

Characterization of the CSN1A^G Allele of the Bovine α_{s1} -Casein Locus by the Insertion of a Relict of a Long Interspersed Element¹

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ABSTRACT

The bovine CSN1A^G allele is associated with a lower proportion of α_{s1} -casein in milk and is characterized by an insertion in the last noncoding exon (19th). According to DNA sequence analysis, the location, length, and origin of the insertion characterizing the bovine CSN1A^G allele have been identified. This insertion interrupts the 19th exon between nucleotides 58 and 59, is 371 bp long, and has a high level of homology with relicts of long interspersed elements of retropositional origin. Northern blot analysis shows that the inserted element is transcribed and that the number of transcripts of the CSN1A^G allele is less than one-half of the number of transcripts of the CSN1A^B allele. The lower amount of α_{s1} -casein in milk obtained from cows carrying the CSN1A^G allele can be explained by a reduced mRNA stability. Furthermore, a method has been developed using polymerase chain reaction for identification of the carriers of the CSN1A^G allele.

(**Key Words:** bovine α_{s1} -casein, CSN1A^G allele, long interspersed element)

Abbreviation key: LINE = long interspersed element, nt = nucleotide, PCR = polymerase chain reaction.

INTRODUCTION

The concentration of α_{s1} -CN in bovine milk is about 10 mg/ml (36% of total casein). Six electrophoretic variants of this protein are known: the CSN1A A variant lacks 13 amino acid residues (from position 14 to 26 inclusive) compared with CSN1A B variant (12); the two most common variants, CSN1A B and C, differ in position 192 (Glu vs. Gly) (28); CSN1A D has a phosphorylated threonine in place of the alanine residue of CSN1A B in position 53 (13); CSN1A E, which is probably specific to the yak (*Bos grunniens*), differs from CSN1A C, having a glutamine instead of a lysine residue in position 59 (11); CSN1A F has been recently identified (7) and has a leucine in place of the phosphorylated serine of CSN1A B in position 66 (31).

Several studies have demonstrated that different CSN1A variants are associated with variations in yield, composition, and processing quality of bovine milk. For example, CSN1A A slows acidification and produces soft curd (37), CSN1A C/C homozygotes produce significantly more α_{s1} -CN than do CSN1A B/B homozygotes (10), CSN1A B/B type is superior for milk yield over CSN1A A/B and B/C, and fat and protein contents are higher with the CSN1A B/C type milk (22, 26, 29, 30).

Southern blot analyses showed several restriction fragment length polymorphisms in the DNA region containing the bovine CSN1A locus (32, 44). One of the observed polymorphisms was determined by an insertion of about 400 bp in the DNA region of the CSN1A gene containing the last, noncoding exon (19th) (34, 35). Milk obtained from homozygous carriers of the insertion, compared with milk obtained

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from CSN1A B/B cows, is characterized by a very low concentration of α_{s1} -CN (about 4.5 mg/ml vs. 10 mg/ml, 16% vs. 36% of total casein). Heterozygotes have intermediate values (24, 25). This allele has been named CSN1A^G and synthesizes an α_{s1} -CN showing the same electrophoretic mobility as the CSN1A B variant (24, 34). According to informative genotypes, this allele is linked to the B allele of the κ -CN and the A² allele of the β -CN (24). Furthermore, the contents of calcium and phosphorus, as well as titratable acidity, are lower in the milk of heterozygous carriers of the CSN1A^G allele; in addition, milk from these cows contains about 6% less true protein and total casein and shows a casein number that is slightly lower than that observed in control cows (25). Effects of the CSN1A^G allele on size distribution of casein micelles in milk have also been analyzed. The freeze-fracturing technique, followed by transmission electron microscopy of milk obtained from a CSN1A G/G cow, shows that such milk, compared with milk obtained from a CSN1A B/B cow, has a higher proportion of submicelles, a lower percentage of micelles with a diameter in the range of 76 to 125 nm, and a higher mean diameter of isolated submicelles (38). Preliminary results indicate that milk obtained from CSN1A B/G cows has a rennet coagulation time and a curd-firming time that are lower than those of milk obtained from CSN1A B/B or B/C cows (23, 33). According to these results, the CSN1A^G allele is a major gene affecting several physicochemical and commercially valuable characteristics of bovine milk.

