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Association between the polymorphism of the goat stearoyl-CoA desaturase 1 (*SCD1*) gene and milk fatty acid composition in Murciano-Granadina goats

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ABSTRACT

Genetic variability of the caprine stearoyl-CoA desaturase 1 (*SCD1*) gene has been investigated by sequencing a 4.7-kb cDNA in 6 goats from the Murciano-Granadina and Malagueña breeds. Sequence alignment revealed the existence of one synonymous polymorphism at exon 5 (c.732C > T) and one nucleotide substitution (c.*3504G > A) at exon 6 that encodes the 3′ untranslated region (UTR). Moreover, the existence of a previously reported 3′UTR polymorphism involving a 3-bp indel (c.*1902_1904delTGT) was confirmed. Single nucleotide polymorphism and haplotype-based association analyses revealed suggestive associations between genetic variability of the *SCD1* locus and lactose, stearic, polyunsaturated, and conjugated linoleic fatty acid contents. Associations with milk fatty acid composition might be explained by the global effects that *SCD1* exerts on mammary gland lipid metabolism through the down-modulation of key transcription factors. Interestingly, the performance of an in silico analysis revealed that the c.*1902_1904delTGT polymorphism involves a considerable change in the secondary structure of the *SCD1* mRNA. Gene reporter assays and quantitative PCR analysis would be needed to assess if this mutation has a causal effect on milk polyunsaturated and conjugated linoleic fatty acid levels by altering the amount of *SCD1* transcripts in mammary epithelial cells.
Key words: goat, stearoyl-CoA desaturase, milk fatty acid, lipid metabolism

INTRODUCTION

Milk fatty acid (**FA**) composition is an important trait for the goat dairy industry because of its influence on cheese yield and the organoleptic properties of dairy products (Chilliard et al., 2003). Goat milk is particularly rich in saturated fatty acids (**SFA**; 55% of total milk triacylglycerols), whereas monounsaturated (**MUFA**, 29%) and polyunsaturated (**PUFA**, 16%) FA are less abundant (Fontecha et al., 2000). Because dietary SFA are associated with the occurrence of coronary and atherosclerotic diseases in humans, increasing the unsaturated FA milk content would be highly beneficial.

Milk FA composition is affected by nutritional and genetic factors. For instance, supplementing a low forage diet with sunflower oil involves a substantial increase in the milk content of C18:1 and C18:2 isomers and a reduction in medium-chain SFA (Ollier et al., 2009). Lactation stage and energy balance (Chilliard et al., 2003), udder health (Jensen, 2002), and seasonal variations in the lipid content of pastures (Mel'uchová et al., 2008) are other factors that influence milk FA composition. From a genetic perspective, Arnould and Soyeurt (2009) have reported moderate heritability values for dairy cattle milk SFA ($h^2 = 0.36$), MUFA ($h^2 = 0.09$–0.17), and PUFA ($h^2 = 0.25$), indicating that there is a considerable amount of additive variance for these traits. Additionally, multiple QTL for milk FA composition phenotypes have been reported in cattle (Schennink et al., 2009; Stoop et al., 2009) and sheep (García Fernández et al., 2010a,b). Candidate gene studies have also shown the existence of significant associations between specific genotypes and milk FA content (Moioli et al., 2007). Taken together, these results suggest that milk FA composition can be modified through selection.

Consistent and significant associations have been found between the polymorphism of the bovine stearoyl-CoA desaturase 1 (***SCD1***) gene and milk FA composition traits (Taniguchi et al., 2004; Mele et al., 2007; Jiang et al., 2008; Schennink et al., 2008; Kgwatalala et al., 2009). The SCD1 enzyme catalyzes the introduction of a Δ^9-*cis* double bond in saturated fatty acyl CoA, a

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fundamental step in the synthesis of MUFA (Miyazaki and Ntambi, 2003). This enzyme also plays an essential role in the synthesis of milk CLA in ruminants (Khanal and Dhiman, 2004). In goats, the sequence and chromosomal location of the *SCD1* gene have been reported and a single TGT indel polymorphism has been identified in the 3′ untranslated region (**UTR**; Bernard et al., 2001; Yahyaoui et al., 2002). The main goal of the current work was to investigate if genetic variability at the goat *SCD1* gene is associated with milk FA content and other milk traits.

