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Review

Real-time RT-PCR and cDNA macroarray to study the impact of the genetic polymorphism at the α_{s1} -casein locus on the expression of genes in the goat mammary gland during lactation

Christine LEROUX^{a,b*}, Fabienne LE PROVOST^a, Elisabeth PETIT^a,
Laurence BERNARD^b, Yves CHILLIARD^b, Patrice MARTIN^a

^aLaboratoire de Génétique biochimique et de Cytogénétique, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy-en-Josas, France

^bUnité de Recherches sur les Herbivores, Équipe Tissu Adipeux et Lipides du Lait, Institut National de la Recherche Agronomique, Theix, 63122 Saint-Genès-Champagnelle, France

Abstract — Milk fat has a large effect on nutritional, technological and sensorial properties of milk products. The milk fat content and composition are modulated by genetics and nutritional factors and imply a large number of enzymes. The regulation of their gene expression in the mammary gland still needs to be clarified. An association between the extensive polymorphism at the α_{s1} -casein (α_{s1} -Cas) locus and both the lipid content and the characteristics of this fraction in caprine milk has been demonstrated. In order to decipher the mechanism responsible for this impact, a quantification of the transcripts of four lipogenic key enzymes (acetyl-CoA carboxylase, fatty acid synthase, lipoprotein lipase and stearoyl-CoA desaturase) was performed using real-time RT-PCR, suggesting an absence of association between the α_{s1} -Cas genotype and expression variability of the studied genes. This approach has been completed by a more global analysis using a first generation of ruminant macroarray gathering 400 gene probes. The comparison of the expression profiles of lactating goat α_{s1} -Cas A/A (strong allele) and F/F (defective allele) mammary gland allowed to confirm the expected variability in the expression of known genes (such as those encoding the α_{s1} -casein) in ruminant mammary tissues as well as to identify up- and down-regulated genes. A second generation of ruminant cDNA macroarray extended to a few thousands of genes is currently in progress and will be applied to study different factors such as the nutritional regulation of gene expression in the mammary gland.

goat milk / casein / fatty acids / genetic polymorphism / macroarray / lipogenesis

1. INTRODUCTION

Because of its commercial and nutritional significance, milk is probably the best known food with respect to its biosynthesis

and composition, and the chemical structure of its components. It is processed to provide a wide diversity of food products, which comprise more than 25%, and between 15 and 25% of total consumed dietary protein [1] and fat [2], respectively.

* Corresponding author: cleroux@clermont.inra.fr

Milk protein composition differs quantitatively as well as qualitatively among species. Total milk protein, which can vary in amounts from 10 (primate) to 200 (lagomorph) g·L⁻¹ milk, comprises two major groups namely the caseins (α_{s1} , α_{s2} , β and κ) and the whey proteins (including mainly β -lactoglobulin, α -lactalbumin, lactoferrin, lysozyme and WAP), and some minor components [1]. In addition to this inter-species variability, intra-species variability has also been described. Thus, Holstein and Jersey cows fed with pasture diet produce milk with 29.4 and 34.3 g·L⁻¹ of protein, respectively [3]. Due to the nutritional and technological impact, the quantitative variability of caseins has been well studied, in particular in ruminants (for review see: [4, 5]). The genetic polymorphism at the α_{s1} -casein (α_{s1} -Cas) locus has been shown to be responsible for a large variability in protein content (25.5 to 31.8 g·L⁻¹) of goat milk [4].

The principal lipids of milk are triacylglycerols (TG) secreted in the form of droplets (or milk fat globule: MFG) of variable size. In cow's milk over 99% of the lipids are contained within MFG and at least 97% comprises TG. Both milk fat content and composition have a large effect on nutritional, technological and sensorial properties of milk, butter and cheeses. Milk fat content can vary from 15 (equine [6]) to ca. 500 (phocids [7]). Bovine, caprine and human milk share quite the same milk fat content ranging between 30 and 45 g·kg⁻¹ [8]. As observed for the protein, the fat content is variable among the same species. Thus, in cattle, quantitative trait loci (QTL) analyses have mapped a QTL with a major effect on milk composition, particularly fat content, to bovine chromosome 14 [9–11]. Recently, a cloning positional candidate gene of this QTL was detected and a non-conservative substitution in the acylCoA: diacylglycerol acyltransferase (DGAT1) gene with a major effect on milk fat content and other milk characteristics was identified [12].

