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Isolation, mapping and identification of SNPs for four genes *(ACP6, CGN, ANXA9, SLC27A3)* from a bovine QTL region on BTA3

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Abstract. On the basis of fine mapping of a quantitative trait loci region of BTA3 for milk fat content, an examination of the comparative map between cattle and human indicates that the annexin 9 protein gene *(ANXA9)* and the fatty acid transport protein type 3 gene *(SLC27A3)* are two strong candidate genes. The objective of the present study is to isolate, map and characterize these genes and identify polymorphisms that could be further utilized in linkage or association studies. Furthermore, two new genes which are in the same region, cingulin protein gene *(CGN)* and lysophosphatidic acid phosphatase protein gene *(ACP6)* were studied. DNA fragments (869, 1778, 1933 and 2618 bp) corresponding to partial sequences of *ACP6*, *CGN*, *ANXA9* and *SLC27A3* genes were isolated. Direct sequencing of PCR products amplified from different cattle breeds revealed 1, 4, 4 and 2 SNPs for *ACP6*, *CGN*, *ANXA9* and *SLC27A3*, respectively. For *ANXA9* one SNP was located in exon 5 ($A \rightarrow G$ 951) resulting in an amino acid change from histidine to arginine. Finally, *ACP6*, *CGN*, *ANXA9* and *SLC27A3* genes were located on chromosome 3 between *ILSTS096* and *BMS819* markers, in a region in which quantitative trait loci (QTL) for several milk traits have been described.

Quantitative trait loci for some milk traits have been reported on bovine chromosome 3 (BTA3) (Zhang et al., 1998; Heyen et al., 1999; Ashwell et al., 2001, 2004; Plante et al., 2001; Olsen et al., 2002; Boichard et al., 2003; Viitala et al., 2003) between microsatellite markers *INRA003* and *INRA006*. Subsequent examination of comparative map between cattle and human (http://bos.cvm.tamu.edu/bovgbase) indicates two candidate genes identified in human but unknown in cattle. These genes are fatty acid transporters and include annexin 9 protein *(ANXA9)* and fatty acid transport protein type 3 *(SLC27A3)*. The annexin 9 protein is a membrane transport channel and a member of the annexin family of Ca^{+2} and phospholipid-binding proteins, with a molecular mass of 37 kDa (Morgan and Fernandez, 1998). Fatty acid transport protein type 3 is a 78-kDa protein which facilitates the transport and acyl-CoA activation of long chain fatty acids (LCFAs) across the plasma membrane (Hirsch et al., 1998; Pei et al., 2004). Two other genes in the same re-gion, cingulin *(CGN)* and lysophosphatidic acid phosphatase *(ACP6)*,

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were also isolated and mapped. Cingulin is a 140-kDa protein localized on the cytoplasmic face of tight junctions (Citi et al., 1991). *ACP6* protein (80 kDa) is membrane bound, and hydrolyzes lysophosphatidic acid (LPA), which is a bioactive phospholipid, and plays an important role in phospholipid metabolism inside the cells (Hiroyama and Takenawa, 1999).

Materials and methods

Genomic walking and genetic polymorphism

Primers designed from human, mouse and rat genomic DNA were used to amplify partial genomic DNA sequences of *ACP6*, *CGN*, *ANXA9* and *SLC27A3* genes. Primers, annealing temperature, GenBank reference information and PCR product size are described in Table 1. Genomic DNA (50 ng) was amplified in a final volume of 25 μl containing 5 pmol of each primer, 200 μM dNTPs, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100 and 0.5 U Taq polymerase (Taq polymerase, Biotools). Standard PCR amplification cycles were used. The exons were identified in the cattle gene sequence according to homology with known sequences of rat, mice and humans.

