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Fine mapping of genes on sheep chromosome 1 and their association with milk traits

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Summary

On the basis of comparative mapping between cattle/sheep and human for milk trait quantitative trait loci (QTL) on BTA3/OAR1, *annexin A9* (*ANXA9*) and *solute carrier family 27* (*fatty acid transporter*), *member 3* (*SLC27A3*) were selected as candidate genes for fat content (FC) in sheep milk. Two other genes in the same region, *cingulin* (*CGN*) and *acid phosphatase 6, lysophosphatidic* (*ACP6*), were also considered. DNA fragments of 1931 and 2790 bp corresponding to *ANXA9* and *SLC27A3* respectively were isolated, and 14 and 6 single nucleotide polymorphisms (SNPs) respectively were found in each gene. *ANXA9*, *SLC27A3*, *CGN* and *ACP6* were localized to chromosome 1 between *INRA006* and *AE57* by linkage mapping using the International Mapping Flock. Across-family analyses of a daughter design comprising 13 sire families revealed significant sire and *SLC27A3* genotype-nested-within-sire effects for FC. Within-family analyses indicated significant regression coefficients for FC in four of six heterozygous sires. These results could reflect the existence of a QTL for FC linked to *SLC27A3* in sheep.

Keywords dairy, quantitative trait loci, ovine chromosome 1, *SLC27A3*, *ANXA9*, *CGN*, *ACP6*.

Introduction

Quantitative trait loci (QTL) for milk traits have been reported on bovine chromosome 3 (BTA3) (Zhang *et al.* 1998; Heyen *et al.* 1999; Ashwell *et al.* 2001, 2004; Mosig *et al.* 2001; Plante *et al.* 2001; Olsen *et al.* 2002; Boichard *et al.* 2003; Viitala *et al.* 2003) and on the homologous region of ovine chromosome (OAR1) (Calvo *et al.* 2004; Barillet *et al.* 2005) between microsatellite markers *INRA003* and *INRA006*. A QTL for protein content has been detected in a Sarda × Lacaune backcross in the same region in OAR1 (Barillet *et al.* 2005).

Previous results in a within-family analysis using the *AMY* gene cluster (located close to *INRA003*) as genetic markers showed significant association with milk yield in a family of Manchega sheep (Calvo *et al.* 2004). Subsequent examination of comparative maps between cattle/sheep and human (http://bos.cvm.tamu.edu/bovgbase; http://rubens.its.unimelb.edu.au/~jillm/jill.htm) indicated two candidate genes identified in human but unknown in sheep. Both are fatty acid transporters and include *annexin A9* (*ANXA9*) and *solute carrier family 27* (*fatty acid transporter*), *member 3* (*SLC27A3*).

Annexin A9 is a membrane transport channel protein, and a member of the annexin family of Ca(+2) and phospholipid-binding proteins, with a molecular mass of 37 kDa (Morgan & Fernandez 1998). The fatty acid transporter, member 3, is a 78-kDa protein that facilitates the transport of long chain fatty acids across the cytoplasmic membrane (Hirsch *et al.* 1998). Two other genes, which are in the same region, *cingulin* (*CGN*) and *acid phosphatase 6, lysophosphatidic* (*ACP6*), were also considered. CGN is a 140-kDa protein located on the cytoplasmic face of tight junctions of polarized epithelia and some endothelia (Citi *et al.* 1991). The ACP6 protein (80 kDa) is membrane bound; hydrolyzes lysophosphatidic acid, which is a bioactive phospholipid; and plays an important role in phospholipid metabolism

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inside cells (Hiroyama & Takenawa 1999). All four of these genes are expressed in various tissues, including secretory tissue and mammary glands (http://www.dsi.univ-paris5.fr/genatlas).

The objective of this paper was to further study this QTL for milk traits on sheep chromosome 1 (OAR1) using a candidate gene approach. Here we present the isolation, characterization and linkage mapping of four genes underlying this QTL (*SLC27A3*, *ANXA9*, *CGN*, *ACP6*).

