Aberrant Low Expression Level of Bovine β -Lactoglobulin Is Associated with a C to A Transversion in the *BLG* Promoter Region

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ABSTRACT

β -Lactoglobulin (β -LG) is the major whey protein in cow's milk. It is well established that the predominant 2 genetic variants, β -LG A and B, are differentially expressed. Extensive investigation of the genetic variation in the promoter region of the *BLG* gene revealed the existence of specific haplotypes associated with the A and B variants, respectively. However, the genetic basis for the differential expression of *BLG* A and B alleles is still elusive. We have previously reported a quantitative β -LG B variant, characterized by a very low β -LG protein expression level. Here, we report that the corresponding *BLG* allele (*BLG* B*) shows a correspondingly low mRNA expression level. Comparative DNA sequencing of 7,670 bp of the *BLG* B* allele and the established *BLG* B allele revealed a unique difference of a C to A transversion at position 215 bp upstream of the translation initiation site (g.-215C>A). This mutation segregated perfectly with the differential phenotypic expression in a paternal half-sib family and could be confirmed in 2 independent cases. The sequence of the *BLG* B allele in the region of the mutation is highly conserved among 4 related ruminant species. The site of the mutation corresponds to a putative consensus-binding sequence for the transcription factors c-Rel and Elk-1 as predicted by searching the TRANSFAC database. The β -LG B* site might be relevant in the natural production of milk of low β -LG content.

Key words: β -lactoglobulin, genetic polymorphism, milk protein, expression

INTRODUCTION

β -Lactoglobulin is the major whey protein in cow's milk. This whey protein is found in a wide variety of species, including all ruminants, but not in the milk of the human and mouse, for example (Pérez and Calvo, 1995). Eleven genetic β -LG variants have been re-

ported, with variants A and B being the most frequent (Farrell et al., 2004). More than 50 yr after the description of the genetic variants A and B of β -LG by Aschaffenburg and Drewry (1955, 1957) the differential expression of these 2 variants is still under investigation. In numerous studies, a higher protein expression level of the β -LG A variant compared with the B variant has been reported in the milk of heterozygous β -LG AB cows from various breeds (Cerbulis and Farrell, 1975; Reimerdes and Mehrens, 1978; Kroeker et al., 1985; Graml et al., 1989; Hill, 1993; Ng-Kwai-Hang and Kim, 1996; Ehrmann et al., 1997; Lum et al., 1997; Robitaille et al., 2002). Wilkins et al. (1995) reported ratios for amounts of the corresponding *BLG* mRNA A to B ranging from 1.37 to 1.87. The genetic variation in the 5'-flanking region was proposed as the cause for the differential expression of the 2 *BLG* alleles (Wagner et al., 1994). Lum et al. (1997) found in vitro that the activator protein-2 transcription factor binds with a 60% higher affinity to the *BLG* A promoter activator protein-2 recognition site compared with the corresponding site in *BLG* B. The most frequent *BLG* promoter variants linked to the respective A and B allele showed an expression level ratio of 1.33:1 in a reporter gene study using murine HC11 mammary gland cells (Folch et al., 1999).

Ten years ago we reported the occurrence of an extreme ratio between the A and a novel quantitative B variant of β -LG in Swiss Brown cattle milk samples (Kim et al., 1996). During routine phenotyping of the genetic variants of milk proteins for the Swiss Brown Cattle Breeder's Federation, an extremely weak β -LG B variant band was observed on the isoelectric focusing polyacrylamide gel. Additional analyses of the same samples at later stages of the lactation confirmed this aberrant β -LG B band. Later, in an independent study, the aberrant β -LG B band was found repeatedly in the milk of offspring from one sire. Pedigree data suggested the existence of a heritable phenotype caused by a novel *BLG* allele. The quantification of the β -LG A and B variants by capillary electrophoresis revealed a ratio between these 2 variants in normal and affected cow's milk of 1.62 and 4.02, respectively (Kim et al., 1996).

Here, we report on the impact of the quantitative *BLG* allele (*BLG* B*) on the major milk protein fractions

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and show that the ratio of the *BLG* mRNA for *BLG* B and *BLG* B* to *BLG* A allele, respectively, reflects the corresponding ratio observed on the protein level. In addition, we present evidence that the *BLG* B* allele is associated with a single nucleotide polymorphism (SNP) in the 5'-flanking promoter region.

MATERIALS AND METHODS

DNA Extraction

Deoxyribonucleic acid was extracted from blood, semen, and milk samples. The DNA from blood was isolated using a phenol-chloroform extraction protocol, that from semen was extracted as described by Lien et al. (1990), and that from milk samples was obtained by using a modified phenol-chloroform extraction procedure. The DNA was extracted from individuals representing a variety of breeds: 25 Swiss Brown, 4 Brown Swiss, 1 Angus, 1 Eringer, 1 Fleckvieh, 1 Jersey, 1 Hereford, 2 Holstein-Friesian, 1 N'Dama, 1 Simmental, and 1 Gayal (Mithun; *Bos frontalis*).