MATERIALS AND METHODS

Animal Material and Phenotype Recording

A total of 452 Murciano-Granadina goats were used to estimate the genotype frequencies of the *SCD1* gene. Murciano-Granadina is a black-coated dairy breed mainly distributed in southeast Spain (Gonzalo et al., 2002). Milk from Murciano-Granadina goats is mainly devoted to the manufacture of fresh, semi-aged, and aged cheese with appellation of origin (cheese of Murcia). Analyses were performed in 2 Murciano-Granadina groups of goats including 4 herds (Supplementary Figure 1; available online at http://www.journalofdairyscience.org/). All herds were under an official milk recording scheme and they were raised under a fully intensive management system. Kidding took place throughout the year with 2 peaks in fall (groups 1 and 2) and winter (group 1). Sampling procedures and traits recorded for group 1 (n = 133) have been reported in Badaoui et al. (2007). With regard to group 2 (n = 176), whole milk produced in the first of the 2 daily milkings was collected and measured approximately every 2 mo (65.5-d average sampling interval), starting around the third month after parturition (average 97.7 d). Milk samples were stored in iceboxes and transported to the laboratory, where protein, fat, lactose, and DM contents and SCC were measured with a CombiFoss 600FC instrument (comprising a MilkoScan FT 6000 for the analysis of milk components and a Fossomatic FC for SCC; Foss Electric, Hillerød, Denmark). A small sample (10 cm^3) of milk from each goat was freeze-dried, stored at –20°C (average 3 mo), and subsequently used for the gas chromatography analyses of FA.

Gas Chromatography Analysis

Separation and quantification of fatty acid methyl esters were carried out using a gas chromatograph Agilent 6890N Network GS System (Agilent, Santa Clara, CA), equipped with a flame-ionization detector and fitted with an HP-88 capillary column (100 m, 0.25 mm i.d., 0.2-μm film thickness). Nonanoic acid methyl ester (C9:0 ME) at 4 mg/mL was used as an internal standard. Extraction and direct methylation were performed in a single-step procedure based on the method published by Sukhija and Palmquist (1998). Individual FA were identified by comparing their retention times with those of an authenticated standard FA mix Supelco 37 (Sigma Chemical Co. Ltd., Poole, UK). Identification of the conjugated linoleic acid (**CLA**) isomers *cis*-9,*trans*-11, *cis*-11,*trans*-13, *trans*-10,*cis*-12, and *cis*-10,*cis*-12 CLA was achieved by comparing retention times with those of another authenticated standard mix (Sigma Chemical Co. Ltd.). Fatty acid content was expressed as the percentage of total methyl esters identified and grouped as follows: SFA, MUFA, and PUFA. Desaturase indices were calculated from specific FA to estimate SCD1 activity. These indices were calculated as a ratio of product/substrate (Lock and Garnsworthy, 2003). The following indices were calculated: C14 index, C16 index, C18 index, and CLA index.

Sequence Analysis and Genotyping of the Goat SCD1 Gene

Total RNA was extracted from liver tissue of Murciano-Granadina (n = 3) and Malagueña goats (n = 3) using the RiboPure Kit (Ambion Inc., Austin, TX), and complementary DNA was synthesized with the ThermoScript RT-PCR kit (Invitrogen S.A., Barcelona, Spain) according to Zidi et al. (2008). The coding sequence and part of 3′UTR (from 141 to 5012 bp, GenBank accession no. AF325499) of the goat *SCD1* gene was amplified by using 6 primer pairs. Amplification protocols (PCR1 to PCR6) are reported in Supplementary Table 1 (available online at http://www.journalofdairyscience.org/). Amplified products were sequenced in both directions. Sequencing reactions were purified by using the Montage SEQ_{96} cleanup kit (Millipore Corporation, Billerica, MA). Sequencing reactions were analyzed in an ABI PRISM 3730 capillary electrophoresis device (Applied Biosystems, Sant Andreu de Llavaneres, Spain).