Materials and methods

Genomic walking and genetic polymorphism

Primers designed from human and mouse genomic DNA were used to amplify parts of *ACP6*, *CGN*, *ANXA9* and *SLC27A3* (Table S1). Genomic DNA (50 ng) was amplified in a final volume of 25 μl containing 5 pmol each primer, 200 nM dNTPs, 2.2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, 0.1% Triton X-100 and 0.5 U *Taq* polymerase (Biotools, Madrid, Spain). Standard amplification profiles were used. Polymerase chain reaction (PCR) products were sequenced using an ABIPrism 3700 (Applied Biosystem, Madrid, Spain).

Sheep primers were designed (Table S1) for sequencing PCR products from six animals (two Manchegas, one each of the Awassi, Assaf and Rasa Aragonesa sheep breeds and one Mouflon). Homology searches were performed with BLAST (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST/). Sequence analysis was performed using DNASIS (Hitachi Software Engineering Company, Ltd), CLUSTALW (http://www.ebi.ac.uk/clustalw/) and GenView (http://www.itba.mi.cnr.it/webgene/) software. Repeat elements were identified using RepeatMasker (http://www.repeatmasker.org/).

Chromosomal location

Linkage mapping was used to map the ovine *ACP6*, *CGN*, *ANXA9* and *SLC27A3* genes against markers on the sheep framework map (Maddox *et al.* 2001) using genotypes obtained for animals in the AgResearch International Mapping Flock (IMF) pedigrees (Crawford *et al.* 1995). Multipoint linkage analysis using CRI-MAP (Lander & Green 1987) was carried out.

PCR-RFLP analysis

Primer sequences, their corresponding product sizes and annealing temperatures for the analysis of genes in sheep are given in Table S1. These reactions included primer sets PCR3, PCR7, PCR10, PCR15 and PCR17 for the *ACP6 C/T*, *CGN C/T*, *ANXA9 C/G*, *SLC27A3 C/T* and *SLC27A3 A/G* polymorphisms respectively, which were detected with the *Ban*I, *Hha*I, *Hin*F1, *Bsa*HI and *Ape*KI restriction enzymes respectively.

Association studies

A daughter design comprising 13 families was used to evaluate candidate gene associations between polymorphisms in the four genes and the estimated breeding values (EBVs) of several milk traits. A total of 378 ewes with an average of 29 daughters (range of 12–62) per sire were genotyped. All animals belonged to a nucleus-breeding scheme for selection in the Spanish Manchega sheep breed and were sampled from flocks connected by artificial insemination. EBVs of milk yield (MY), fat content (FC) and protein content (PC) from the latest genetic evaluation (2004) were used in the analyses. Statistical analyses were carried out as a regression of EBVs of milk traits on the *ACP6*, *CGN*, *ANXA9*, or *SLC27A3* genotypes using the GLM procedure of SAS (v 6.12, SAS Institute Inc., Cary, NC, USA). Analyses were performed across and within sire half-sib families for which the sire was heterozygous.

The model for the across-family analysis was:

$$y_{ij} = \mu + s_i + bx_j + b_i x_j + e_{ij}$$

where y_{ij} = predicted breeding value of animal j within sire i, μ = population mean, s_i = sire i, x_j was a variable that took values of 1, 0 and −1 for the *AA* homozygous, *AB* heterozygous and *BB* homozygous genotype respectively; b was the regression coefficient that corresponded to the additive value, $b_i x_j$ was the interaction effect between family and genotype, and e_{ij} = residual error. The model for the within-family analysis was similar to the previous model but without the sire effect. The sire effect was treated as a fixed effect and genotype as a covariate. Type III sum of squares was used in all F-tests. Separate analyses were carried out for each of the four genes described.

Results and discussion

Isolation and genomic structure of the sheep ACP6, CGN, ANXA9 and SLC27A3 genes

Partial ovine genomic DNA sequences of 0.851 kb for *ACP6* (AY85288), 1.742 kb for *CGN* (AY85290), 1.931 kb for *ANXA9* (AY85286) and 2.790 kb for *SLC27A3* (AY996127) were obtained. The sheep exons were identified based on comparisons to known sequences of rats, mice and humans.

The 851-bp ovine *ACP6* DNA fragment contained exons 9 through 10 and encoded 67 amino acids. The ovine *CGN* DNA fragment spanned 1742 bp, containing exons 19 through 21 and a partial 3′-UTR sequence. The three exons encoded 137 amino acids. The 1931-bp ovine *ANXA9* DNA fragment contained exons 3 through 7, encoding 140 amino acids, with the start codon in exon 3. The ovine *SLC27A3* DNA fragment spanned 2790 bp, contained exons 3 through 10 and encoded 365 amino acids. Sequence analyses of the introns using RepeatMasker revealed a repetitive element in intron 19 (SINE/MIR) of *CGN* and

Table 1 Percent identity of ovine *ACP6*, *CGN*, *ANXA9* and *SLC27A3* to corresponding genes and proteins in humans, mice, rats and cattle.