RNA Extraction

Ribonucleic acid was extracted from a mammary gland biopsy taken at the Veterinary Hospital Zurich and from control samples from a slaughterhouse using Trizol (Invitrogen, Carlsbad, CA) and subsequently treated with DNase I (Ambion, Austin, TX). A first-strand cDNA synthesis kit (Amersham Biosciences, Uppsala, Sweden) was used to reverse-transcribe the RNA. The reaction was subsequently purified with a QIAquick column (Qiagen, VWR International AB, Stockholm, Sweden).

Milk Samples

Milk samples were obtained from the Swiss Brown Cattle Breeder's Federation. The major protein fractions of milk were determined as described previously (Braunschweig et al., 2000). Fifteen first-lactation heifers phenotyped as β -LG AB were selected based on the observed aberrant ratio between the 2 β -LG variants in their milk. Four to 5 milk samples of these heifers were analyzed and compared with the corresponding milk samples from 15 randomly selected heifers of the same sire showing a normal ratio between the β -LG A and B protein variants.

Semiquantification of Allele-Specific Expression

Reverse transcription (RT)-PCR was performed using the *BLG*_A_B forward primer CCCCCCTGAGAGTGTATGTGGAG and the *BLG*_A_B reverse primer T

GGGTTGGGTTGAAGGACAGCCG, with the forward primer *BLG*_A_B being radioactively end-labeled with [γ - 32 P]ATP (Amersham Biosciences). T4 polynucleotide kinase (New England Biolabs, In Vitro Sweden AB, Stockholm, Sweden) for 5' end labeling was used for the labeling reaction according to the manufacturer's protocol. The reaction was purified through a micro spin column 30 (Bio-Rad, Hercules, CA). The RT-PCR fragments were digested with *Hae*III, loaded on a polyacrylamide gel, and subsequently exposed to an x-ray film overnight at -80°C . The ratio between the bands from allele A and B was quantified with a 425F PhosphorImager (Molecular Dynamics, Little Chalfont, UK). One mRNA sample from an affected cow and one sample from a control cow were analyzed 5 times and the average was calculated.

DNA Sequencing

To determine the DNA sequence from a heterozygous *BLG* AB* animal, 2 large fragments covering the entire *BLG* gene were cloned into pGEM-T vectors (Promega, Madison, WI). This animal was selected based on the aberrant differential expression of the *BLG* gene on the protein and the mRNA level. The 2 large fragments were amplified with 28-bp primers, *BLG*_678 forward and *BLG*_3868 reverse and *BLG*_3634 forward and *BLG*_7662 reverse, respectively using the Expand Long Template PCR system (Roche Diagnostics GmbH, Mannheim, Germany). The numbering in the primer names corresponds to the nucleotide position of the 5' end of the primer as referred to in the GenBank *BLG* sequence with accession number Z48305. The *BLG* promoter region was screened for SNP by amplifying a 807-bp fragment spanned by the primer pair *BLG*_2222 forward and *BLG*_3028 reverse, with the numbering in the primer names also corresponding to the annotation of the sequence with accession number Z48305. A number of additional primers were designed to obtain the *BLG* sequence of the B and the B* alleles as well as to confirm the sequence from the clones. Additional sequence information from the 5'- and 3'-flanking region of the *BLG* gene was obtained by sequencing PCR products with primers designed based on the *Bos taurus* chromosome 11 genomic contig (accession number NW_928426). The DNA was sequenced with a MegabACE (Amersham Biosciences) or an ABI 3730 DNA Analyzer (ABI, Rotkreuz, Switzerland). The obtained sequences were analyzed with the Sequencher 4.5 software (Gene Codes Corporation, Ann Arbor, MI).

Bioinformatic Analysis

Bioinformatic analysis was performed using the GenBank *BLG* gene sequences from cattle (accession num-

Table 1. Content of protein fractions and the CN number in normal and aberrant β -LG AB milk from progenies of a single sire (mean value $\pm$ SD)¹

Item	Normal (n = 15)	Aberrant (n = 15)
True protein, %	3.07 $\pm$ 0.16	3.00 $\pm$ 0.21
CN, %	2.49 $\pm$ 0.14	2.48 $\pm$ 0.16
Whey protein, %	0.58 $\pm$ 0.05	0.52 $\pm$ 0.07**
CN number, %	81.08 $\pm$ 1.33	83.75 $\pm$ 1.37**

¹Significance of difference (Kruskal–Wallis rank sum test, 2-sided).