Genomic DNA was extracted from blood samples of all 452 goats (Supplementary Figure 1) following Caravaca et al. (2009). Polymorphic regions were amplified as described in Supplementary Table 1 (PCR7 to PCR9). The PCR products were purified by using the ExoSAP-IT kit (Amersham Biosciences Europe GmbH, Barcelona, Spain). Genotyping was performed either with the SNaPshot ddNTP Primer Extension kit (Applied Biosystems) or with the Sequenom MassARRAY iPLEX platform at the Spanish National Genotyping Centre (CeGen, Santiago de Compostela, Spain). The extension primers used in the primer-extension geno-

typing protocol were 5′-GTG AAA CGT TTC AAA A-3′ (polymorphism c.732C > T), 5′-GCC GAA TGC TCA TGT-3′ (c.*1902_1904), and 5′-TTT TAT AAA GCA TCT TGG A-3′ (c.*3504G > A). The existence of Hardy-Weinberg equilibrium for the 3 polymorphisms was tested with the Hardy-Weinberg equilibrium online tool (http://www.oege.org/software/hwe-mr-calc.shtml; Rodríguez et al., 2009).

In Silico Analyses of mRNA Secondary Structure and 3'UTR Functional Motifs

An in silico search of functional sequence motifs and microRNA target sites in the 3′UTR of the goat *SCD1* gene was performed by using programs RNA Analyzer (Bengert and Dandekar, 2003; http://wb2x01.biozentrum.uni-wuerzburg.de), UTRscan (Pesole and Liuni, 1999; http://www.ba.itb.cnr.it/BIG/UTRScan), and TargetScan 5.1. (http://www.targetscan.org). Moreover, the effects of the 3 polymorphisms on mRNA secondary structure were analyzed. Fragments encompassing 50- to 80-bp sequences flanking each polymorphism were evaluated with RNAfold (Gruber et al., 2008) and Mfold (Zuker, 2003) under default parameters.

Statistical Analysis

Associations between *SCD1* genotypes and traits under study were analyzed with the SAS software (SAS 9.2, SAS Inst. Inc., Cary, NC) using a mixed model for repeated measurements (Littell et al., 1998). The number of goats with records for milk yield, somatic cell count logarithmic (logSCC) and milk protein, fat, lactose, and dry matter contents was 309 (133 goats from group 1 and 176 from group 2), whereas for milk FA composition, data from 176 goats (group 2) were employed (Supplementary Figure 1). Statistical analyses were performed taking into consideration single polymorphisms or haplotypes as well as fixed factors with significant effects on the traits under study. Haplotypes were inferred using the PHASE program v. 2.0 (Stephens et al., 2001). Haplotype substitution effects were exclusively calculated for the 2 most frequent haplotypes; that is, T/DelTGT/G and C/TGT/A. They were estimated considering the number of copies of haplotype T/DelTGT/G as a variable that can take values of 1 (goats homozygous for the T/DelTGT/G haplotype), 0 (heterozygous goats: T/DelTGT/G-C/TGT/A), and −1 (C/TGT/A homozygous goats). The model used for analyzing dairy traits (milk yield, and protein, fat, lactose, and DM contents) included as fixed factors *SCD1* genotype/haplotype, ordinal number of lactation (1, 2, 3, and >3), number of kids born (1, 2, and ≥3), month of lactation (1 to 9), herd (4 levels), season of sampling (4 levels), the random animal effect, and the residual error term. Log SCC was used as a covariate in the statistical model. The statistical model used to analyze milk FA composition was similar to the one described previously, but there were 2 main differences: 1) the herd effect was not included in the model (because all goats from group 2 belonged to a single herd) and 2) season of sampling had 3 levels: fall, winter, and spring (the number of summer records was very low so they were added to the spring class). LogSCC was also used as a covariate in the statistical analysis of milk FA data (Jensen, 2002). Bonferroni threshold of significance was computed as the ratio between the original significance level (e.g., 0.05) and the number of hypothesis tests. The Bonferroni thresholds of significance for FA composition and milk yield traits were 0.0013 and 0.0083, respectively. Results with *P*-values between the Bonferroni threshold and 0.01 have been considered as highly suggestive and those with *P*-values between 0.01 and 0.05 as suggestive.