Thus, the milk protein and fat contents are modulated by genetic factors. Further-

more, in ruminants, a selection to increase the protein content has a positive impact on the fat content. In order to decipher the mechanism involved in this association of protein and fat contents in milk, studies have been undertaken in the goat, which presents an extensive polymorphism at the α_{s1} -Cas locus. After a brief overview of the impact of this polymorphism on different traits, we present thereafter preliminary studies, which rely upon a candidate gene approach followed by a gene expression profiling approach using a restricted ruminant macroarray, in order to better understand the regulation of protein and lipid biosynthesis and secretion in the goat mammary gland.

2. GOAT α_{s1} -CASEIN POLYMORPHISM AND ITS IMPACT ON MILK COMPOSITION AND MAMMARY EPITHELIAL CELL FUNCTION

An extensive polymorphism detected at the α_{s1} -Cas locus was shown to be responsible for a structural and quantitative variability of the relevant protein, in goat milk. The 18 alleles described up to now, are distributed among 11 different protein variants (A to M without D and J [13]) associated with four different levels of expression defining 4 quantitative classes. These four classes of expression are called "strong", "medium", "low" and "null" alleles associated with ca. 3.6, 1.6, 0.6 and 0 α_{s1} -casein g·L⁻¹ per allele, respectively [4, 14]. Thereafter, we will mainly focus on two alleles, named *A* and *F*, which are representative of "strong" and "low" classes, respectively. α_{s1} -Cas*A* is considered as the reference allele whereas α_{s1} -Cas*F* is a defective allele. These two alleles have been particularly well studied at a molecular level as well as for their effects on milk properties. Thus, the establishment of the overall organisation of the goat gene and the characterisation of the α_{s1} -Cas*F* allele at the genomic level together with its

expression products (mRNA and protein) have demonstrated the occurrence of alternative splicing events. Indeed, this variant is characterised by an internal deletion of a 37 amino acid residue long peptide segment starting at position 59 and including a multiple phosphorylation site (SerP₆₄-SerP-SerP-SerP-SerP-Glu-Glu₇₀) [15]. This deletion was shown to be due to the out-splicing of three exons (9 to 11). The splicing of α_{s1} -CasF primary transcripts seems to be deeply disturbed since at least 9 different mRNA have been detected [16]. Nevertheless, alternative splicing also exists for α_{s1} -CasA allele but is much less complex and involves only two sequences of 24 nucleotides corresponding to exons 13 and 16 which are out-spliced during the course of the maturation of the primary transcripts [17].

At the end of the 1980's, defective alleles were preponderant in French flocks. Before applying a selection for "strong" alleles in breeding programmes, it was necessary to evaluate the impact of such alleles on milk properties. The effects of the α_{s1} -Cas allele on casein (26.7 g·L⁻¹ for A/A versus 20.7 g·L⁻¹ for F/F) and protein (31.8 g·L⁻¹ versus 25.5 g·L⁻¹) contents have been confirmed as well as the association of a strong allele with smaller micelles [18]. Higher cheese yields and firmer curds have also been shown to be associated with strong alleles. Furthermore, an unexpected impact of the α_{s1} -Cas genotype on the lipid fraction of milk has been recorded (Fig. 1). Casein, milk fat (34.5 g·L⁻¹ for A/A versus 30.1 g·L⁻¹ for F/F) and cream fat (350 g·L⁻¹ versus 180 g·L⁻¹) content are together positively correlated whereas lipase activity (42 nmol·mL⁻¹·min⁻¹ versus 72 nmol·mL⁻¹·min⁻¹) and post-milking milk fat lipolysis are negatively correlated to the other traits [19]; reviewed in [4, 20]. However, the effect of the α_{s1} -Cas genotype is less pronounced on milk fat lipolysis than on milk fat content in early lactation goats [20]. Likewise, a comparison of the milk fat globule (MFG) structure from homozygous A/A and F/F goats revealed that the

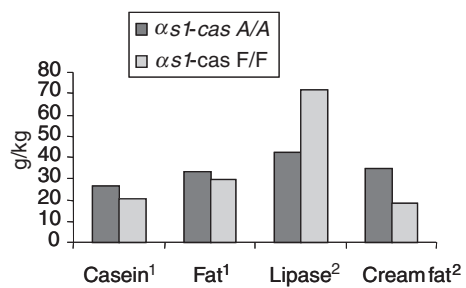


Figure 1. The effects of A/A and F/F α_{s1} -cas variants on Alpine goat milk composition¹, lipase activity and creaming ability². ¹From [4] and [47]. ²From [46].