	Percent identity (DNA/protein[1])			
Sheep	Human	Mice	Rat	Cattle
ACP6 (AY785288)	86/81,89[1] (NM_016361)	86/77,91 (NT_039238)	85/75,90 (XM_215655)	96/91,95 (AY785289)
CGN (AY785290)	91/94,98 (NT_004487)	89/94,98 (NT_039238)	90/96,98 (XM_227472)	97/98,99 (AY785291)
ANXA9 (AY785286)	84/82,89 (NT_037496)	86/85,90 (NT_039238)	87/85,90 (XM_227442)	97/97,98 (AY785287)
SLC27A3 (AY996127)	89/93,97 (NT_004487)	84/89,94 (NT_039238)	86/94,94 (XM_215605)	96/98,99 (AY995157)

ANXA9, *annexin A9*; *SLC27A3*, *solute carrier family 27*; *CGN*, *cingulin*; *ACP6*, *acid phosphatase 6*, *lysophosphatidic*.
[1]For protein comparisons, the first number is the percent amino acid identity, and the second number is percent identity when conservative amino acid substitutions are not considered.

repetitive elements in intron 4 (SINE/MIR) and intron 6 (three repeats of a SINE/BovA core repetitive element) of *ANXA9*. Percentage identities for the DNA and predicted amino-acid sequences are presented in Table 1. Maximum identity was found with bovine DNA sequences.

Detection and characterization of genetic polymorphisms

Direct sequencing of PCR products amplified from DNA samples of different sheep breeds and mouflon for the *ACP6* fragment revealed one single nucleotide polymorphism (SNP) in intron 9. For ovine *CGN*, three SNPs were detected: one in intron 19 and two in the 3′-UTR. Fourteen SNPs were found in ovine *ANXA9*: one in the 5′-UTR, one in intron 3, six in intron 4, two in intron 5 and four in intron 6. For ovine *SLC27A3*, six SNPs were detected that did not result in an amino acid change: two in intron 3, one in intron 4, two in intron 5 and one in exon 9. Two SNPs of *SLC27A3* and one SNP from each of the *ACP6*, *CGN* and *ANXA9* genes were used for linkage mapping and association studies.

The *ACP6* polymorphism included a *Ban*I restriction site polymorphism located at position 472 in intron 9 (AY785288). This polymorphism had two alleles: *C* (471 and 300 bp) and *T* (771 bp). The *CGN* polymorphism is a *Hha*I restriction site polymorphism located at position 1232 in the 3′-UTR region (AY785290). This polymorphism had two alleles: *C* (510 and 303 bp) and *T* (813 bp). The *ANXA9* polymorphism is a *Hin*F1 RFLP located at position 843 in intron 4. This polymorphism had two alleles: *C* (328, 227, 187, 139 and 137 bp) and *G* (366, 328, 187 and 137 bp). The *SLC27A3* polymorphism is detectable with *Bsa*HI and located at position 963 in intron 4. This polymorphism had two alleles: *C* (444 and 240 bp) and *T* (684 bp). Another polymorphism was analysed for *SLC27A3* located at position 2198 in exon 9, detectable with *Ape*KI. This polymorphism had two alleles: *A* (301, 205, 71, 49, 43 and 41 bp) and *G* (301, 246, 71, 49 and 43 bp). The SNP in *SLC27A3* at position 2198 was tested rather than the SNP at position 963 because of its limited informativeness in the AgResearch IMF pedigree (115 *CC*, 10 *CT* and 0 *TT* animals). In the Manchega population the SNP in intron 4 was highly polymorphic (64 *CC*, 183 *CT* and 37 *TT* animals) and so was used for association studies. Codominant segregation of the polymorphisms was verified in the IMF population.