In this paper, we report the identification of the precise location, length, and origin of the insertion located in the 19th exon of the bovine CSN1A^G allele and discuss the possible causes of its reduced efficiency of synthesis. Furthermore, a fast and economical method, based on polymerase chain reaction (PCR), has been developed for identification of carriers of the CSN1A^G allele.

MATERIALS AND METHODS

DNA Samples

Genomic DNA was extracted from leukocytes (9) obtained from 260 Italian Brown, 155 Italian Friesian, 150 Modicana, 61 Podolian, 56 Jersey, 30 Age-rola, 23 Italian Red Pied, and 22 Reggiana individuals.

Primers Synthesis

Two 18-bp long primers were synthesized by using a 391 DNA Synthesizer (Applied Biosystems, Foster

City CA): AS1-1, forward primer, 5'-CATGCA-GCATAACTAACC-3' and AS1-2, reverse primer, 5'-TACTACTGCTGTTCTC-3'. Sequences of the two primers correspond to nucleotides (nt) 19213 to 19230 and to the complementary nt 19878 to 19895, respectively, of the sequence of the bovine α_{s1} -CN gene deposited at EMBL (accession no. X59856) (20).

PCR and Analysis of PCR Product

From 100 to 200 ng of genomic DNA were amplified in a total volume of 50 μ l using a Gene Amp PCR System 9600 (Perkin-Elmer Corp., Norwalk, CT). Each reaction contained 5 μ l of a 10-fold concentrated PCR buffer (Promega Corp., Madison, WI), 150 nmol of MgCl₂, 20 nmol of deoxynucleotide-triphosphate, 20 pmol of each primer, 10 μ g of bovine serum albumin, and 2.5 U of *Taq* DNA polymerase (Promega Corp.). Samples were amplified for 31 cycles set at the following conditions: denaturation at 97°C for 2 min, primer annealing at 54°C for 45 s, and primer extension at 72°C for 3 min, 1 cycle; denaturation at 94°C for 45 s, primer annealing at 54°C for 45 s, and primer extension at 72°C for 3 min plus 4 s of ramping for each cycle, 29 cycles; and a final extension cycle of 10 min at 72°C. Amplified fragments were analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide (27).

DNA Sequencing and Computer Analyses

Nucleotide sequencing of the amplified fragments was performed using the dideoxynucleotide chain-termination technique (40) with the *fmol*[®] DNA Sequencing System (Promega). Homology searches in EMBL and GenBank databases, RNA secondary structure analyses, comparisons among sequences, and multiple alignments were accomplished by means of MacDNASIS II software (Hitachi Software Engineering Co., San Bruno, CA).

Northern Blot Analysis

Total RNA was extracted from biopsies of mammary glands of three lactating cows with known genotypes (CSN1A B/B, B/G, and G/G) and at comparable lactation stages by using the guanidinium isothiocyanate method (3). Agarose gel electrophoresis was accomplished by using formaldehyde as the denaturing agent (39). The concentration of RNA in samples was spectrophotometrically determined be-

fore denaturation and loading. Transfer to nylon membranes (Hybond N; Amersham International plc, Buckinghamshire, England) was according to directions of the supplier. The bovine α_{s1} -CN cDNA probe C184 (43) (a kind gift from A. G. Mackinlay, University of New South Wales, Australia) was labeled with [α -³²P] dCTP (3,000 Ci/mmol) by means of nick translation. Hybridization conditions were as previously described (32).

RESULTS

In order to establish the precise location, length, and identity of the insertion, a DNA segment ranging from nt 19213 to 19895 of the bovine α_{s1} -CN gene sequence deposited at EMBL database (accession no. X59856) (20) was amplified by using as a template the genomic DNA that had been obtained from two CSN1A G/G cows. This segment contains the 19th exon (spanning nt 19255 to 19639).