Specific bovine primers were designed (Table 1) for direct sequencing of PCR products from ten animals (six Holstein and two each of the Pirenaica and Brown Swiss cattle breeds) using an ABIprism 3700 automatic sequencer (Applied Biosystem) and standard protocols. Holstein cattle corresponded to the highest Estimated Breeding Values (EBV) for milk fat content (three animals) and the lowest EBV (three animals) for milk fat content. Pirenaica and Brown Swiss animals were selected because of the low selection pressure on milk production and also alternative alleles could be fixed in these breeds.

Homology searches were performed with BLAST (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/BLAST/). Sequence analysis was performed using DNAsis (1991, Hitachi software engineering, Ltd.), CLUSTALW (http://www.ebi.ac.uk/clustalw/) and Geneviewer (http://www.itba.mi.cnr.it/webgene/) software. Repeat elements were identified using the RepeatMasker software (www.repeatmasker.org/).

Chromosomal location

PCR screening (Table 1: PCR 3, PCR 8, PCR 10, and PCR 15 for *ACP6*, *CGN*, *ANXA9* and *SLC27A3*, respectively) of a bovine BAC library (Eggen et al., 2001) was performed, and BAC clones containing the bovine *ACP6*, *CGN*, *ANXA9* and *SLC27A3* were identified. BAC clones of the bovine BAC library belong to contigs previously mapped (Schibler et al., 2004). This location was confirmed for *ACP6*, *ANXA9* and *CGN* by a bovine × hamster radiation hybrid panel analysis. PCR reactions were performed on the 94 radiation hybrid cell lines that constitute the developed 3,000 rad bovine panel (Williams et al., 2002). Primers and PCR conditions for radiation hybrid mapping were the same as those used for screening the BAC library. Carthagene software and two point analysis was used to assign the genes to cattle chromosomes (de Givry et al., 2005).

Results and discussion

Isolation and partial genomic structure of cattle ACP6, CGN, ANXA9 and SLC27A3 genes

Using human, mouse and rat genomic DNA sequences to design primers (Table 1), partial genomic DNA regions of 0.860, 1.742, 1.931 and 2.790 kb for *ACP6* (EMBL: AY85288), *CGN* (EMBL: AY85290), *ANXA9* (EMBL: AY85286) and *SLC27A3* (EMBL: AY996127) genes were obtained, respectively. The sequence data have been submitted to GenBank.

The cattle exons were identified based on comparisons to known sequences of rat, mice and humans. The 860-bp *ACP6* DNA fragment contained exons 9 through 10, and encoded 70 amino acids. The *CGN* DNA fragment spanned 1,742 bp, containing exons 19 through 21 and a partial 3′-UTR sequence. The three exons encoded 137 amino acids. The 1,931-bp *ANXA9* DNA fragment contained exons 3 through 7, encoding 140 amino acids, with the start codon in exon 3. Finally, the *SLC27A3* DNA fragment spanned 2,790 bp, and contained exons 3 through 10. These resulting eight exons encoded 319 amino acids. The exon sizes were similar to those of human. Homology searches were performed with BLAST (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/BLAST/), finding the maximum percentage of identity of the coding regions both for the DNA sequence as well as the predicted amino-acid sequence with ovine DNA sequences (AY85288, AY85290, AY85286, AY996127) (Table 2). The sequences were also compared to the emerging bovine genome sequences, finding between 99 and 100% identity of the coding regions with bovine *ACP6*, *CGN* and *SLC27A3* mRNA sequences (XM_593810, XM_613217 and XM_880607). No significant identities were found for intron sequences of the four genes and bovine *ANXA9*. Sequence analysis of the introns using RepeatMasker software (http://ftp.genome.wasington.edu/cgi-bin/RepeatMasker) revealed a repetitive element in intron 19 (SINE/MIR) of *CGN*, three repetitive elements in intron 3 (low complexity AT rich repetitive element), intron 4 (SINE/MIR) and intron 6 (three repeats of a SINE/BovA core repetitive element) of *ANXA9*, and one low complexity GA rich repetitive element in intron 4 of *SLC27A3*.