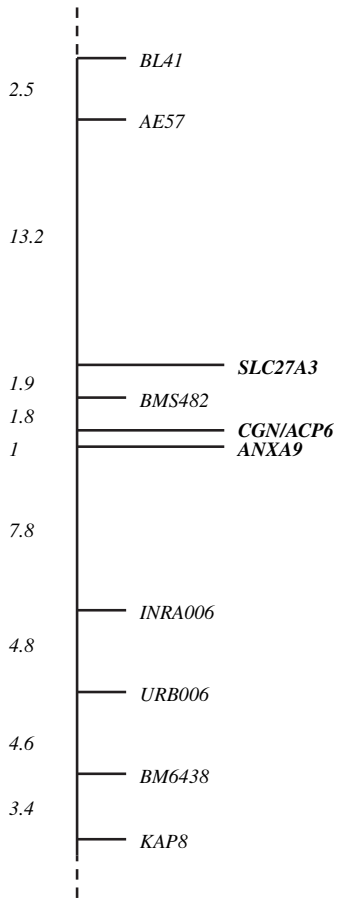

Figure 1 Linkage map of *ACP6*, *CGN*, *ANXA9 and SLC27A3* genes on ovine chromosome 1. Distances between genes are in cM.

Chromosomal location of ovine ACP6, CGN, ANXA9 and SLC27A3

Linkage mapping was used to assign *ACP6*, *CGN*, *ANXA9* and *SLC27A3* to OAR1 between *INRA006* and *AE57* over a

4.7-cM interval (Fig. 1). Support for multipoint linkage to the framework map exceeded a LOD of 13 for each marker. These assignments are consistent with comparative mapping information as human *ACP6*, *CGN*, *ANXA9* and *SLC27A3* map to HSA1, which is partly homologous to OAR1. In human and mice the gene order is *ACP6-ANXA9-CGN-SLC27A3*, over an interval of 5.17 Mb.

Association studies

The comparative region on HSA1/OAR1 contains a limited number of genes related to lipid and protein metabolism. Two of these candidate genes are *ANXA9* and *SLC27A3*, both of which are related to lipid transport. Genotyped animals from the Manchega breed sheep revealed a low degree of polymorphism in *ACP6*: only 10 of 374 genotyped animals were homozygous for the *T* allele, and only four sires were heterozygous. For the other three genes, intermediate allele frequencies were found for the polymorphisms. The SNPs of each of the four genes were tested only in the offspring of heterozygous sires. In this analysis a direct effect of the genes or strong linkage disequilibrium between the genes and a QTL would result in a significant effect of the paternal allele. In contrast, the effects of QTL more distantly linked to the loci would be exhibited as a significant interaction effect, as different sires would have different phases for the marker and QTL.

Regression effects for the across-family analyses of EBVs of milk traits on *SLC27A3* polymorphisms are presented in Table 2. The sire effects were highly significant ($P < 0.001$) for all analysed traits. Genotype nested within sire was significant only for FC. No significant genotype or genotype-within-sire effects were found for *ACP6*, *CGN* or *ANXA9*.

In the within-family analyses (Table 3), only two of eight informative families were significant for an effect of the *ANXA9* genotype on protein content (PC) (family 1) and milk yield (MY) (family 3). The estimated additive effect of genotype *CC* was 0.058 for PC and 16.590 for MY. Another family had an effect near significance ($P < 0.10$) for MY. For *SLC27A3*, three of six informative families were significant for FC while another family was nearly significant at

Table 2 Regression analyses across families of heterozygous sires considering genotype of the *SLC27A3* gene and estimated breeding values (EBV) of milk yield (MY), fat content (FC) and protein content (PC).

Trait	Mean	R^2	Effect	d.f.	*F*	Pr > *F*
MY	28.382	0.538	Sire	5	36.94	0.0001***
			Genotype	1	0.78	0.3793
			genotype(sire)	5	1.17	0.3266
FC	−0.044	0.314	Sire	5	9.83	0.0001***
			Genotype	1	1.04	0.3093
			genotype(sire)	5	3.58	0.0042**
PC	−0.125	0.241	Sire	5	6.60	0.0001***
			Genotype	1	0.12	0.7242
			genotype(sire)	5	1.46	0.2073

R^2, coefficient of determination; d.f., degrees of freedom; **, significance $P < 0.01$; ***, significance $P < 0.001$.

Table 3 Within-family regression analyses of heterozygous sires considering genotypes of *ANXA9* and *SLC27A3* and estimated breeding values (EBV) of milk yield (MY), fat content (FC) and protein content (PC).