** $P < 0.01$.

bers Z48305, X14710, NW_928426, Z11996, X63139, and U31361), yak (*Bos grunniens*; accession number AF194981), goat (accession numbers Z33881, DQ417346, and AJ292058), and sheep (accession numbers M32233, X68105, and X12817). The DNA sequences were aligned with the ClustalW program (<http://www.ebi.ac.uk/clustalw/>). By searching the transcription factor binding sites database TRANSFAC (<http://www.gene-regulation.com>), the presence of potential regulatory elements was evaluated.

Definition of *BLG* Alleles and Nomenclature of Polymorphisms

The newly discovered *BLG* allele was termed *BLG* B* because it encodes a translation product with the same AA sequence as the common *BLG* B allele. Numbering of polymorphisms was done with respect to the translation initiation codon (+1 corresponds to the adenosine of the ATG).

RESULTS AND DISCUSSION

The effects of the *BLG* B* allele on concentrations of total milk protein, CN, and whey protein, and on the CN number, respectively, are presented in Table 1. Significant differences were found between the whey protein content of the β -LG AB and the β -LG AB* milk. The difference in the AB* milk from progenies of a single sire suggests that this is a consequence of the aberrantly low expressed *BLG* B* allele. No significant difference could be demonstrated for the protein and the CN content between the 2 milk types. Thus, the CN number differed significantly between the 2 types of milk. We should emphasize that the sample number used in this study was small, and previous studies have suggested that a lower whey protein content is associated with a higher CN content (e.g., Braunschweig et al., 2000). Kuss et al. (2003) reported pronounced pleiotropic effects of a polymorphic position in the *BLG* promoter (R10) on CN fractions. This R10 polymorphism is most likely in linkage disequilibrium with the respective genetic variants A and B of the *BLG* gene. This

Figure 1. Restriction digest of a 355-bp reverse transcription (RT)-PCR fragment with *Hae*III performed for the semiquantitative expression analysis of *BLG* alleles. Lane 1, undigested 355-bp fragment; lane 2, sample from a *BLG* BB animal (fragment size 243 bp); lane 3, sample from a *BLG* AB* animal; lane 4, sample from a normal *BLG* AB animal (fragment size of the A allele is 285 bp); molecular weight marker (M) is an *Hpa*II digested pUC19 cloning vector, which was radioactively end labeled.

indicates that a lower β -LG content, and therefore possibly also the whey protein content, can be balanced by a higher CN content to some extent. Whether this holds true for the *BLG* B* allele presented here has yet to be demonstrated. However, the DNA sequence in or near the *BLG* B* allele is a QTL for whey protein content.

The semiquantitative expression analysis of mRNA samples from mammary biopsies from a *BLG* AB and a *BLG* AB* cow using RT-PCR with subsequent restriction digest with *Hae*III and x-ray quantification revealed mRNA ratios of 1:4 (range 1:2.8 to 1:5.3) and 1:9 (range 1:6.4 to 1:12.6) for *BLG* B and *BLG* B* to the *BLG* A allele, respectively (Figure 1). The completion of the restriction enzyme digest was monitored with the aid of a second restriction site for the endonuclease *Hae*III present in both alleles. In this experiment both alleles of the heterozygote *BLG* AB or *BLG* AB* samples were assumed to be reverse transcribed and amplified similarly, and therefore assumed to correlate with their respective abundance. All 5 replicates of the quantification of the *BLG* mRNA A to B ratio for the cow with the *BLG* B were lower than those obtained for the cow with the *BLG* B* allele. This strongly indicates that the low content of β -LG B protein in the milk of the cow with the *BLG* AB* genotype was due to the low expression of *BLG* B* mRNA.

We hypothesized that the low mRNA expression might be caused by a mutation in a regulatory sequence

of the *BLG* gene. Clones and PCR products from the *BLG* gene of a heterozygous *BLG* AB* animal were sequenced and compared with the *BLG* B reference sequence in GenBank with accession number Z48305. The DNA sequence contained 2,133 bp of the region upstream of the translation initiation site and 187 bp downstream of the polyA signal as referred to Z48305. An additional 100 bp further upstream of the 5'-flanking region and 568 bp further downstream of the 3'-flanking region of *BLG* were identical between the *BLG* B* allele, the *BLG* A and B alleles, and the reference sequence, respectively. Furthermore, adjacent to the allele-specific sequence, another 919 bp at the 5' and 919 bp at the 3' end were amplified by the PCR and sequenced, each from a heterozygous *BLG* AB and *BLG* AB* animal. No sequence differences were found between these 2 samples. In total, 7,670 bp of the B* allele were sequenced, including 2,250 bp of the 5'-flanking region upstream of the translation initiation site and 755 bp of the 3'-flanking region downstream from the polyA signal (accession number DQ489319). Two substitutions (g.-215C>A, g.4303T>C), 2 deletions (g.369delC, g.445delT), and 2 insertions (g.960_961insC, g.2349_2350insT) were found when compared with Z48305. All but one of these sequence differences could be excluded as being potentially causative for the observed low *BLG* B* expression, however, because they were also present in the *BLG* B allele from the control sample. The unique C to A transversion (g.-215C>A) is associated with the *BLG* B* allele. This substitution was subsequently found in the founder sire and in all 4 affected daughters analyzed. Furthermore, the mutation was found in 2 affected cows of unknown ancestry that were phenotyped for milk protein variants for the Swiss Brown Cattle Breeder's Federation.

