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Polymorphisms in the goat β-lactoglobulin gene

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β-lactoglobulin polymorphisms have been reported in the milk of different goat breeds, although no genetic variants affecting the protein have been characterized. In the present study, we amplified and sequenced the proximal promoter and the first six exons containing the entire coding region for the β-lactoglobulin gene in eleven goat breeds from Spain, France, Italy, Switzerland, Senegal and Asia to identify genetic variants. Fifteen polymorphisms were detected, nine in the promoter region and six in the exons of the β-lactoglobulin gene. All polymorphisms were single nucleotide substitutions with the exception of one deletion/insertion in the promoter region. The polymorphisms in the coding region did not produce any amino acid change. In addition, pyrosequencing technology was used to genotype four polymorphisms in the promoter region in 200 goats belonging to eleven breeds. Differences in allelic frequencies for these polymorphisms between breeds are described and a specific polymorphism for the Italian populations was identified. Finally, the analysis of association between these four promoter point mutations was investigated resulting in five haplotypes, GCGC being the most frequent haplotype in all breeds analysed.

Keywords: Promoter, pyrosequencing, caprine, haplotype.

β Lactoglobulin (β-lg) is the major whey protein in the milk of ruminants. It is also found in the milk of most mammals with the exception of man, rodents, and lagomorphs (Pérez & Calvo, 1995). Although no clear physiological function has been defined for this protein, a role in the transport of retinol and fatty acids has been suggested (Pérez & Calvo, 1995; Flower, 1996).

Since Aschaffenburg & Drewry (1955) detected the first polymorphism in the bovine β-lg protein, polymorphisms have been characterized for all the major milk proteins. To date eleven variants of bovine β-lg have been described at the DNA level (Godovac-Zimmermann et al. 1990, 1996; Ng-Kwai-Hang & Grosclaude, 1992) variants A and B being the most frequent. These variants are associated with quantitative effects on milk composition and cheesemaking properties (reviewed in Ng-Kwai Hang & Grosclaude, 1992). For instance, the B variant correlates with higher casein content in milk, which results in increased cheese yield (Lundén et al. 1997). Milk from β-lg AA genotype cows contains more β-lg than milk from β-lg BB genotype cows (Hill, 1993). In fact, quantification of β-lg A and B in the milk of heterozygous (AB) animals has shown a relative proportion of 60 : 40 between the A and B variants (Lum et al. 1997) which correlates with higher levels of mRNA for β-lg A compared with B (Wilkins et al. 1995). This differential allelic expression has been explained by nucleotide polymorphisms in the bovine β-lg promoter region associated with these two alleles (Wagner et al. 1994; Lum et al. 1997; Folch et al. 1999). In sheep, several transcription factors are known to bind to recognition sequences of the β-lg promoter (Watson et al. 1991). Comparison of ovine and caprine β-lg promoter sequences reveals a conservation of these binding sites (Folch et al. 1994). Three variants (A, B, C) have been reported in sheep (Gaye et al. 1985; Erhardt, 1989) and variants A and B are also associated with milk production and milk composition (reviewed in Moioli et al. 1998). In goat, no variants producing amino acid change have been characterized at the DNA level, but polymorphisms in the 3′ untranslated region (exon 7) and in the β-lg proximal promoter region of the Spanish and French goats have been described (Pena et al. 2000; Yahyaoui et al. 2000). Differences in β-lg content, ranging from 43% to 63% of major whey proteins, have been detected in the milk of the Italian Girgentana goats (Chianese et al. 2000). A polymorphism in the promoter region of individuals of this breed, with reduced β-lg content, has recently been identified but it was not correlated with the β-lg content (Graziano et al. 2003).