α_{s1} -CasF/F genotype is associated with a higher proportion of phospholipids in total lipids and smaller MFG [21].

In addition, morphological observations performed at the cellular level, on mammary tissue sections, have revealed that goats homozygous for defective alleles display epithelial cells with a dramatic dilatation of the rough endoplasmic reticulum (RER) primarily due to an accumulation of proteins, strongly suggesting that secretion mechanisms are impaired [22]. This accumulation affects the caseins and other proteins such as immunoglobulin heavy chain binding protein (BiP) and protein disulphide isomerase (PDI) [23], which are involved in facilitating the assembly of multimeric protein complexes inside the RER and in the rearrangement of both intra-chain and inter-chain disulphide bonds in proteins, respectively.

3. IMPACT OF GOAT α_{s1} -CAS GENETIC POLYMORPHISM ON THE MAMMARY EXPRESSION OF LIPOGENIC CANDIDATE GENES IDENTIFIED ON THE BASIS OF THEIR FUNCTION

In order to decipher the mechanism involved in the relationship between protein and fat contents in goat milk, studies on

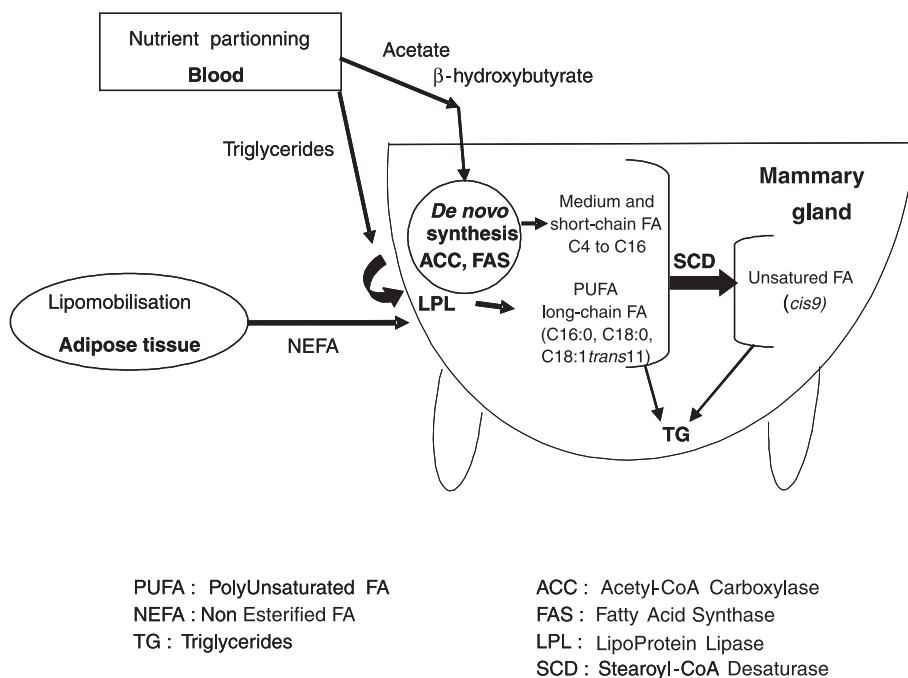


Figure 2. Mammary synthesis and secretion of lipids in ruminant milk (adapted from [48]).

lipogenic genes have been undertaken according to the α_s1 -*Cas* genotype of the goat.

Four key enzymes have been identified on the basis of their role in fatty acid (FA) synthesis (Fig. 2). Thus, the expression of the genes encoding Acetyl-CoA Carboxylase (ACC), FA Synthase (FAS), LipoProtein Lipase (LPL) and Stearoyl-CoA Desaturase (SCD) has been studied through mRNA quantification by real-time RT-PCR.