RESULTS AND DISCUSSION

The variability of the goat *SCD1* gene was assessed by amplifying and sequencing 4.7 kb of cDNA. This sequence encompassed most of the coding region (95%) and the 3′UTR (96%) of the caprine *SCD1* gene. Alignment of 6 goat *SCD1* sequences revealed the existence of 3 polymorphisms: one synonymous c.732C > T polymorphism at exon 5, one TGT indel at the 3′UTR (c.*1902_1904delTGT, exon 6), that was previously reported by Bernard et al. (2001), and a c.*3504G > A substitution also located in the 3′UTR. Genotype and haplotype frequencies are shown in Table 1. These data demonstrate the existence of linkage between c.*1902_1904delTGT and c.*3504G > A polymorphisms. No significant departure from Hardy-Weinberg equilibrium was detected. PHASE analysis revealed the existence of 8 haplotypes, of which 2 are the majority ones with a global frequency of 0.91 (Table 1).

Phenotypic measurements obtained in the Murciano-Granadina resource population are presented in Supplementary Table 2 (all supplementary tables available online at http://www.journalofdairyscience.org/). The phenotypic means for dairy traits presented in the current work are within the range of what has been published so far in the Murciano-Granadina breed (Gonzalo et al., 2002; Fernández et al., 2005). Means of milk FA composition (Supplementary Table 2) were similar to those reported by Salama et al. (2005) in Murciano-Granadina dairy goats. However, several differences were observed. For instance, milk C6:0 content was 0.32% in the current experiment, whereas Salama et al. (2005) describe means of 1.12 to 1.45% for this

Table 1. Genotype and haplotype frequencies of the stearoyl-CoA desaturase 1 (*SCD1*) gene in Murciano-Granadina goats (n = 452)

Polymorphism	Genotype	Frequency
c.732C > T	TT	0.49
	CT	0.40
	CC	0.11
c.*1902_1904delTGT	DelTGT/DelTGT	0.55
	TGT/DelTGT	0.38
	TGT/TGT	0.07
c.*3504G > A	GG	0.55
	GA	0.38
	AA	0.07
Haplotype		
T/DelTGT/G		0.68
C/TGT/A		0.23
C/DelTGT/G		0.05
T/TGT/G		0.01
Remaining haplotypes[1]		0.03

[1]T/DelTGT/A + T/TGT/A + C/DelTGT/A + C/TGT/G.

trait, which are 4-fold greater than ours. These differences among studies might be due to environmental factors (e.g., nutrition) or for technical reasons.

Performance of association analyses between *SCD1* single nucleotide polymorphisms versus dairy traits revealed suggestive associations with lactose percentage (c.732C > T, $P = 0.02$; c.*1902_1904delTGT, $P = 0.009$; c.*3504G > A, $P = 0.02$) as shown in Supplementary Table 3. Conversely, no association ($P > 0.05$) was observed between goat *SCD1* haplotypes and milk traits (Supplementary Table 4). The biological basis of these associations is unclear because *SCD1* is not functionally related to lactose metabolism in the mammary gland. Thus, it is possible that the associations detected in this work are the result of linkage disequilibrium with other causative mutations yet to be found. In this regard, it is worth mentioning that Schennink et al. (2008) did not find any significant association between an A293V substitution in the bovine SCD1 enzyme and protein and fat percentages and yields or milk production in Dutch Holstein-Friesian cattle.

The caprine *SCD1* genotype showed suggestive associations on milk FA composition (Table 2; Supplementary Tables 5 to 7). These associations were consistently found in both the single nucleotide polymorphism and haplotype-based analyses. In the latter, haplotype T/DelTGT/G was suggestively associated with a lower milk stearic FA content and with higher percentages of PUFA and total CLA compared with haplotype C/TGT/A. Unexpectedly, none of the associations found involved MUFA content or desaturase indices (Supplementary Table 8). This negative result combined with the existence of associations with other FA is intriguing because the primary function of SCD1 is to synthesize MUFA. In fact, association analyses between bovine *SCD1* genotype and milk FA traits have demonstrated the existence of effects on C10:1, C12:1 (Schennink et al., 2008; Kgwatalala et al., 2009), and C14:1 (Mele et al., 2007; Schennink et al., 2008). However, Bionaz and Loor (2008) failed to find associations between mammary gland *SCD1* mRNA levels and desaturase indices, concluding that many other factors regulate milk FA output. These discrepancies among studies might be explained by the fact that *SCD1* mRNA expression varies throughout lactation, beginning to decay once the first 2 mo of milking have elapsed (Bionaz and Loor, 2008). This feature needs to be taken into account when evaluating our results because our sampling procedures began 3 mo after parturition.