The nt sequence of the amplified fragment from the two CSN1A G/G individuals (Figure 1) showed an insertion of 371 bp between nt 58 and 59 of the 19th exon. As a consequence, the 19th exon of the CSN1A^G allele is 756 bp long rather than 385 bp long, and the region amplified by the two primers is 1054 bp long. The inserted segment is characterized by flanking direct short repeats of 11 nt (CAAACCATTCT) because of the duplication of the CSN1A 11 nt sequence upstream or downstream of the insertion site and by a stretch of thymine residues, about 60 nt long, interrupted by some adenine residues. This structure is typical of relicts of long interspersed elements (LINE) of retropositional origin (42, 46). In fact, homology searches in the GenBank and EMBL databases show that the 360 nt characterizing the insertion share strong homology with five sequences corresponding to the 3' end of a LINE (Table 1). Figure 2 shows multiple alignment of the segment inserted

			intron 18		↓	exon 19	
catgcagcat	aactaaccac	atatttcttt	tttggtttac	agatggttct		gaaaattcca	
tgctctacat	gtcttttcat	ctatcatgtc	<u>aaaccattct</u>	TTTTTTTTTT		TTTTTTTTTT	
TTTTTTTTTT	TATTATTTT	TTAATTTTAT	TTTATTTTTA	AACTTTACAT		AATTGTATTA	
GTTTTGCCAA	ACATCAAAGT	GAATCCACGA	CAGGTATACA	TGTGTTCCCC		ATCCTGAACC	
CTCCTCCCTC	CTCCCTCCCC	ATACCATCCC	TCTGGGTCGT	CCCAATGCAC		TAGCCCCAAG	
CATCCAGTAT	CGTGCATCGA	AGGTGGACTG	GCAACTCGTT	TCTTACATGA		TATTTTACAT	
GTTACAATGT	CATTCTCCTA	AATCTTCCCA	CCCTCTCCCT	TCTCCCACAG		AGTCCATAAG	
ACTGTTCTAT	ACATCAGTGT	CTCTTTTGCT	GTCTCGTACA	<u>CAAACCATTCT</u>		<u>Tatccaaagg</u>	
cttcaactgc	tgtttttagaa	tagggcaatc	tcaaattgaa	ggcactcctt		cttcttgagt	
tctctactgt	atttttagata	gtgtaacatc	cttaagtgaa	attgtcctaa		cagcttgtta	
cctaaattcc	agtagtatca	tgctggtata	aaggccactg	agtcaaaggg		aattaaagtc	
ttcattaaat	ttctgtatgg	aaaatgtttt	aaaagccttt	gaatcacttc		tcctgtaagt	
gccatcatat	caaataattg	tgtgcattaa	ctgagatttt	gtctttcttc		ttttcaataa	
attacatttt	aaggcactat	tcctattttt	tgtcattatt	ccattggaag		gaatttacac	
aaccttggtga	gtttgtgtgt	atataacatt	ttgttttcac	taaattttta		tgacattttc	
aaccacattt	taatgaaaaa	attcaaagt	tcacttctag	ctgatcctgg		tagattataa	
actgagtcta	agatctttca	tttgaagtca	actgtttata	gaatattttc		catgtgaaca	
tggacgtggc	tgacagagaga	acagcagtgt	agta				

Figure 1. The nucleotide sequence of the polymerase chain reaction (PCR) product obtained by amplifying genomic DNA samples from two CSN1A G/G cows. The insertion is in capital letters; arrows indicate the first and the last nucleotide of the 19th exon; direct repeats at both sides of the insertion are underlined.

TABLE 1. The DNA sequences homologous to the 360 nucleotides (nt) characterizing the insertion in the 19th exon of the bovine CSN1AG allele.

Description of LINE ¹	Accession number	Reference	Homologous region	Similarity
First intron of the bovine lysozyme-1 gene	M95097	(16)	From nt 4178 to 4535 (complementary)	89.2% > 361 nt
Intron B of the bovine mitochondrial ATPase γ subunit gene	M22463	(6)	From nt 1749 to 2074	86.6% > 321 nt
Upstream (Lac1) of a bovine pseudo- α -LA gene	M96455	(45)	From nt 222 to 432	85.8% > 212 nt
Downstream (Lac2) of a bovine pseudo- α -LA gene	M96455	(45)	From nt 1809 to 2159	92.9% > 353 nt
19th Exon of the goat CSN1A ^E allele	X72221	(19)	From nt 850 to 1210	71.3% > 356 nt

¹Long interspersed element.

