. We also observed that the environmental factors without herd influence had little impact on the variance of individual milk proteins. On the other hand, environment had a large impact on the daily secretion of milk proteins.

Taken together, our evidence suggests direct effects of the R10 variants of the AP-2 binding site on differen-

Table 3. Associations between R10 genotypes of the AP-2 binding site in the 5'-flanking region of the β -Lg gene and contents of protein fractions (% of total milk protein) and total milk protein (% of whole milk).

Genotype (Frequency)			LS-Means $\pm$ SE											
a) Sm:	No. of animals	No. of milk samples	α -La [%]		β -Lg [%]		α_{S1} -Cn [%]		β -Cn [%]		κ -Cn [%]		Total milk protein [%]	
CC (0.23)	14	69	2.42 ^a	0.06	9.96 ^a	0.19	39.91 ^a	0.34	44.29 ^a	0.27	3.37	0.09	3.47 ^a	0.03
CG (0.44)	27	131	2.59 ^b	0.04	14.23 ^b	0.14	37.03 ^b	0.24	42.19 ^b	0.20	— ²		3.65 ^b	0.02
GG (0.33)	20	95	2.88 ^c	0.04	14.79 ^c	0.15	38.27 ^c	0.26	40.47 ^c	0.21	3.57	0.07	3.52 ^a	0.02
		<i>P</i> ¹	***		***		***		***		n.s.		***	
b) HF:														
CC (0.56)	44	232	4.34 ^a	0.06	12.57 ^a	0.20	37.92 ^a	0.24	40.74 ^a	0.23	4.39	0.07	3.23 ^a	0.01
tCG (0.34)	27	136	3.48 ^b	0.07	15.78 ^b	0.23	36.79 ^b	0.27	39.98 ^a	0.26	— ²		3.26	0.01
GG (0.10)	8	40	5.18 ^c	0.13	17.92 ^c	0.46	35.00 ^c	0.54	37.61 ^b	0.53	4.26	0.17	3.33 ^b	0.03
		<i>P</i> ¹	***		***		***		***		n.s.		***	

¹****P* < 0.001 (F-Test); significant differences (*P* < 0.01; t-Test) between genotypes are indicated by different letters; n.s.: not significant.

²Not considered, due to low precision of trait evaluation.

Figure 2. Contribution of the factors genotype, animal, and environment to the variance as explained by the statistical model.

tial expression of the β -Lg gene. Experiments that have been carried out by Folch et al. (1999) in the murine HC11 cell line showed allelic differences in gene expression between promoter segments including R10 variants. Reciprocal point mutation of R10 alone, however, had very little effect on gene expression results in their study. The authors therefore suggest that this might be due to cooperative effects of the AP-2 element and other polymorphic sites. But in view of the fact that these observations have been made in a murine system in which the bovine β -Lg promoter has also been shown to be unresponsive to hormonal control (Geldermann et al., 1996), additional evidence needs to be obtained from future studies in the bovine system.

We conclude that the positive association of the R10 G variant with β -Lg expression is caused by differences in the protein binding capacity of the corresponding AP-2 binding site (Lum et al., 1997; Kock, 1998) while the observed associations with total protein characteristics and with traits of other milk proteins can be interpreted as the results of indirect effects of gene regulation or linkage. Thus our study clearly links a DNA polymorphism of molecular function very closely with in vivo expression parameters of the same locus.

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