Veterinary Immunology and Immunopathology 134 (2010) 279–283

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Technical report

Molecular cloning, promoter analysis and SNP identification of Italian Nicastrese and Saanen lactoferrin gene

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ARTICLE INFO

Article history:
Received 13 January 2009
Received in revised form 3 September 2009
Accepted 16 September 2009

Keywords:
Lactoferrin
Iron-binding protein
Gene promoter
Transcription factor
AP2
Goat

ABSTRACT

Lactoferrin (Lf) is an iron-binding glycoprotein found in exocrine secretions including milk. High levels of lactoferrin may have a role in the prevention of microbial infection of the mammary gland. In this report we sequenced and characterized goat lactoferrin cDNA and its promoter region in two different breeds of goat. The complete cDNA comprised 2356 nucleotides, including 38 bp at the 5′-UTR and 194 bp at the 3′-UTR. The open reading frame is 2127 bp long and it encodes a mature protein of 689 aminoacids. A total of 19 nucleotide differences, 11 of them being responsible for 8 aminoacid changes, were identified through the comparison with French, Korean and Tibetan goat lactoferrin cDNAs. About 1700 bp of the lactoferrin gene promoter were sequenced. Sequence analysis revealed a non-canonical TATA box, multiple SP1/GC elements, and other putative binding sites for transcription factors, such as NF-κB, STAT3 and AP2. Two SNPs were identified, one of which would seem to create a new putative AP2 consensus sequence. The presence of an additional AP2 binding site could be associated with quantitative differences of such protein fraction, which could enhance all the activities related to such protein, and improve mammary gland defence against bacterial infections.

Lactoferrin is a bioactive iron-binding glycoprotein of ~80-kDa which has been found in milk and other exocrine secretions. Many functions have been attributed to lactoferrin (for a review, González-Chávez et al., 2009), however, its best known role is the primary defence against microbial infection, mainly through iron sequestration required for microbial growth, direct interaction with bacterial surface (Legrand et al., 2008) and direct bactericidal activity due to lactoferricins (Bellamy et al., 1992; van der Kraan et al., 2006).

The antimicrobial action of lactoferrin and its role in the natural defence mechanism of the mammary gland make it a candidate gene for increasing resistance against bacterial infections in farm animals. In dairy cattle, its concentration dramatically increases during the dry period and a mastitis infection (Kutila et al., 2003), suggesting that this protein plays important physiological roles in the reduction of mastitis incidence (Hagiwara et al., 2003). In the goat, the same concentration of lactoferrin secreted from two different genetic types showed different antibacterial activity. In particular, Saanen goat lactoferrin exhibited no activity against *Escherichia coli* O111, even at 7.5 mg/ml, whereas Korean native goat lactoferrin was already effective at 5 mg/ml. These observations suggested that the different antibacterial activities may be the result of differences in the protein conformation caused by the polymorphisms in the goat lactoferrin gene (Lee et al., 1997). This gene has been mapped on chromosome 22; its main feature is the extremely split architecture consisting in 17 exons

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doi:10.1016/j.vetimm.2009.09.002

(Le Provost et al., 1994). Although the functions of this protein have received adequate attention, the molecular mechanisms of gene expression and regulation remain relatively unknown.

Studying mastitis incidence in Southern Italy goat breeds, we noticed that its occurrence in Nicastrese goat was lower compared to the other breeds as, for example, Saanen goat (unpublished data). Our interest in these data prompted us to speculate that lactoferrin could play a more active role in mammary gland innate defence mechanisms against infections. Aim of this work was, therefore, to characterize the lactoferrin cDNA, the regulatory regions of gene promoter in the Italian Nicastrese and Saanen breeds and to identify mutations which could potentially affect milk lactoferrin concentration or produce aminoacid changes.

Genomic DNA was isolated from leukocytes obtained from individual blood samples, using conventional phenol–chloroform extraction method. The DNA was then resuspended in 100 μl TE pH 7.6 (10 mM Tris, 1 mM EDTA).

According to Chomczynsky and Sacchi (1987), total RNA was extracted from milk somatic cells of five goats per breed, at the end of the lactation stage. cDNA synthesis was performed using Improm-II™ Reverse-Transcriptase (Promega) as follows: 1 μg of total RNA was incubated with 10 μM of the reverse primer LF17R: 5′-AGGGAATGAAAATCAACAGCA-3′ in a volume of 10 μl according to the manufacturer's guidelines conditions.