BT CSN1A G	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATTATTATTTTAAATTTTATTATTATTTT-A	60
BT α -LA Lac2	-----*****C**C**T**T**A*****A*****A**	
BT α -LA Lac1	*AT**ACT*A*****C**CA*****CT**T**GA*****T-	
BT Lys1	-**A**G**G**G**A**TA**T*****-*	
BT ATPase B	-----T**T*****-*	
CH CSN1A E	*****C**AC**GAA**GAC*****A**T*****ATT*****-*****T	
BT CSN1A G	AACTTTACATAATGTATAGTTTGGCCAAACATCAAAG-TGAATCCACGACAGGTATACA	120
BT α -LA Lac2	*****T*****A-*****G**C**C*****	
BT α -LA Lac1	*****AT*****G*****T**T**G**A-*****TG**C*****	
BT Lys1	*****AT*****G*****A**T**G**AA*****G**C*****C	
BT ATPase B	*****AT*****G*****T***-**AA*****TG**C*****	
CH CSN1A E	TTA**TTT**T**TA**TT**AA**TTT**T**T**T**T**AATC**T**TCT**	
BT CSN1A G	TGTGTTCCTCCCTGAACCTCCTCCCTCCTCCCTCCCATACCATCCCTCTGGGTCGTC	181
BT α -LA Lac2	*****G*****	
BT α -LA Lac1	*****G*****_*****A**	
BT Lys1	***C*****C*****	
BT ATPase B	*****C*****T*****T	
CH CSN1A E	**C*****A**A*****C*****A*****C**A*****T*****A**	
BT CSN1A G	CCAATGCACCTAGCCCAAGCATCCAGTATCGTGCATCGAAGGTGGACTGGCAACTCGTTTC	242
BT α -LA Lac2	*****CC*****T*****	
BT α -LA Lac1	***T*****C**T*****	
BT Lys1	***G*****C*****CC*****G**T*****	
BT ATPase B	-**G*****C*****T**CC-*****G*****	
CH CSN1A E	**C*****A**C*****AG**T**C**C**A**G**AG**CA*****G**T**AA**	
BT CSN1A G	TTACATGATATTTACATGTTACAAT-GTCATTCTCCTAAATCTTCCCACCTCTCCCTTC	302
BT α -LA Lac2	A*****C**T***_*C*****C*****_*	
BT Lys1	A*****G**A*****T***_*CA*****C*****C*****_*	
BT ATPase B	A*****A*****T***_*C**C**C*****C*****_*G*****_*	
CH CSN1A E	*****C**G**A*****T**TCC*****C*****A*****_*	
BT CSN1A G	TCCCACAGAGTCCATAAGACTGTTCTATACATCAGTGTCTCTTTGCTGTCTCGTACA	360
BT α -LA Lac2	*****T*	
BT Lys1	*****A-*****C*****TA**G*	
BT ATPase B	*****C*****CGTATA**G**TAT**G**A**CA**CTTCTA*	
CH CSN1A E	****T**T*****A**T**GTA**C*****T*****T**CC*****T**C**T*	

Figure 2. Multiple sequence alignment of the DNA segment inserted in the 19th exon of the bovine CSN1AG allele (BT CSN1A G) with long interspersed elements (LINE) in the following locations: upstream and downstream of a bovine pseudo α -LA gene (BT α -LA, Lac1, and Lac2, respectively), in the first intron of the bovine lysozyme-1 gene (BT Lys 1), in intron B of the bovine mitochondrial ATPase γ subunit gene (BT ATPase B), and in the 19th exon of the goat CSN1A^E allele (CH CSN1A E). Asterisks and dashes indicate nucleotide identities and deletions, respectively.

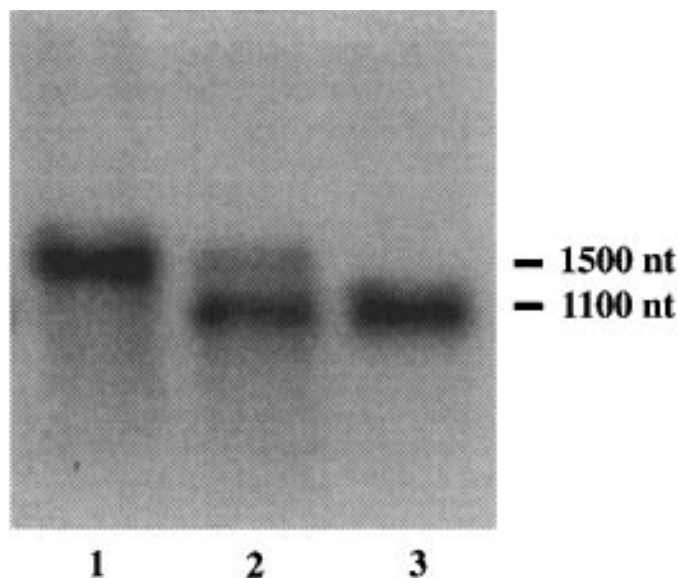


Figure 3. Northern blot analysis of RNA samples extracted from mammary glands of three lactating cows: 1) 10 μ g, genotype CSN1A G/G; 2) 4 μ g, genotype CSN1A B/G; and 3) 4 μ g, genotype CSN1A B/B. nt = Nucleotide.

in the 19th exon of the bovine CSN1A^G allele with these sequences.