Interestingly, associations between goat *SCD1* genetic variability and PUFA have been found (Table 2). Polyunsaturated fatty acids are essential FA not synthesized endogenously but acquired through the diet. Despite this feature, there is evidence that milk C18:2 and C18:3 contents are regulated not only by nutritional factors but also by genetic factors because heritabilities for milk PUFA content are low to moderate (C18:2, $h^2 = 0.11$ to 0.27; C18:3, $h^2 \approx 0.09$; Arnould and Soyeurt, 2009). To the best of our knowledge, genetic factors determining milk PUFA levels remain to be identified. Interestingly, Reh et al. (2004) engineered goats transgenic for the *SCD1* gene and observed that, among other changes, milk PUFA content was augmented. These findings suggest the existence of some type of interaction between expression levels of this desaturase and other genes, or biochemical pathways, influencing the uptake, esterification, or secretion of PUFA in the mammary gland. This interpretation agrees well with the multiple roles of SCD1 in the regulation of lipid and carbohydrate metabolism (Paton and Ntambi, 2009). In this way, microarray analysis of *SCD1* knockout mice fed a low-fat diet revealed that many genes involved in the hepatic biosynthesis, elongation, desaturation, oxidation, and transport of FA were differentially expressed compared with controls, an effect that is probably mediated through the down-modulation of key lipid metabolism transcription factors such as peroxisome proliferator-activated receptor-α, coactivator 1 of the peroxisome proliferator-activated receptor-γ, and sterol regulatory element-binding transcription factor 1 (Flowers et al., 2008). The highly suggestive association between *SCD1* genetic variability and milk *trans*-10,*cis*-12 CLA levels should also be understood in the light of the aforementioned interpretation, because this CLA isomer is exclusively produced by ruminal bacteria (Khanal and Dhiman, 2004). In contrast, suggestive associations with *cis*-9,*trans*-11 CLA (Table 2) can be understood more easily because this FA is mostly synthesized in

the mammary gland by SCD using *trans*-vaccenic acid as a substrate (Griinari et al., 2000). In goats, the high correlation between CLA and *trans*-vaccenic acid in milk fat suggests the predominance of mammary gland endogenous versus ruminal synthesis (Chilliard et al., 2003).

The 2 *SCD1* polymorphisms with suggestive associations are located in the 3′UTR, a region that plays a fundamental role in the posttranscriptional regulation of gene expression and usually contains sequence motifs influencing translation efficiency and mRNA stability (e.g., AU-rich elements, selenocysteine insertion sequence elements, microRNA target sites; Grzybowska et al., 2001). Similarly, Jiang et al. (2008) have reported significant associations between 2 polymorphisms located in the 3′UTR of the bovine *SCD1* gene and muscle CLA content. These authors argued that these polymorphisms might affect a functional motif. In the current work, an in silico search of functional elements and microRNA target sites in the 3′UTR of the goat *SCD1* gene has been made with the RNA Analyzer, UTRscan, and TargetScan 5.1 software. This analysis did not yield evidence suggesting that the c.*1902_1904delTGT and c.*3504G > A polymorphisms might involve the creation or suppression of either a functional sequence motif or a microRNA target site. However, local analyses of mRNA secondary structure with RNAfold allowed us to infer that the c.732C > T and the c.*1902_1904delTGT polymorphisms alter this parameter, producing a conformational change that, in the case of c.*1902_1904delTGT, has dramatic consequences (Figures 1A to 1D). In this way, RNAfold analysis showed that the TGT deletion promotes the formation of a long stacked pair terminated in 2 small hairpin loops (Figure 1C and 1D). The in silico predictions obtained with Mfold were quite similar to the ones calculated with RNAfold (data not shown). Changes in 3′UTR mRNA secondary structure might have important consequences at many stages of posttranscriptional regulation (Chen et al., 2006). For instance, a polymorphism located in the 3′UTR of the protein phosphatase-1 regulatory subunit 3 (*PPP1R3*) gene, which involves a 5-bp deletion plus 3 nucleotide substitutions, causes a severe reduction in the amount of *PPP1R3* mRNA because of an increased degradation rate (Xia et al., 1999). This alteration has been associated with an increased susceptibility to type 2 diabetes in Pima Indians (Xia et al., 1998). Moreover, a 1-bp deletion in the 3′UTR of the natriuretic peptide receptor A (*NPR1*) gene has been shown to produce a 3-fold decrease in the amount of *NPR1* transcripts possibly due to the loss of a binding site for a mRNA-stabilizing *trans*-acting factor (Knowles et al., 2003). In summary, it would be worth investigating if the