Detection of genetic polymorphisms

Direct sequencing of PCR products amplified from DNA samples of Holstein, Pirenaica and Brown Swiss cattle from all four cattle genes revealed some SNPs. One SNP in intron 9 was found for the bovine *ACP6* fragment. For the bovine *CGN* four SNPs were detected, two in intron 20, and two in the 3′-UTR. Bovine *ANXA9* revealed four SNPs. One SNP was in each of intron 3, intron 4, intron 5 and exon 5 (A→G 951) which resulted in an amino acid change from histidine to arginine. For the bovine *SLC27A3* two SNPs were detected in intron 7.

Chromosomal location of cattle ACP6, CGN, ANXA9 and SLC27A3

PCR screening of a bovine BAC library (Eggen et al., 2001) was performed, and BAC clones containing the bovine *ACP6* (0317H10 and 0974B07), *CGN* (0804E09, 0094E12 and 0500D05), *ANXA9* (0543C01 and 0875H07) and *SLC27A3* (0138F03 and 0290E08) were identified. *ACP6*, *CGN*, *ANXA9* and *SLC27A3* BAC clones belong to contigs previously mapped to BTA3 between *ILSTS096* and *BMS819* markers (Schibler et al., 2004) (Fig. 1). This location was confirmed for *ACP6*, *ANXA9* and *CGN* by a bovine × hamster radiation hybrid panel analysis. Using two point analysis in Carthagene software, the three genes were assigned to BTA3