The whole cDNA and the promoter of the goat lactoferrin gene were amplified using an iCycler-IQ (Bio-Rad). Primers were designed by DNASIS-Pro (Hitachi) using, as templates, the sequence of the goat lactoferrin cDNA (EMBL Acc. No.: X78902) for the couple LF1F 5′-CGGAGTCGCCCCAGG-3′ and LF17R (amplicon A) and the sequence of the cattle lactoferrin gene and promoter (EMBL Acc. No.: AY319306) for the following primers LFPRF 5′-TCCTTTTCATTGGCAAATGAG-3′ and LF1R 5′-GGCGGGGACGAAGAG-3′ (amplicon B); LF5′F 5′-AGATACAAAGATGCTTCA-3′ coupled with a primer designed on newly determined goat promoter sequence LFPRR 5′-TGGCAGAGGCAATAT-3′ (amplicon C).

The PCR reaction mix (50 μl) comprised: 100 ng of genomic DNA or cDNA, 1× PCR Buffer (Promega), 2.5 mM $MgCl_2$, 5 pmol of each primer, dNTPs each at 400 μM, 2.5 U of *Taq* DNA Polymerase (Promega). PCR conditions included an initial cycle of denaturation at 95 °C for 4 min followed by 35 cycles at 95 °C for 60 s, 58 °C (amplicons A and B)–54 °C (amplicon C) for 45 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min. PCR products were purified with QIAquick columns, inserted into pDrive vector (Qiagen), and then used to transform chemically QIAGEN EZ competent cell following the manufacturers' guidelines. Screening of the recombinant clones was performed by PCR using the plasmid primers M13. More than 10 clones of each PCR product were sequenced on both strands by automated sequencer (ABI-PRISM, Applied Biosystems) using standard cycle conditions by Sanger's dideoxy-chain termination method.

Comparison among sequences and multiple alignments were accomplished using DNASIS-Pro. The putative regulatory motifs were searched by AliBaba 2.1 software.

The complete goat lactoferrin cDNA was sequenced for the two investigated breeds. It comprised 2356 nt (EMBL Acc. No. FM875929). Analysis of the sequence revealed one ORF 2127 bp long (positions 39–2162), plus 38 and 194 nucleotides in the 5′- and 3′-UTR, respectively. The signal-peptide (19 aminoacids) is encoded by the nucleotides 39–95, the stop-codon (TAA) is realized between the nucleotides 2163–2165, whereas the polyadenylation-signal is in positions 2322–2327. No nucleotide differences were

Table 1
Comparison among the Italian goat LTF cDNA sequence and French, Korean native and Tibetan goat LTF cDNA.

		Italian goat		French goat		Korean goat		Tibetan goat	
		EMBL No. FM875929		EMBL No. X78902		EMBL No. U53857		EMBL No. DQ387456	
Exon	Position	Mutation	Aminoacid	Mutation	Aminoacid	Mutation	Aminoacid	Mutation	Aminoacid
2	123-125	**GTG**	V	**GTG**	V	**ATA**	***I***	**GTG**	V
3	56	**CTG**	L	**CGG**	***R***	**CTG**	L	**CTG**	L
4	54	**CAG**	Q	**AAG**	***K***	**CAG**	Q	**CAG**	Q
4	74	**GGC**	G	**GGT**	G	**GGC**	G	**GGC**	G
4	90-91	**ATG**	M	**ATG**	M	**ATG**	M	**GCG**	***A***
4	144-145	**TTC**	F	**CCC**	***P***	**TTC**	F	**TTC**	F
8	28	**CGC**	R	**CGC**	R	**AGC**	***S***	**CGC**	R
8	84	**TCT**	S	**TCT**	S	**TCT**	S	**TCC**	S
9	12	**GTG**	V	**TTG**	***L***	**TTG**	***L***	**GTG**	V
10	29	**GAT**	D	**GGT**	***G***	**GAT**	D	**GAT**	D
14	64	**ACA**	T	**ACA**	T	**ACG**	T	**ACG**	T
16	9	**TTC**	F	**TTT**	F	**TTC**	F	**TTC**	F
16	33	**CCG**	P	**CCG**	P	**CCG**	P	**CCA**	P
17	104	**G**	-	**G**	-	**A**	-	*	-
17	122-123	**GA**	-	**GA**	-	**AC**	-	*	-
17	205	**A**	-	**T**	-	**A**	-	*	-
Molecular weight			~75.48KDa		~75.43KDa		~75.44KDa		~75.42KDa

A total of 19 nucleotide differences (bold letters) were detected, 11 of them are responsible for 8 aminoacid changes (bold and italic letters). Grey cells correspond to the same coding triplets, asterisk shows unavailable sequence, dash indicate non-translated sequences. The different nucleotide combination does not determine remarkable difference in the estimated molecular weight of the deduced mature protein (689 aminoacids).