Northern blot analysis of RNA extracted from mammary glands of three lactating cows (genotypes: CSN1A B/B, B/G, and G/G) shows that the inserted LINE is transcribed because α_{s1} -CN mRNA from the CSN1A G/G cow shows an increase in length (about 400 nt) that corresponds to the insertion size (Figure 3). Also, the number of transcripts of the CSN1A^G allele is about 40% that of the CSN1A^B allele because 10 μ g of RNA from the CSN1A G/G cow are necessary to obtain a band intensity corresponding to 4 μ g of RNA from the CSN1A B/B cow (Figure 3). As a consequence, the lower amount of α_{s1} -CN in milk obtained from cows carrying the CSN1A^G allele can be explained by a lower transcription rate, by a reduced mRNA stability, or both.

Computer analysis of the possible secondary structure of the 3'-untranslated region of the mRNA of the CSN1A^G allele after addition of 60 adenine residues (considered as the mean size of the poly (A) tail of cytoplasmic mRNA) (1) shows a highly stable secondary structure that has a much shorter poly (A) tail and more than twice the amount of free energy as the 3'-untranslated region of the CSN1A^B allele (Figure 4).

The amplification of samples of bovine DNA with the two primers allows a fast and economical identification of carriers of the insertion (Figure 5). In fact,

PCR typing of DNA samples of 187 cows with known phenotypes demonstrated that, as expected, CSN1A B/B, B/C, and C/C individuals (140 cows) show a fragment of 683 bp; samples of CSN1A G/G individuals (4 cows) show a fragment of 1054 bp. Samples from heterozygous carriers of the CSN1A^G allele (43 cows) show both fragments.

Population analysis indicated that the CSN1A^G allele is absent in 155 Italian Friesian, 23 Italian Red Pied, 56 Jersey, and 22 Reggiana individuals. The frequency of the CSN1A^G allele was 0.125 in 260 Italian Brown (3 homozygotes and 50 heterozygotes), 0.017 in 150 Modicana (5 heterozygotes), 0.033 in 61 Podolian (1 homozygote and 2 heterozygotes), and 0.033 in 30 Agerola individuals (2 heterozygotes). Furthermore, all carriers of the insertion were also genotyped (not shown) for the Glu vs. Gly (i.e., B vs. C electrophoretic variant) mutation in codon 192 of the α_{s1} -CN gene by means of allele-specific PCR (4). Informative genotypes show that insertion is always linked to the Glu codon in position 192. This result confirms that the insertion characterizing the CSN1A^G allele occurred in a gene coding for the CSN1A B electrophoretic variant.

DISCUSSION

The LINE have been found in the genome of several mammalian species (5). Apparently LINE are randomly distributed in the genome as a result of RNA-mediated movement of genetic material from one chromosomal location to another (retroposition) (21, 46), which usually generates short (7 to 21 bp) direct flanking genomic repeats. A full-length LINE (6 to 7 kb) contains a 5'-untranslated region, two open reading frames (ORF-1 and ORF-2), a 3'-untranslated region, a poly (A) tail, and no intron sequences (15). However, because of the failure of reverse transcriptase to synthesize a complete cDNA first strand or to rearrangement phenomena, most of them are truncated at the 5' end (8). The LINE sequence found in the bovine CSN1A^G allele is flanked by two genomic direct repeats and contains only a 3'-untranslated region, some possible polyadenylation signals, and a poly (A) tail [actually, a poly (T) according to the direction of transcription of the CSN1A gene].