The aim of this work was to study the genetic polymorphism in the caprine β-lg gene. We describe new

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Table 1. Primers sequence

Primer†	Position‡	Size (bp)	Sequence (5′-3′)
GOAPF3	promoter (−588)	20	GTCACTTTCCCGTCCTGGGG
GOAPF1	promoter (−187)	21	AGAGGGGGACTTCCTGCTTGG
GoatE1R2	exon 1 (100)	22	GCCTTTCATGGTCTGGGTGACG
GoatI1R	intron 1 (226)	20	GCTGCCCTAGCTGACTGATG
GoatI1F	intron 1 (675)	20	CATTCCCCAGGGTGCAGAGT
GoatI3R	intron 3 (1960)	21	TCATCACCACCAGCCCCTTAG
GoatI3F	intron 3 (2900)	20	GCTGGTCGTGGAGGGTGCTG
GoatI6R	intron 6 (4203)	20	GACCCCAGGGCCTAATGTGG
BLG7§	exon 7 (4646)	22	GCGGATCCGAGCAGGAGGCCAC

† F: Forward, R: Reverse
‡ Position is indicated using as reference the goat β-lg gene sequence (Folch et al. 1994)
§ Two nucleotides are modified to generate a *Bam*HI restriction site

polymorphisms in the proximal promoter region and in exons 1, 2, 3, and 6 of the goat β-lg gene. In addition, a protocol to genotype four polymorphisms of the proximal promoter region was developed using pyrosequencing technology. The distribution of allelic frequencies was evaluated in 200 goats belonging to eleven breeds.

Materials and Methods

Animal samples

A total of 200 goat DNA samples were chosen at random from the following breeds: Alpine (n=22, France), Saanen (n=22, Switzerland), Malagueña (n=21, Spain), Murciano-Granadina (n=21, Spain), Tinerfeña (n=14, Canary Islands, Spain), Palmera (n=14, Canary Islands, Spain), Majorera (n=11, Canary Islands, Spain), Teramana (n=22, Italy), Girgentana (n=19, Italy), Cashmere (n=18, Asia) and Sahelian (n=16, Senegal) breeds. Genomic DNA was isolated from blood samples using standard protocols (Ausubel et al. 1987).

Amplification of the caprine β-lactoglobulin gene

Genomic DNA samples from different goat breeds were used to amplify two overlapping fragments of the β-lg gene. Primers GOAPF3 and GoatI1R were used to amplify an 833 bp caprine fragment that includes: 588 bp of the proximal promoter, 136 bp of exon 1, and 109 bp of intron 1. Primers GOAPF1 and BLG7 were used to amplify a 4854 bp fragment containing the entire coding region of the gene (from −187 position in the proximal promoter to exon 7). All primers were designed over the goat β-lg gene (Folch et al. 1994) and are indicated in Table 1.

PCR reactions were performed with Expand High Fidelity PCR System (Roche, Barcelona, Spain) in a 50-μl final volume. The reaction mixture contained 2·6 units of Expand High Fidelity PCR System enzyme mix, 1× PCR buffer, 1 mM-$MgCl_2$ for the 833 bp fragment amplification or 1·5 mM-$MgCl_2$ for the 4854 bp fragment amplification, 200 μM of each dNTP, 0·3 μM of each primer and approximately 125 ng of goat genomic DNA. For the 4854 bp fragment amplification the thermal cycling conditions were: 95 °C for 3 min, 10 cycles of 95 °C for 30 s, 70 °C for 1 min, and 72 °C for 2 min 30 s, followed by 25 cycles of 95 °C for 30 s, 70 °C for 1 min, and 72 °C for 2 min 30 s increasing 20 s extension in each cycle and a final extension of 72 °C for 10 min. Thermal conditions for the 833 bp fragment amplification were similar with the exception of the annealing temperature (60 °C) and the extension time (90 s, increasing 20 s extension in each cycle).

DNA sequencing reactions

PCR products from two animals per breed (Alpine, Saanen, Malagueña, Murciano-Granadina, Tinerfeña, Palmera, Teramana, Girgentana, Cashmere, and Sahelian) were sequenced for the 833 bp fragment of the proximal promoter. In addition, the 4854 bp fragment was sequenced from two animals per breed indicated above with the exception of Cashmere and Sahelian breeds. Primers used for sequencing reactions are detailed in Table 1.