The main metabolic pathway involves ACC, which is a rate-limiting enzyme for de novo biosynthesis of FA and which catalyses the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, the activated donor of two-carbon units for FA chain elongation. In ovine adipose tissue, multiple forms of ACC mRNA ranging from 9.0 kb to 9.4 kb are generated by the casual occurrence of an insertion in the 5' UTR and the use of 2 different polyadenylation signals [1]. Due to the multiplicity

of 5' and 3' extremities, real-time RT-PCR tools to quantify ACC mRNA have been developed in the translated region.

The second key enzyme is FAS, which catalyses the synthesis of long-chain FA from acetyl-CoA, malonyl-CoA and NADPH. In animal tissues, the active synthase is a homodimer of a multifunctional protein that is organised in a head-to-tail fashion, generating two active catalytic centers [25]. The tissue concentration of FAS is a key determinant for the maximal capacity of a tissue to synthesise FA by the de novo lipogenic pathway. FAS activity and mRNA levels as well as gene transcription are sensitive to nutritional and hormonal manipulations [26]. FAS mRNA, in bovine and ovine adipose tissues [27] and the caprine mammary gland [28] is about 8.5 kb in size. Recently, the sequencing of caprine cDNA has allowed a real-time RT-PCR quantification technique [Leroux et al., in

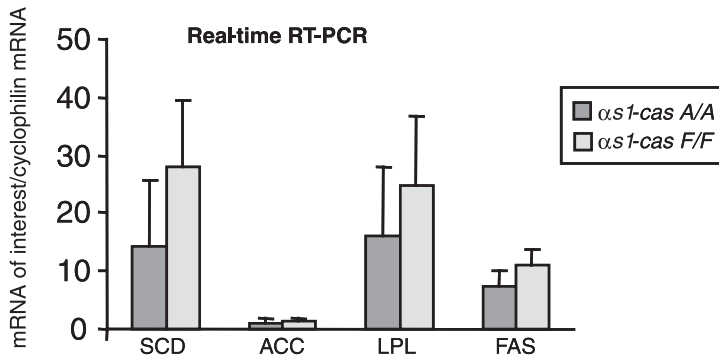


Figure 3. Expression of Stearoyl-CoA Desaturase (SCD), Acetyl-CoA Carboxylase (ACC), LipoProtein Lipase (LPL) and Fatty Acid Synthase (FAS) gene in the mammary gland of lactating α_s1 -Cas A/A ($n=3$) and F/F ($n=3$) goats determined by real-time RT-PCR. mRNA of interest quantifications were normalised by mRNA of cyclophilin, a housekeeping gene. Values (arbitrary units) are means for 3 goats per genotype with their standard errors. Differences between genotype at the α_s1 -Cas on mRNA quantification were tested using the nonparametric Wilcoxon-U test.

preparation] of ruminant FAS mRNA to be developed.

Triglycerides are hydrolysed in mammary blood vessels by LPL to yield monoglycerides and free FA, which are then taken up by secretory cells. In bovine, two major LPL mRNA (3.4 and 3.8 kb in size) have been identified in adipose tissue [27] as well as in the mammary gland [29]. The regulation of LPL gene expression is complex. A tissue-specific expression pattern between adipose tissue and cardiac muscle has been described in ewes and cows [27]. Moreover, quantification by real-time RT-PCR of the two major LPL mRNA [30] has shown that nutritional factors sharply regulate the expression of the LPL gene in adipose tissue and cardiac muscle.

Finally, the 4th key enzyme considered was SCD, which catalyses the $\Delta 9$ desaturation of a spectrum of fatty acyl-CoA substrates from C14 to C19. SCD is the rate-limiting component in the *cis* double bond introduction in the $\Delta 9$ position. Its activity is regulated by a large number of factors such as nutrients and hormones [31, 32]. In goats, mammary SCD mRNA is 5.1 kb long and presents an unusually long (3.8 kb) 3' UTR

sequence derived from a single exon [33]. The knowledge of the SCD mRNA structure has allowed the development of a quantification protocol based on real-time RT-PCR [34].

Quantification of these four-lipogenic-enzyme mRNA was performed starting from lactating mammary gland total RNA samples extracted from 3 homozygous α_s1 -CasA/A and 3 α_s1 -CasF/F goats. At the slaughter, these goats were multiparous and in mid-lactation but the nature and intake of feed were not controlled. The "lipogenic" mRNA level was normalised by the level of cyclophilin (a housekeeping gene) mRNA [35]. Variations observed in the level of "lipogenic" mRNA were independent from the α_s1 -Cas genotype of these six animals.