The bovine CSN1A^G allele is the second example of insertion of a genetic mobile element that has had dramatic effects on the expression of a casein gene and the characteristics of milk in ruminants. In fact, the caprine CSN1A^E allele is also characterized by an insertion in the last noncoding exon (19th) and is

associated with both a reduction (to about 40%) of the amount of α_{s1} -CN and strong variations of the physicochemical and commercially valuable properties of milk [for a review, see (14)]. Both insertions are LINE relicts of retropositional origin, show a similarity of 71.3%, and are in the same orientation according to the direction of transcription of the CSN1A gene. Although homology between the two inserted segments is high, some differences exist. In the bovine CSN1A^G allele, the insertion is 371 bp long, it contains a 3'-untranslated region and a poly (A) tail of a retroposon, it is located between nt 58

and 59 of the 19th exon, and it is flanked by two short direct repeats of 11 bp. In the caprine CSN1A^E allele, the insertion is longer (457 bp); it contains a longer poly (A), a 3'-untranslated region and the codons of the 20 C-terminal residues of the ORF-2 protein of a retroposon; it is located between nt 124 and 125 of the 19th exon; and it is not flanked by repeated sequences. Based on these differences, the two insertions are probably the result of two independent mutational events.

In general, insertions or deletions inside coding regions lead to the loss of gene function, but inser-