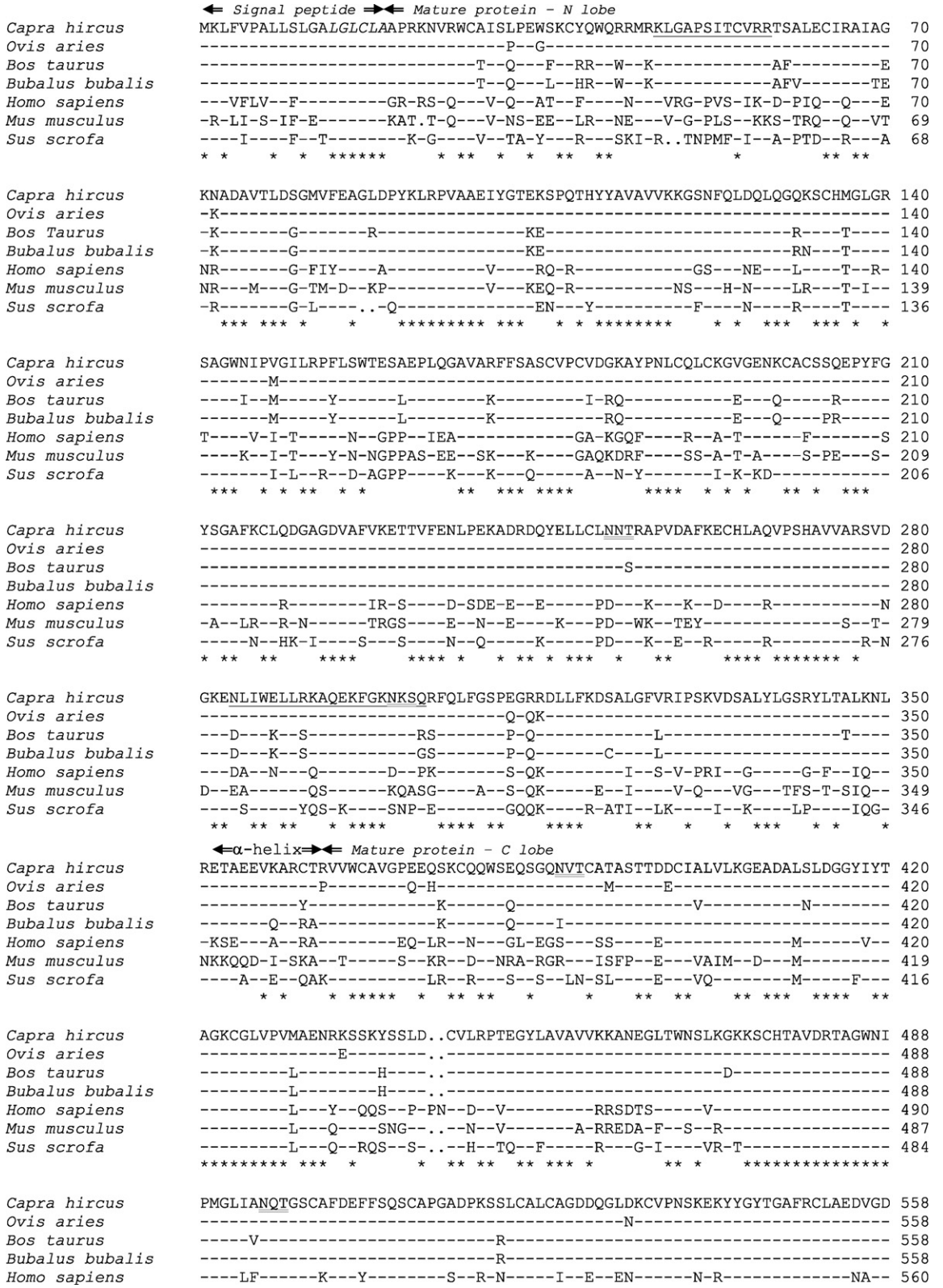

Fig. 1. Alignment of deduced goat lactoferrin aminoacid sequence with other lactoferrin sequences deposited in GenBank. It is a polypeptide chain folded into two symmetrical lobes (N lobe: aminoacids 1–332 and C lobe: aminoacid 344–689 of the mature protein), connected by a hinge region containing an α-helix (aminoacids 333–343), which provides flexibility to the molecule. Gaps were introduced in the sequences indicated by dots (·). Identity of aminoacids is denoted by hyphens (-). Full conserved aminoacids among the species are symbolized by asterisk (*). The signal peptide cleavage site is in italics. Bioactive peptides are underlined. The potential N-glycosylation sites in goat lactoferrin are double-underlined. Percentage of identity, calculated by DNASIS, are in bold.