This technique revealed a large individual variation of SCD and LPL mRNA contents in the mammary glands of goats without a relationship with the α_s1 -Cas genotype (Fig. 3). Such a variation could be due in part to different nutritional status. Regarding SCD mRNA, a possible association with a previously detected polymorphism (involving 3 nucleotides in the 3'UTR) [33, 34] is currently under investigation.

4. IMPACT OF GENETIC VARIABILITY ON GOAT MAMMARY GLAND EXPRESSION PROFILING USING FUNCTIONAL GENOMIC TOOLS

High-throughput gene expression profiling using array technologies is becoming a powerful way to simultaneously analyse the expression pattern of hundreds or thousands of genes in a variety of biological samples. These technologies have been used to compare transcriptional activity within very different samples such as normal human mammary epithelial cells and breast tumours [36] proposing a generic approach for cancer classification based on gene expression monitoring by DNA arrays [37, 38]. Organogenesis has also been studied using murine arrays to develop a picture of the biological programs used during mammary gland development [39–41]. Murine mammary organogenesis studies demonstrated a coordinate regulation of genes involved in β -oxidation of FA, which reflects the presence of brown adipose tissue in the mammary gland [39]. Up to now, little work has been devoted to evaluate the effects of the genotype on the transcriptome. Using oligoarray, a comparison of adipose tissue expression profiles of mice with different leptin genotypes (wild-type versus leptin deficient *ob/ob*) revealed a differential expression for 7% of 1875 detected signals, which correspond to 2% of 6500 genes contained on the array used [42]. Unexpectedly, this study revealed that the expression of several genes that play a role in FA or cholesterol biosynthesis decreased, whereas adipose tissue mass increased in *ob/ob* mice.

In our study, a first macroarray has been designed and constructed using (i) one independent clone representing each different cDNA from a caprine lactating mammary gland cDNA library [43] and (ii) goat or bovine DNA fragments amplified using a set of specific primer pairs available in our laboratory. PCR products of this working set of genes were regularly arranged at least in

triplicate on high-density filters. Then, 4 nylon membranes were hybridised with the same radioactive complex targets obtained from reverse transcription of mRNA. Hybridisation signals of the 4 membranes were analysed using XdotsReader software (Cose, France) [44]. Signal intensities validated on 3 or 4 membranes as proposed by [44] were analysed. Since each cDNA was spotted in triplicate, each value represents a mean of 9 to 12 intensities. Thus, a panel of ca. 400 gene expression profiles was compared between different α_{s1} -*Cas* genotypes (a comparison of homozygous associated with high α_{s1} -casein content in milk = α_{s1} -*CasA/A* vs. low = α_{s1} -*CasF/F*). Thus, with the aim to identify genes whose expression is modulated according to the genotype at the α_{s1} -*Cas* locus, especially those responsible for the association with milk fat content (MFC), mammary mRNA from 3 homozygous lactating goats of each genotype (α_{s1} -*CasA/A* and α_{s1} -*CasF/F*) were pooled in 2 samples named *A* and *F*. The goats used in this study were the same as those used in the RT-PCR experiments described before. Global expression analysis by scatter plots demonstrates few differences in the detected gene expression pattern between *A* and *F* samples. For each gene, we compared the mean intensity values (*Im*) obtained from the 4 membranes and the triplicated spots. Only *Im* higher than 2 fold intensities of background were taken into account. Moreover, the expression of each gene was considered potentially differential when the ratio of the intensity signals was higher than 1.5 in the comparison between *A* and *F* allele.

Before considering up- or down-regulated “mammary” genes associated with the α_{s1} -*Cas* genotype, these first macroarray results had to be validated. To perform such a validation, genes whose expression was known in our model were analysed.