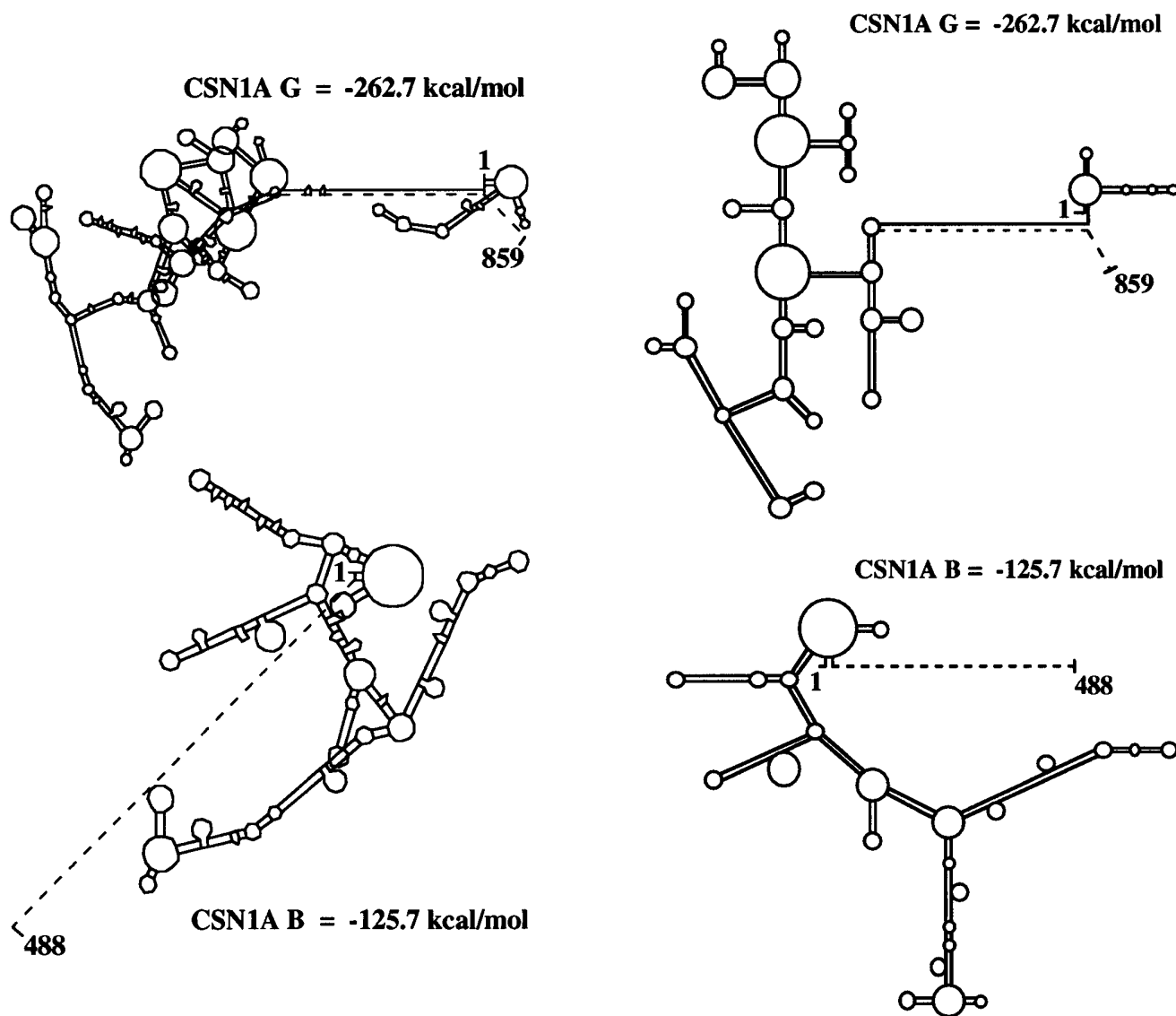


Figure 4. Computer-predicted secondary structure and total energy (expressed in kilocalories per mole) of the 3'-untranslated region of bovine CSN1A^G (up) and CSN1A^B (down) mRNA. The 60-nucleotide long poly (A) tail is indicated by a dashed line.

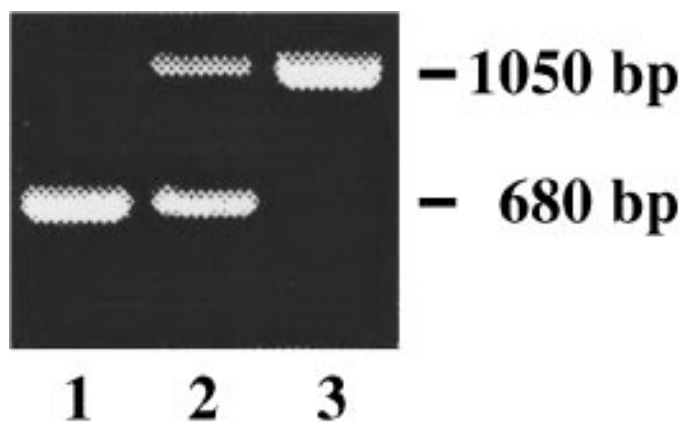


Figure 5. Polymerase chain reaction (PCR) amplifications of genomic DNA samples from cows with the following genotype: 1) CSN1A B/C, 2) CSN1A B/G, and 3) CSN1A G/G. In lane 2, competition in amplification efficiency between the long and the short fragment can be observed.

tions or deletions inside noncoding regions can modify the mechanisms that regulate gene expression, resulting in quantitative variations in gene product (15). In particular, possible explanations for the reduced expression of the caprine CSN1A^E allele have been detailed by Jansa Perez et al. (19) and could apply as well to the bovine CSN1A^G allele. That is, transcription products of these two alleles show sequences rich in A + U in the 3'-untranslated region, which can reduce the stability of transcripts (2, 17). Furthermore, the presence of the 5'-AUUUA motif, which has been found in several unstable mRNA (41), could contribute to a reduced stability of transcripts. Finally, the poly (U) in the 3'-untranslated region, together with the poly (A) tail of the messenger RNA, creates a highly stable terminal structure (19) that could interfere with the modulation of translation efficiency accomplished by the 3'-untranslated region and the poly (A) tail (18). Such a structure, which is similar to a poly (A)⁻mRNA, is responsible for fast degradation of the mRNA (36, 47).

At present, data on the transcription efficiency of caprine CSN1A^E and bovine CSN1A^G genes are not available. Therefore, other mutations could be responsible for a lower transcription rate in both genes. However, this possibility seems unlikely because 1) two independent mutational events (insertions) are associated with the same phenotypic effect (an identical reduction of α_{s1} -CN in milk) in two different species. As a consequence, the insertions themselves or features common to both insertions are likely

responsible for such an effect; 2) except for the insertion, there is no evidence for additional mutational events at the origin of the bovine CSN1A^G allele. In fact, the haplotype (seven markers spread from the 5' to the 3' end of the gene) characterizing the DNA region containing the CSN1A^G allele is identical, except for the insertion, to the most frequent haplotype characterizing the DNA region containing the CSN1A^B allele in six breeds (34).

In conclusion, retroposons, together with other repetitive sequences found throughout the genome of eukaryotes, could represent an important source of variability and serve as tools in understanding the nature of at least part of the genetic variation observed in animal populations. It might be fruitful to search for the polymorphisms caused by insertion or deletion of these elements in order to find genes with important major effects on economic traits of animal populations.

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