Table 2. Suggestive associations between stearoyl-CoA desaturase 1 (*SCD1*) genotypes (single-SNP and haplotype analyses) and milk fatty acid composition traits (LSM ± SE; number of individuals and records are shown in parentheses)

	c.*1902_1904delTGT				c.*3504G > A				Haplotype[1]	
Milk trait	DelTGT/DelTGT (89, 250)	DelTGT/TGT (76, 207)	TGT/TGT (11, 32)	*P*-value	GG (88, 247)	AG (74, 201)	AA (10, 26)	*P*-value	T/DelTGT/G vs. C/TGT/A[1]	*P*-value
C18:0	10.55 ± 0.21	10.72 ± 0.22	11.55 ± 0.44	0.08	10.57 ± 0.48	10.67 ± 0.22	11.51 ± 0.48	0.15	0.50 ± 0.18	0.005
C18:2n-6 *cis*	2.46 ± 0.04[a]	2.37 ± 0.05[ab]	2.24 ± 0.10[b]	0.05	2.47 ± 0.04[a]	2.36 ± 0.05[ab]	2.27 ± 0.11[b]	0.03	−0.08 ± 0.04	0.03
c-9,t-11 CLA[2]	0.42 ± 0.02	0.37 ± 0.02	0.36 ± 0.05	0.14	0.43 ± 0.02	0.37 ± 0.02	0.34 ± 0.04	0.06	−0.046 ± 0.02	0.03
t-10, c-12 CLA[3]	0.15 ± 0.008[a]	0.13 ± 0.009[a]	0.10 ± 0.01[b]	0.0024	0.15 ± 0.008[a]	0.13 ± 0.009[a]	0.09 ± 0.02[b]	0.0051	−0.03 ± 0.007	0.006
Other CLA[4]	0.14 ± 0.005	0.13 ± 0.005	0.12 ± 0.01	0.14	0.14 ± 0.005	0.13 ± 0.005	0.12 ± 0.01	0.21	−0.01 ± 0.004	0.02
PUFA[5]	4.08 ± 0.07[a]	3.95 ± 0.07[b]	3.71 ± 0.15[b]	0.03	4.10 ± 0.07[a]	3.93 ± 0.07[b]	3.73 ± 0.17[b]	0.02	−0.16 ± 0.06	0.01
Total CLA	0.71 ± 0.02[a]	0.64 ± 0.03[b]	0.58 ± 0.06[b]	0.01	0.71 ± 0.03[a]	0.63 ± 0.03[b]	0.55 ± 0.06[b]	0.0071	−0.08 ± 0.02	0.001

[a,b]Means within rows with different superscripts show either highly suggestive ($0.0013 < P < 0.01$) or suggestive ($0.01 < P < 0.05$) differences.

[1]Effect of replacing haplotype T/DelTGT/G by haplotype C/TGT/A (only the 2 most frequent haplotypes were compared).

[2]*cis*-9, *trans*-11 conjugated linoleic acid (CLA).

[3]*trans*-10, *cis*-12 CLA.

[4]Geometric and position isomers of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA.

[5]PUFA = polyunsaturated fatty acids.