Table 1. Primer sequences and GenBank reference information

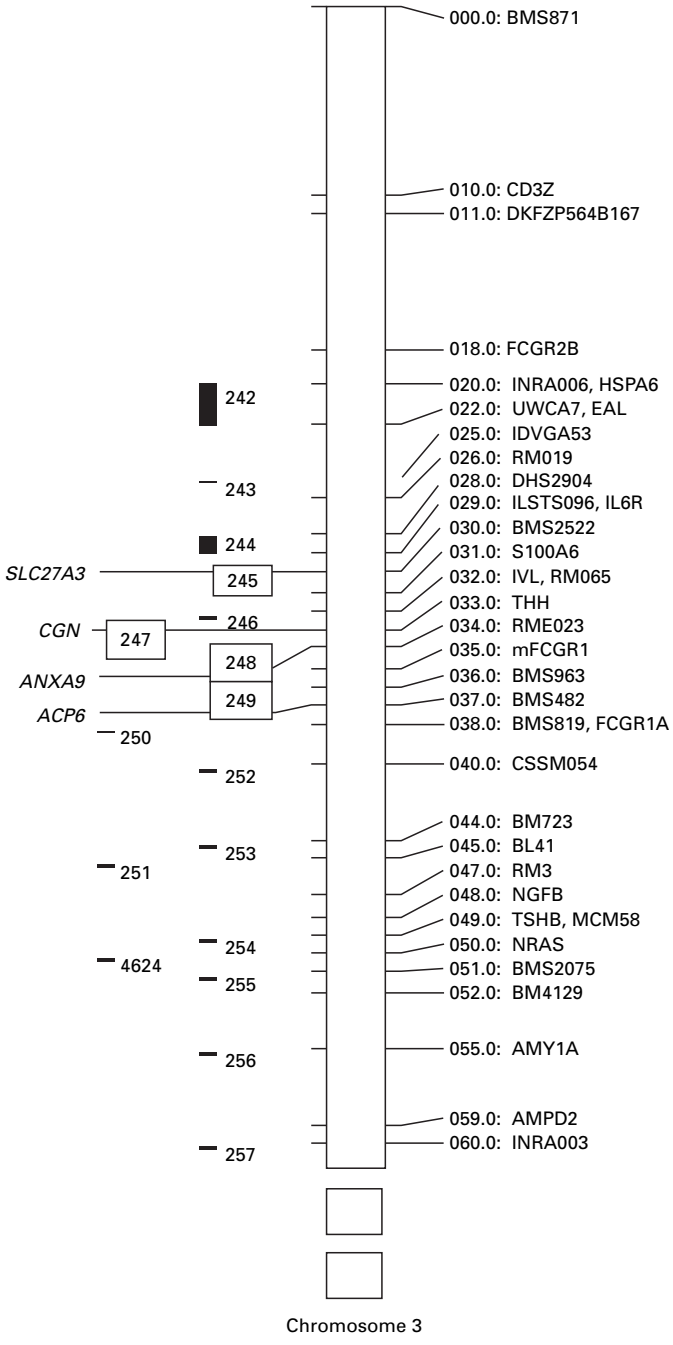
PCR primer pair	Primer sequence (5′→3′)	Gene/Site	GenBank	T_{ann}[a]	Size (kb)
1	AAAGMTGTAYCTCTATGC	*ACP6*/exon 9	NM_016361 (human)/XM_287273 (mice)	50 °C	
	GGTATTTTTCTGGGCTTA	*ACP6*/exon 10	NM_016361 (human)		0.909
2	TCATGCCTCTCTTAATGA	*ACP6*/exon 9	AY785289 (cattle)	52 °C	
	ACTGATATGGTGTTCAAG	*ACP6*/exon 10	AY785289 (cattle)		0.846
3	TCATGCCTCTCTTAATGA	*ACP6*/exon 9	AY785289 (cattle)	52 °C	
	GAGACGTAACTCAGCAGG	*ACP6*/intron 9	AY785289 (cattle)		0.771
4	GAGAAGACAGTTCTGCAGTC	*CGN*/exon 19	NT_004487 (human)	57 °C	
	GCTGACTCAGCAGCTGAGC	*CGN*/exon 21	NT_004487 (human)		0.969
5	TCCATTGCATCACTGCTTAC	*CGN*/exon 21	NT_004487 (human)	55 °C	
	GCTGGTCTTTGGAGAGGTA	*CGN*/3′UTR	NT_004487 (human)		0.766
6	CTGCAGTCTACCAACCGAA	*CGN*/exon 19	AY785291 (cattle)	54 °C	
	CCCTTGTGAACCACAAGCTA	*CGN*/exon 21	AY785291 (cattle)		1.083
7	ACTCAGGCGCAAAGCTTCC	*CGN*/exon21	AY785291 (cattle)	54 °C	
	GGCAAGAAGAGGCATCCC	*CGN*/3′UTR	AY785291 (cattle)		0.823
8	GAGAAGACAGTTCTGCAGT	*CGN*/exon 19	AY785291 (cattle)	54 °C	
	T GCTCTAGTT TCTCTGTCC	*CGN*/intron 20	AY785291 (cattle)		0.243
9	CCAGYWGCACCATGTCTG	*ANXA9*/exon 3	NT_037496 (human)/AF437742 (mice)	57 °C	
	CTGCCAGGCACTCCTGCAG	*ANXA9*/exon 7	NT_037496 (human)		1.968
10	GTGACCCACGGGAAGATGG	*ANXA9*/exon 3	AY785287 (cattle)	56 °C	
	GTACAGAGGCTGCTGAAGG	*ANXA9*/exon 4	AY785287 (cattle)		0.327
11	GAGCTTCAGTGCGGACAAGG	*ANXA9*/exon 4	AY785287 (cattle)	60 °C	
	GCATCGAGATGGGCTGCAGG	*ANXA9*/exon 6	AY785287 (cattle)		1.019
12	GTAGGACCTACTGAAGTCC	*ANXA9*/exon 6	AY785287 (cattle)	57 °C	
	CTGCCAGGCACTCCTGCAG	*ANXA9*/exon 7	NT_037496 (human)		0.739
13	GTACCACATGTCCGGCTC	*SLC27A3*/exon 3	AY996127 (sheep)	55 °C	
	GGCACMGTGACTCCATAG	*SLC27A3*/exon 8	NT_004487 (human)/NT_039234 (mice)		2.018
14	CCTTCTCCTTGATTCGRTA	*SLC27A3*/exon 6	NT_004487 (human)/NT_039234 (mice)	53 °C	
	CCAGGAGGGCGCTGTACCG	*SLC27A3*/exon 10	NT_004487 (human)		1.378
15	GTACCACATGTCCGGCTC	*SLC27A3*/exon 3	AY996127 (sheep)	52 °C	
	GATGTACTGGAACACTGTC	*SLC27A3*/exon 4	AY995157 (cattle)		0.710
16	GACGGTGTTCCAGTACATC	*SLC27A3*/exon 4	AY995157 (cattle)	56 °C	
	CGTTCACCTCCTGAAGAAAG	*SLC27A3*/exon 8	AY995157 (cattle)		1.308
17	CTTGGAGGCCCTGGACTTTC	*SLC27A3*/exon 8	AY995157 (cattle)	57 °C	
	ATGTAGAGCGGGTCGGACAGA	*SLC27A3*/exon 10	AY996127 (sheep)		0.677