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Mus musculus      ----L----R--K-N---------------N-----I--EK-EN--A-----R-Q-----L-----KA-N 557
Sus scrofa        ----LV------K-----------SQ-G-N-----V-N---VN------N-R-------------NA-- 554
                  ****  *** ** * * ********  * * ***** *   *  ** *** * * ***** *****  *

Capra hircus      VAFVKNDTVWENTNGESSADWAKNLNREDFRLLCLDGTTKPVTEAQSCYLAVAPNHAVVSRSDRAAHVEQ 628
Ovis aries        ------------------------------------------------------------------------ 628
Bos taurus        ----------------T--------------------R---------H-------------------K- 628
Bubalus bubalis   ----------------T--------------------R---------H----------L-E------- 628
Homo sapiens      -----DV--LQ--D-NNNEA---D-KLA--A------KR------R--H--M---------M-KVERLK- 630
Mus musculus      ---L-DS--LQ--D-KNTEE--R--KLK--E-----D-R------KN-H--I---------T-KVEVLQ- 627
Sus scrofa        -----DV--LD----QNTEE--RE-RSD--E-------R-------N-H-----S------KEK--Q--- 624
                  *** *  **  ** *     **  *   ** *****   ******  * ** ** *****        *

Capra hircus      VLLHQQALFGKNGKNCPDQFCLFKSETKNLLFNDNTECLAKLGGRPTYEKYLGTEYVTAIANLKKCSTSP 698
Ovis aries        ------------------------------------------------------------------------ 698
Bos taurus        ----------------K-----------------------------E---------------------- 698
Bubalus bubalis   ----------E-------K-----------------------------E---------------------- 698
Homo sapiens      -------K--R--SD---K----Q----------------R-H-KT-------PQ--AG-T--------- 700
Mus musculus      ---D--VQ--R--QR--GE----Q-K---------------IP-KT-S-----K---I-TER--Q--S-- 697
Sus scrofa        ---TE--Q--RY--D---K----L----------------Q-Q-KT-------S----------Q--V-- 694
                  ***  *  **  *  **  **** * **************   *  * * ***  **     ** ** **

Capra hircus      LLEACAFLTR 708                    EMBL Acc. No. FM875929
Ovis aries        ---------- 708      97.7%        EMBL Acc. No. AY792499
Bos taurus        ---------- 708      91.9%        EMBL Acc. No. NM_180998
Bubalus bubalis   ---------- 708      91.6%        EMBL Acc. No. AJ005203
Homo sapiens      -----E--RK 710      65.6%        EMBL Acc. No. NM_002343
Mus musculus      ---------Q 707      56.4%        EMBL Acc. No. NM_008522
Sus scrofa        --G----MM- 704      68.1%        EMBL Acc. No. AY306198
                  ** ** *
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Fig. 1. (*Continued*).

found in the two investigated breeds, whereas the comparison with the lactoferrin cDNA of French (EMBL Acc. No.: X78902), Korean native (U53857) and the Tibetan goat (DQ387456), showed 19 nucleotide differences (respectively 9, 8 and 5), 11 of them being responsible for 8 aminoacid changes. Consequently, the Italian goat lactoferrin cDNA appears to be a new and particular allelic combination, in fact, the first part (ex1–ex8) appears to be more similar to the Korean and Tibetan cDNAs, conversely the second fraction (ex9–ex17) more homologue to the French counterpart (Table 1).