The first validation was obtained by quantifying the α_{s1} -*Cas* mRNA. Indeed, the 6 goats were chosen on the basis of their

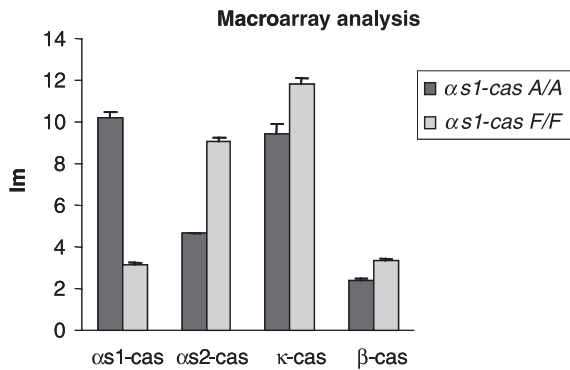


Figure 4. Comparison of mRNA level of genes encoding caseins using macroarray. Each cDNA was spotted in triplicate. Hybridisations were realised using a pool of mRNA extracted from 3 lactating goats of each genotype (3 goats α_{s1} -Cas A/A and 3 goats α_{s1} -Cas F/F). Signal intensity (Im) values are means for validated intensities obtained with 4 membranes and for the 3 spots of each cDNA, with their standard errors (vertical bars).

α_{s1} -Cas genotype, with a level of α_{s1} -Cas gene expression being high (A) or low (F). The comparison of the Im obtained with α_{s1} -Cas cDNA was in agreement with the animal genotype and followed the same pattern as the means of α_{s1} -Cas mRNA real-time RT-PCR quantification performed on the same mammary gland RNA samples. However, it was underlined that the intensity of fold changes was underestimated on the macroarray, as already observed in comparison with northern-blot analyses [42]. In our preliminary study, we can suspect that the

low number of cDNA (400) on macroarray and the very large representation of the major milk protein mRNA could be responsible for this underestimation. In another hand, macroarray results obtained on the expression of BiP and PDI genes are in agreement with those obtained by semi-quantitative RT-PCR previously performed (unpublished data) showing an absence of differential expression between A and F samples.

In order to understand the mechanisms for the association between the biosynthesis of protein and milk fat content, we analysed

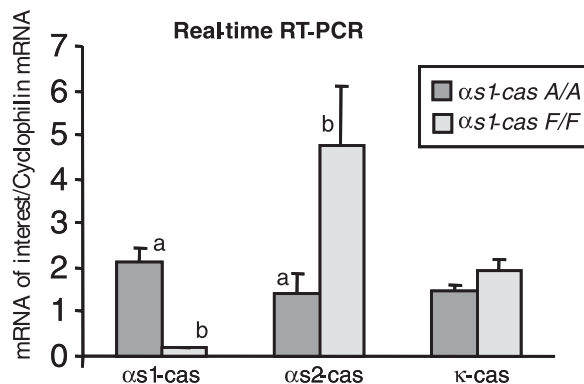


Figure 5. Comparison of the means of mRNA content encoding 3 (α_{s1} , α_{s2} and κ) of the 4 caseins in mammary gland of α_{s1} -Cas A/A ($n = 3$) and F/F ($n = 3$) lactating goats determined by real-time RT-PCR. Caseins mRNA quantifications were normalised by mRNA of cyclophilin, a housekeeping gene. Values (arbitrary units) are means for 3 goats per genotype with their standard errors. Differences between genotype at the α_{s1} -Cas on mRNA quantification were tested using the nonparametric Wilcoxon-U test.

the expression of genes implied in these syntheses in relation with the α_{s1} -*Cas* genotype. For the casein genes, as expected and in accordance with the absence of variation in milk content of the other caseins associated with the α_{s1} -*Cas* genotype, β -*Cas* and κ -*Cas* mRNA levels were not significantly different between the *A* and *F* samples (Fig. 4). Surprisingly, a negative correlation was observed between the α_{s1} -*Cas* and α_{s2} -*Cas* mRNA contents using macroarray. Such a result was subsequently confirmed by real-time RT-PCR analyses (Fig. 5), although a discrepancy between the mRNA level in mammary epithelial cells and casein content in milk remains questionable. It was, however, suggested that some interactions between IGFBP and several proteins present in milk, including α_{s2} -*Cas*, could be a key process in apoptotic cell death and tissue remodelling [45]. Elsewhere, a dysfunction of mammary epithelial cells of α_{s1} -*CasF/F* goats was described [23]. Taken together these results pointed out the interest in studying the interactions between these 2 mechanisms and raised the question: could α_{s2} -*Cas* be involved in apoptosis of α_{s1} -

CasF/F mammary epithelial cells due to the dramatic dilatation of RER?