[a] T_{ann}: annealing temperature.

Table 2. Identity percentage of DNA sequence and protein of bovine *ACP6*, *CGN*, *ANXA9* and *SLC27A3* genes

	Human DNA/protein GenBank Acc. N°	Mice DNA/protein GenBank Acc. N°	Rat DNA/protein GenBank Acc. N°	Sheep DNA/protein GenBank Acc. N°
Bovine *ACP6*	89/87–92[a]	88/82–94[a]	86/78–94[a]	96/91–95[a]
AY785289	NM_016361	NT_039238	XM_215655	AY785288
Bovine *CGN*	92/94–97[a]	89/94–97[a]	90/96–97[a]	97/98–99[a]
AY78785291	NT_004487	NT_039238	XM_227472	AY785290
Bovine *ANXA9*	85/84–89[a]	89/94–97[a]	87/85–90[a]	97/97–98[a]
AY785287	NT_037496	NT_039238	XM_227442	AY785286
Bovine *SLC27A3*	89/93–98[a]	86/88–94[a]	87/70–75[a]	96/98–99[a]
AY995157	NT_004487	NT_039238	XM_215605	AY996127

[a] Conservative amino-acid identity.



on the basis of a bovine marker typed previously on the panel at a lod score level of 3.0 (Table 3). The mapping results indicated that the most likely gene order is *SLC27A3–CGN–ANXA9–ACP6*. These assignments are consistent with comparative mapping information as human *ACP6*, *CGN*, *ANXA9* and *SLC27A3* map to HSA1, which is partly homologous to BTA3. In human and mice the gene order was *ACP6–ANXA9–CGN–SLC27A3*, over an interval of 5.17 Mb.

Quantitative trait loci (QTL) for some milk traits have been reported on the BTA3 region in which *ACP6*, *CGN*, *ANXA9* and *SLC27A3* genes have been located (Heyen et al., 1999; Olsen et al., 2002; Ashwell et al., 2004; Viitala et al., 2003). The BTA3 comparative region in human contains a limited number of known genes related to lipid and protein metabolism. Two of these candidate genes are *ANXA9* and *SLC27A3*, both of which are related to lipid transport. Therefore, *ANXA9* and *SLC27A3* could be studied as positional and functional candidate genes and tested in a QTL design for milk traits.

Table 3. Radiation hybrid mapping and two point analysis results for *ACP6*, *ANXA9* and *CGN* genes at a lod score level of 3.0

Gene	BTA	Markers	LOD score
ACP6	3	*BMS819*	12.2091629
	3	*CSSM054*	10.1719754
	3	*RM019*	4.3996576
	3	*IDVGA53*	3.9284131
	3	*RM065*	3.63649905
	3	*ILSTS096*	3.54809142
	3	*BMS2904*	3.19405763
CGN	3	*RM065*	11.6878766
	3	*RM019*	7.5069506
ANXA9	3	*RM065*	7.98938784
	3	*RM019*	4.81473798

Fig 1. Physical map of BTA3 showing the location of *ACP6*, *CGN*, *ANXA9* and *SLC27A3* genes and the contigs in which they have been located. The distances are in cM.

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