By comparing the deduced lactoferrin aminoacid sequence with the known Lf sequences available in GenBank, we found that the degree of conservation among species varies from 56.4% *vs* mouse to 97.7% *vs* sheep (Fig. 1). The analysis of the primary structure of the goat lactoferrin showed: (a) the signal-peptide, which is fully conserved among the ruminants with a cleavage site at positions 19 and 20 (*LGLCLA*↓AP); and (b) the mature protein (689 aminoacids). Other features include: (a) three potential bioactive peptides: one lactoferricin (KLGAPSITCVRR), which is one of the main peptides responsible for antibacterial properties of lactoferrin in cattle (Bellamy et al., 1992) and appears to be strongly conserved among ruminants; and two lactoferrampins (NLIWELLRKAQEKFGKNKSQ and WELLRKAQEKFGKNKSQ), which in cattle show strong bactericidal activities against Gram-positive and Gram-negative bacteria (van der Kraan et al., 2006); (b) five potential glicosilation sites (Asn-X-Ser/Thr) one of which (NQT) at the Asn495 residue is fully conserved among the species (Fig. 1).

In order to understand whether lactoferrin could affect the different mastitis incidence between these two breeds even in absence of polymorphisms in the coding regions, we decided to extend the sequencing to the 5′-flanking region and to analyze the putative regulatory regions. About 1700 bp of the lactoferrin gene was amplified and sequenced (EMBL Acc. No. AJ784283). The lactoferrin promoter contains a non-canonic TATA-box located, with reference to the first nucleotide of the first exon, at nucleotides −28/−24; at least three GC-boxes (−1369/−1364, −948/−934, −164/−159) and two SP1 binding sites (−200/−187, −69/−55). The high GC content (64.9%) is a typical feature of housekeeping genes promoters and the presence of these last sites could contribute to the constitutive expression of the lactoferrin in various tissues. In particular, a non-canonic TATA-box (ATAAA) and a SP1 binding site at position −69/−55 are almost perfectly conserved in other 7 species (cattle, human, pig, mouse, rat, camel and buffalo), suggesting that these two sites might play a fundamental role in the regulation of lactoferrin gene *in vivo* (Zheng et al., 2005). The region spanning from nucleotides −1009 to −841 contains three potential LPS-responsive elements: one STAT3 motif (−1009/−991), and two NF-κB sites (−960/−949, −852/−838). STAT3 is known as a response factor to acute inflammatory phases, whereas NF-κB plays a central role in immunitary response and it can be activated by Toll-like receptors (Muzio et al., 2000).

Compared to the Saanen conterpart, the promoter sequence of the Nicastrese lactoferrin gene, is characterized by two SNPs: a transition A → G (nt −333) and a

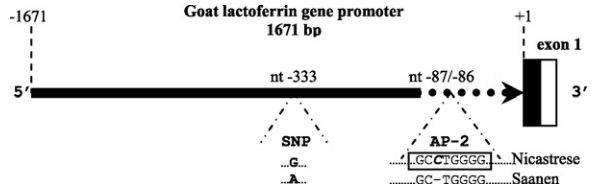

Fig. 2. Schematic representation of the 5′ regulatory region, of the exon 1 and polymorphisms detection in the goat lactoferrin gene. Dots represent CpG islands of the promoter. The 5′ untranslated region (UTR) of the exon 1 is shown as black box.

cytosine insertion realized between the nucleotides −87/−86 (Fig. 2). The first one, apparently does not interest any known regulatory site, whereas the cytosine insertion would create a new putative AP2 binding site (GCCTGGGG) (Fig. 2), located 53 bp upstream the TATA-box, and between two SP1 motifs. The estrogen receptor AP2 is known to be an important transcription factor regulating the differential expression of lactoferrin gene in pigs and mice (Wang et al., 1998; Liu and Teng, 1991). Furthermore, the new AP2 falls into the CpG island of the gene promoter. Such region is known to facilitate a rapid response to microbial infection by inducing the production of IL-6 in milk, prompting the release of the TNF-α, reducing *E. coli* counts in milk (Zhu et al., 2007). The insertion of a cytosine and the creation of an additional motif for the AP2 in such CpG region could enhance the protein production and its correlated effects. An example of how an AP2 can enhance the transcription is shown in bovine lactating mammary gland, where the different binding affinity of the AP2 to the gene promoter of another whey-protein (β-lactoglobulin) brings to the creation of a more or less efficient transcriptional complex, which is responsible for differences in gene expression, even associated with total milk protein content (Kuss et al., 2003). Thus, as observed for such whey-protein, it would be reasonable to think that the presence of an additional AP2 binding site for the goat lactoferrin could increase the transcriptional activity and be associated to quantitative differences in gene expression. This could enhance all the activities related to such protein and improve mammary gland defence against bacterial infections.

Acknowledgements

This research was financially supported by the Italian Ministry for Agriculture and Forestry Policy—MiPAF (SELMOL project).

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