Gene/family[1]	Trait	Sire EBV	Accuracy	Mean	R^2	*F*	Estimate[2]	Pr > *F*
ANXA9/1 ($n = 60$)	MY	27.763	0.99	21.600	0.002	0.12	0.703	0.7357
	FC	0.386	0.99	0.178	0.060	1.91	0.097	0.0608**
	PC	0.172	0.99	0.070	0.066	4.12	0.058	0.0470*
ANXA9/3 ($n = 13$)	MY	30.155	0.94	26.921	0.463	9.51	16.590	0.0104*
	FC	0.043	0.94	0.016	0.074	0.88	−0.093	0.3673
	PC	−0.090	0.95	−0.047	0.091	1.11	−0.100	0.3145
SLC27A3/2 ($n = 29$)	MY	35.190	0.97	26.886	0.003	0.08	0.857	0.7804
	FC	−0.274	0.96	−0.159	0.141	4.45	0.125	0.0444*
	PC	−0.245	0.97	−0.132	0.002	0.07	0.009	0.7999
SLC27A3/3 ($n = 12$)	MY	30.150	0.94	26.134	0.400	6.64	9.470	0.0275*
	FC	0.043	0.94	0.023	0.378	6.09	−0.133	0.0332*
	PC	−0.090	0.95	−0.054	0.266	3.64	−0.108	0.0857**
SLC27A3/5 ($n = 13$)	MY	−22.720	0.80	−6.078	0.076	0.91	−4.435	0.3596
	FC	0.065	0.79	0.080	0.273	4.15	0.172	0.0665**
	PC	0.131	0.85	0.046	0.150	1.93	0.107	0.1920
SLC27A3/9 ($n = 57$)	MY	47.330	0.97	31.372	0.003	0.20	−0.883	0.6551
	FC	0.076	0.97	0.062	0.091	5.55	0.075	0.0221*
	PC	−0.245	0.98	−0.122	0.004	0.25	0.011	0.6173

[1]Only families with significance were included (n = number of daughters per sire).

[2]Estimated additive value.

R^2, coefficient of determination; **, significance $P < 0.10$; *, significance $P < 0.05$.

the $P < 0.10$ level (Table 3). The estimated additive effects of the *CC* genotype were 0.125, −0.133, 0.172 and 0.075 for families 2, 3, 5 and 9 respectively. In family 3 significant regression coefficients were found for the three milk traits, and the additive effect was positive for MY (9.470; $P < 0.05$) and negative for FC (−0.133; $P < 0.05$) and PC (−0.108; $P < 0.10$), in accordance with genetic correlations estimated among these traits. This family showed a negative estimate (−0.133 for *GG* genotype) and therefore, an opposite additive effect compared with the other three families. In this family the *CC* genotype was associated with an increase in milk yield and a decrease in fat and protein content. In family 3, *ANXA9* and *SLC27A3* polymorphisms had the same phase; thus, the *CC* genotype was associated with an increase of milk yield and a decrease in fat and protein contents.

These results indicate that *ANXA9* and *SLC27A3* are probably not directly responsible for variability in milk traits but are linked with a QTL with some effect on these traits. The results provide evidence for the presence of a QTL close to *SLC27A3* that has an effect on milk traits. Although both *ANXA9* and *SCL27A3* are involved in human fatty acid metabolism, in our study no direct associations were found between polymorphisms in these genes and milk FC. However, in some families linkage between *SLC27A3* and one or more segregating QTL responsible for genetic variability in FC was detected. The moderate significance of some tests does not allow definitive conclusions. The putative QTL linked to *SLC27A3* and *ANXA9* in the present study further support several cattle QTLs related to milk yield (Heyen *et al.* 1999; Viitala *et al.* 2003) and FC (Heyen *et al.* 1999; Ashwell *et al.* 2004) on BTA3, a homologue of OAR1.

This is the first QTL described in dairy sheep using a candidate gene approach. More studies are necessary to confirm these results with an experimental design more appropriate for detecting genetic associations (selective genotyping), and using other functional candidate genes and ESTs located in this region.

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Supplementary Material

The following supplementary material is available online at http://www.blackwell-synergy.com:

Table S1. Primer sequences and GenBank accession information.