PCR products were purified with QIAquick PCR Purification Kit (QIAGEN, Milano, Italy). Sequencing was carried out with BigDye® Terminator v3.1 Cycle Sequencing Kit in an ABI PRISM 3100 Avant (Applied Biosystems, Foster City CA, USA). MultAlin software (Corpet, 1988) was used to analyse the nucleotide sequences.

Genotyping by pyrosequencing

Genotyping was done by pyrosequencing in a PSQ HS 96A system (Pyrosequencing AB, Uppsala, Sweden). This DNA sequencing technique is based on indirect bioluminometric quantification of released pyrophosphate (Ppi) as a result of nucleotide incorporation during DNA synthesis. In a cascade of enzymic reactions, visible light is generated, being proportional to the number of incorporated nucleotides (Ronaghi et al. 1996, 1998).

A 181 bp fragment of goat β-lg proximal promoter region (Folch et al. 1994) was amplified from 200 genomic DNA samples to genotype four polymorphisms at

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Table 2. Polymorphisms in the goat β-lg gene

Location	Nucleotide position†	Polymorphism	Breed‡
promoter	−341	T/C	Gir, Alp, Saa, Tin, Mur, Cac, Sah
	−283	C/T	Ter, Pal, Mur
	−197	G/A	Mal, Cac
	−134	G/T	Ter, Pal, Mur
	−118	C/T	Alp
	−114	—/G deletion/insertion	Gir, Alp, Tin, Mur, Cac, Sah
	−64	G/A	Gir
	−60	C/T	Ter, Saa, Mur
	−22	G/A	Cac
exon 1	100	C/T	Cac
	121	C/T	Pal
exon 2	834	G/A	Alp
	840	C/T	Ter, Saa, Mur, Mal
exon 3	1806	C/T	Gir, Alp, Saa, Tin, Pal
exon 6	4122	T/C	Gir, Ter, Alp, Saa, Tin, Pal, Mur, Mal

† Position of polymorphic sites relative to the goat β-lg gene (Folch *et al.* 1994)
‡ Gir: girgentana; Ter: teramana; Alp: alpina; Saa: saanen; Tin: tinerfeña; Pal: palmera; Mur: murciano-granadina; Mal: malagueña; Cac: cashmere; Sah: sahelian

positions: −134, −118, −64, and −60 relative to transcription start. PCR amplification was performed in a 25-μl reaction mixture containing 0·625 units of Taq DNA polymerase (Life Technologies, Rockville MD, USA), 1 × PCR buffer, 1·5 mM-$MgCl_2$, 200 μM of each dNTP, 0·5 μM of each primer and approximately 125 ng of goat genomic DNA. The thermal cycling profile was: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 90 s, with a final extension of 72 °C for 5 min. Primers used were: forward BLGPFpyro (5′-TGG AAG AAG GCC TCC TAT TGT C-3′) and reverse BLGPRpyro (5′-biotin-CTT CTG AGC TCT GCA GGG AGT-3′). A total of 20 μl of the PCR product was used in the pyrosequencing reaction, using the SNP reagent kit (Pyrosequencing AB, Uppsala, Sweden) according to the manufacturer's instructions. The sequencing primers were designed on the forward strand: primer BLGSNP134-114 (5′-GGC CTC CTA TTG TCC T-3′) to genotype polymorphisms at positions −134 and −118 and primer BLGSNP64-60 (5′-CTC GTG GCT GGG G-3′) to genotype polymorphisms at positions −64 and −60 in the proximal promoter of goat β-lg gene.