Elsewhere, we considered the genes involved in the biosynthesis and secretion of milk fat. The Im obtained with the four genes of key lipogenic enzymes (FAS, ACC, LPL and SCD) revealed an absence of variability in relation to the α_{s1} -*Cas* genotype, as suggested by RT-PCR. Moreover, genes encoding proteins anchored in the milk fat globule membrane (MFGM), showed slightly lower intensities (between 20 and 40%) in *F* than in *A* samples but these differences were not significant according to the criteria aforementioned. However due to the lower creaming ability [44] and the smaller MFG observed with allele *F* vs. *A*, suggesting a modified MFG secretion process associated with defective α_{s1} -*Cas* allele, the expression of some genes encoding MFGM proteins, using real-time RT-PCR technique, is under our investigation.

In addition to the analysis of the genes involved in milk protein or fat biosynthesis, we also considered the genes exhibiting, between alleles *A* and *F*, a differential expression according to the criteria

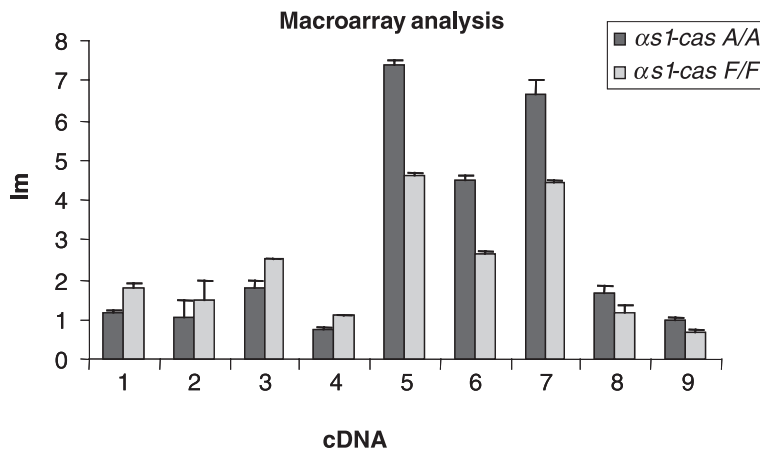


Figure 6. Profiles of nine up- or down-expressed genes in the mammary gland of lactating α_{s1} -*Cas A/A* and *F/F* goats determined by macroarray hybridisation. Im values are means for validated intensities obtained with 4 membranes and for the 3 spots of each cDNA, with their standard errors (vertical bars).

aforementioned. Thus, the comparison of the profiles has allowed the identification of 3% of the 400 genes as being up- or down-regulated in the mammary gland in relation with the α_{s1} -Cas genotype (Fig. 6). Some of them are involved in protein biosynthesis (Fig. 6: 2, 4 and 5), DNA damage repair (Fig. 6: 3), cell trafficking (Fig. 6: 7) or the cell cycle (Fig. 6: 9). Moreover, some EST were also differentially expressed (Fig. 6: 1, 6 and 8).

5. CONCLUSION

The extensive polymorphism at the caprine α_{s1} -Cas locus is now well documented as well as its impact on milk, cheese-making and more recently on mammary epithelial cell morphology. Preliminary, real-time RT-PCR and macroarray analyses suggest that the association between this polymorphism and milk fat content are not due to a difference in the expression of the four key enzymes of lipogenesis (ACC, FAS, LPL and SCD) but could be linked to the expression of some genes encoding MFGM proteins which are under investigations using RT-PCR technique. Moreover the use of a first ruminant macroarray gathering ca. 400 cDNA to study the impact of genetic polymorphism at the α_{s1} -Cas locus on gene expression in the mammary gland has allowed to identify several up- or down-regulated genes in relation with the α_{s1} -Cas genotype.

Despite the small set of cDNA on the macroarray used, these first results point out several interesting genes whose expression varies among α_{s1} -CasA and F alleles. Currently, extended cDNA macroarrays with a thousand genes were in progress. Gene expression profiling in the mammary gland will facilitate our understanding of its biology as well as providing candidate genes for milk production traits. In the same way, the large impact of nutritional factors on milk composition is under investigation to explore the putative effects of nutrients at the

molecular level in the mammary gland using the same technologies.

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