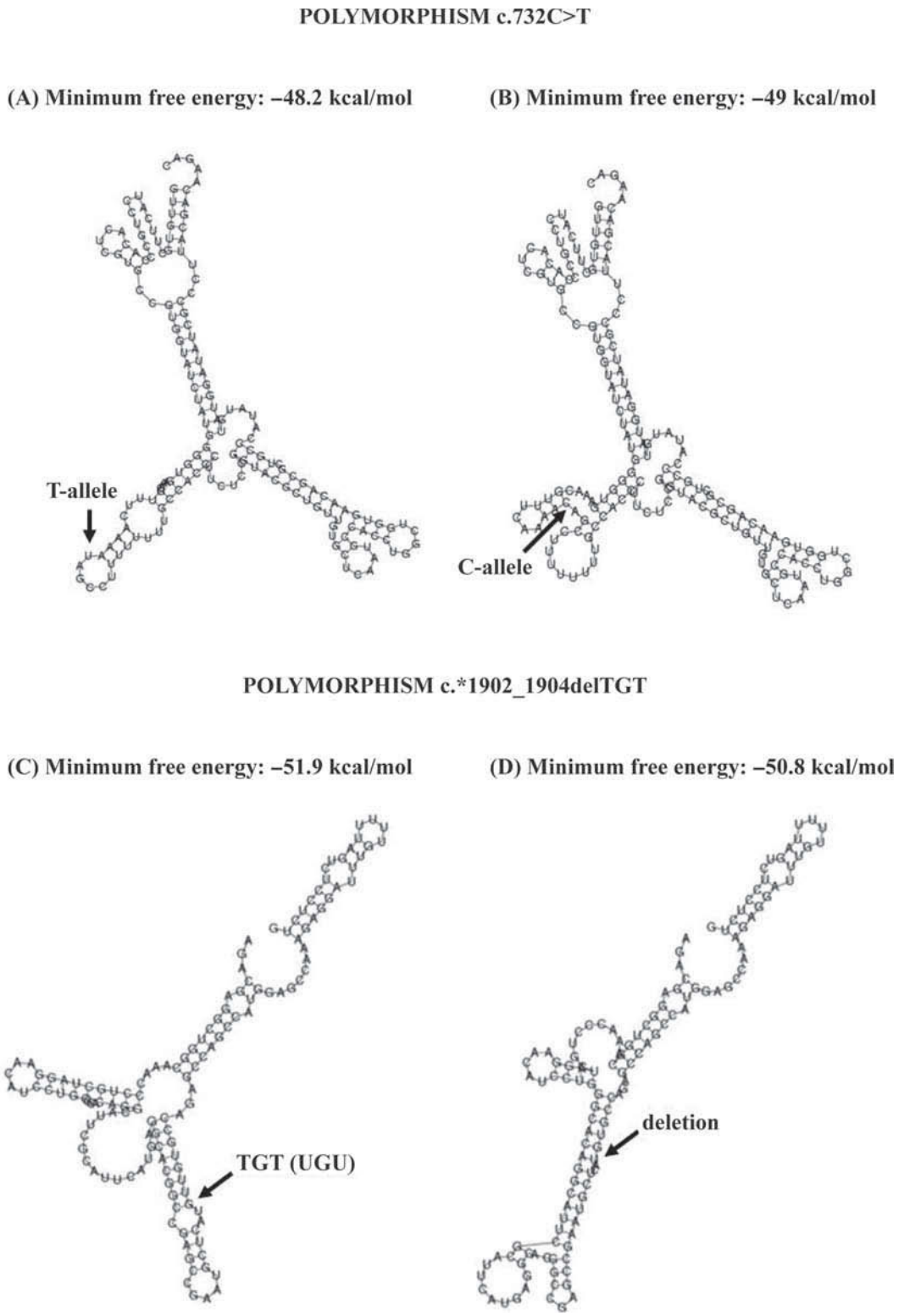

Figure 1. RNAfold (Gruber et al., 2008) prediction of the minimum free energy secondary structure of goat stearoyl-CoA desaturase 1 (*SCD1*) mRNA regions surrounding polymorphisms c.732C > T (A = T-allele, B = C-allele) and c.*1902_1904delTGT (C = TGT-allele, D = 3-bp deletion allele).

c.*1902_1904delTGT polymorphism influences the amount of goat *SCD1* mRNA through a change in the secondary structure of the 3′UTR. An experimental approach relying on the utilization of gene reporter assays and quantitative PCR would be needed to analyze this issue. From a human health perspective, the T/DelTGT/G haplotype would be advantageous because it is associated with an increased amount of milk total CLA ($P = 0.001$), which has been associated with reduced susceptibility to cancer and atherosclerosis in diverse clinical studies (Bhattacharya et al., 2006).

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