Results and Discussion

Identification of polymorphisms in the goat β-lg gene

The proximal promoter region (an 833 bp PCR fragment) was sequenced in animals from 10 different goat breeds. The alignment and analysis of these sequences allowed the identification of nine polymorphic sites, eight of which were base substitutions and one was a deletion/insertion of one nucleotide (Table 2). Interestingly, all the base substitutions are located in CpG dinucleotides suggesting that these polymorphisms are originated by 5-methylcitosine deamination (Duncan & Miller, 1980). This result indicates that the goat β-lg proximal promoter region is probably methylated. The polymorphism at position −60 has been reported previously in Spanish and French goats (Yahyaoui et al. 2000) and the polymorphism at position −341 has been characterized recently in Alpine, Saanen and different Italian breeds (Graziano et al. 2003).

No genetic variants affecting the coding region of the β-lg gene have been described in the caprine species, although different protein variants of faster mobility have been reported (Macha, 1970). To detect polymorphisms in the coding region of the goat β-lg gene, we amplified and partially sequenced a 4854 bp fragment corresponding to all the β-lg coding region from different breeds. Comparative sequence analysis revealed six single nucleotide substitutions in exon 1, 2, 3, and 6 (Table 2). Five of these polymorphisms occurred in the coding region and were synonymous substitutions, suggesting a selection pressure reducing diversity at this DNA region. Mutations at nucleotide position 100, 121, and 834 were only found in five samples from Cashmere, Palmera and Alpine goats, respectively. The rest of the polymorphisms were widely distributed among breeds. The sixth mutation was found in the non-coding region of exon 6 (4122) in all studied breeds. Further studies are required to determine whether this polymorphism affects the stability of the β-lg mRNA.

Genotyping and allelic frequencies of the β-lg proximal promoter region polymorphisms

Two-hundred goat genomic DNA samples from eleven breeds were genotyped for four polymorphisms at positions: −134, −118, −64, and −60 by pyrosequencing technology using two independent reactions (Fig. 1). Analysis of these four point mutations was performed both

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Table 3. Allelic frequencies of the −134, −118, −64 and −60 polymorphisms of the goat β-lg promoter region in different breeds

		allelic frequencies							
		Position−134		position−118		position−64		position−60	
Breed	n° of animals	T	G	T	C	A	G	T	C
Alpine	22	0·18	0·82	0·10	0·90	0	1	0·05	0·95
Saanen	22	0·07	0·93	0·02	0·98	0	1	0·34	0·66
Malagueña	21	0·12	0·88	0·02	0·98	0	1	0·12	0·88
Murciano-Granadina	21	0·12	0·88	0	1	0	1	0·21	0·79
Tinerfeña	14	0·11	0·89	0	1	0	1	0	1
Palmera	14	0·18	0·82	0	1	0	1	0	1
Majorera	11	0·32	0·68	0	1	0	1	0	1
Teramana	22	0·25	0·75	0	1	0·05	0·95	0·32	0·68
Girgentana	19	0·03	0·97	0	1	0·21	0·79	0	1
Cashmere	18	0·17	0·83	0	1	0	1	0·03	0·97
Sahelian	16	0	1	0	1	0	1	0·19	0·81
Total	200				1				