The promoter region around the mutation was then sequenced from 50 chromosomes from a random sample of Swiss Brown and 8 chromosomes from Brown Swiss animals. All of the DNA sequences obtained were homozygous for the g.-215C *BLG* allele. In addition, 20 chromosomes from different cattle breeds and from a gayal were also homozygous for the g.-215C *BLG* allele.

The DNA sequence of the promoter region of the *BLG* gene has been studied extensively in the past (Wagner et al., 1994, Lum et al., 1997). Wagner et al. (1994) found 14 single-point mutations in the 5'-flanking region and 2 in the 5'-untranslated region of exon 1 of the *BLG* gene by sequencing 11 DNA samples from 6 breeds, including German Brown Swiss. By genotyping 60 samples, they found that 82% of cows from several different breeds showed only 3 different genotype combinations of variants in the 5'-flanking region linked to β -LG AA, AB, and BB, respectively. The results led them to suggest that the distinct haplotypes in the 5'-

flanking region might be responsible for differential β -LG expression and concluded that further investigation of the potential binding sites for *trans*-acting factors and of the 5'-untranslated region of exon 1 is needed to find explanations for the observed phenomenon. However, it would be interesting to study the protein expression of the β -LG A and B variants associated with the remaining 18% of haplotypes, which might be recombinants or results of different mutations. Lum et al. (1997) found 13 SNP in the promoter region when investigating Holstein, Jersey, and Brown Swiss cattle. The C to A transversion reported herein was not encountered in the studies of Wagner et al. (1994) and Lum et al. (1997).

The 240-bp sequence (204 bp for the yak sequence) around the mutation shows at least 90% sequence identity with goat, sheep, yak, and gayal sequences. The bovine wild-type g.-215C is conserved among these ruminants (Figure 2A). The sequence comprising the mutation was analyzed for the presence of potential regulatory elements by searching the transcription factor binding sites database TRANSFAC. The bioinformatic results with the matrix or profile selection using all groups of matrices, minimizing the error rates of false positives and false negatives and using the predefined profile best_selection.prf, revealed the transcription factors c-Rel and Elk-1 (Figure 2B). In the mutant allele the potential DNA binding sites for c-Rel and Elk-1 are abolished. The c-Rel-p65 (RelA) was shown to be an inducible and very potent transcriptional activator that is differentially activated in a cell type-specific manner (Hansen et al., 1994). Elk-1 is a member of the ternary complex factor subfamily of E26 domain transcription factors, which form ternary complexes with the serum response factor and the *c-fos* serum response element (Ling et al., 1997). It was demonstrated that the formation of the Elk-1-serum response factor-DNA ternary complex in vitro correlates with their ability to respond to serum growth factors in vivo (Latinkic et al., 1996). The sequence analysis showed no consensus sequence for a known transcription factor that is involved in the regulation of milk protein gene expression. However, there is evidence that other E26 domain factors are involved in gene regulation in the mammary gland (Galang et al., 2004). The SNP presented might be a good candidate for further functional studies to elucidate its significance.

CONCLUSIONS

We found a unique C to A transversion associated with a rare quantitative β -LG B genetic variant (*BLG* B*) in Swiss Brown cattle. However, this finding does not explain the differential expression of the common

Figure 2. (A) Sequence alignment of the cattle, yak, gayal, goat, and sheep promoter sequence encompassing the C to A transversion located 215 bp upstream of the translation initiation site of the bovine *BLG* gene. The arrow indicates the substitution g.-215 C>A. (B) Potential binding sites of transcription factors c-Rel and Elk-1 and their orientation. The relative identities of the query sequence to the binding site consensus sequences are given in parentheses. The consensus sequences of the transcription factors c-Rel and Elk-1 are given on the right side.

BLG A and B allele. The newly described β -LG B* might be relevant to naturally produce milk with a low β -LG content. Whether the identified g.-215C>A substitution is causative for the observed low β -LG B content remains to be proven.

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