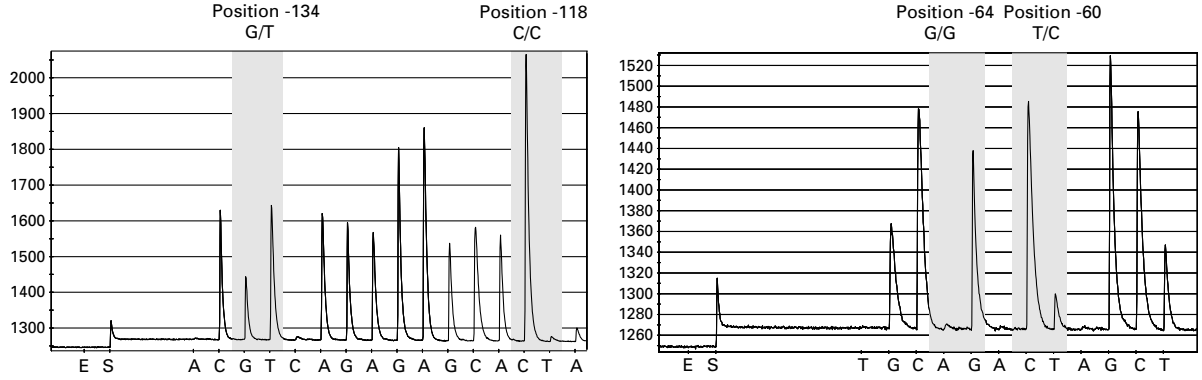


Fig. 1. Genotyping of the polymorphisms at positions −134, −114, −64 and −60 of goat β-lg proximal promoter. Pyrograms for one animal from the Malagueña breed.

because of their distinct distribution among breeds and their location in a region where different transcription factor binding sites have been reported (Folch et al. 1994). We used the pyrosequencing technology for SNP analysis because the technique offers an extremely accurate and easy distinction between the various genotypes. The reaction is performed in real-time and the raw data are directly analysed allowing the genotyping of large numbers of samples in a short period of time. Moreover, closely located polymorphisms can be identified in a single extension reaction and information about the adjacent nucleotides is also obtained. Finally, this method is time- and cost-competitive when compared with other sequencing methods such as primer extension analysis used for genotyping.

The allelic frequencies are shown in Table 3. No significant deviations from Hardy-Weinberg equilibrium were observed, calculated using an exact test (Guo & Thompson, 1992) in the GENEPOP software (Raymond & Rousset, 1995).

The −64 (A/G) polymorphism was only found in Italian breeds with a higher frequency for the −64 A allele (0·21) in Girgentana goats. Although this breed originally comes from the geographic areas of Asia, including Afghanistan and the Himalayan regions, it was raised in Sicily Island where has been used in familiar livestock farming. Nowadays, this breed is in danger of extinction (FAO, 2002) with a population size less than 500 individuals. So, it seems more plausible that allelic distribution of this breed-specific polymorphism might be explained by genetic drift and founder effects. In addition, this polymorphism is localized in an AP-2 consensus binding site (CCCMNSSS; TRANSFAC database, Wingender et al. 1996), being a good candidate to explain the differences in β-lg content observed in animals of this breed. On the other hand, allelic frequencies for the −60 C allele agree with the results previously described by Yahyaoui et al. (2000) for the Murciano-Granadina, Canary, Malagueña and Saanen breeds. Moreover, Graziano et al. (2003) have analysed this polymorphic position in different Italian

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breeds and T allele, that was not present for the Girgentana breed in our study, is found at low frequency. The −60 T allele was not found in the Canary breeds, this difference could be explained by the long-term isolation in the Canary Islands of these breeds (Amills et al. 2004). The −118 (T/C) polymorphism that was only found at low frequencies in the Alpine, Saanen and Malagueña breeds is also located in an AP-2 consensus binding site (Wingender et al. 1996). Finally, −134 (T/G) polymorphism was widely distributed among all the analysed breeds with the exception of Sahel.

Subsequently, we have investigated the association of these four polymorphisms that results in sixteen theoretical haplotypes. Five haplotypes only were identified and their frequencies were computed using an expectation-maximization algorithm (Lange, 1997) (Arlequin software, Schneider et al. 2000). The haplotype GCGC may correspond to the ancestral type as it was found in all the analysed breeds with a frequency of 0·702. The second most frequent haplotype TCGC (0·133) was found in all the breeds analysed with the exception of Sahel breed, followed by the haplotype GCGT (0·125) that was only found in Girgentana and Canarian breeds. Haplotypes less frequent were GCAC (0·025) and GTGC (0·015) from Italian breeds and Alpine and Saanen breeds, respectively.

Although further studies are required to evaluate the effect of these variants in the expression of β-lg gene and therefore in the milk protein composition, these novel polymorphisms can be used as molecular markers and in the association studies with milk production traits.

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