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Short communication: Translational efficiency of casein transcripts in Mediterranean river buffalo

G. Cosenza,*[1] A. Pauciullo,* A. Coletta,† A. Di Francia,* M. Feligini,‡ D. Gallo,* D. Di Berardino,* and L. Ramunno*

*Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, Università degli Studi di Napoli "Federico II", 81100, Portici, Italy
†Associazione Nazionale Allevatori Specie Bufalina (ANASB), 81100, Caserta, Italy
‡Istituto Sperimentale Italiano Lazzaro Spallanzani, 26900, Lodi, Italy

ABSTRACT

Buffalo milk is characterized by the presence of all 4 casein fractions (α_{S1}, β, α_{S2}, and κ) encoded by the 4 tightly linked autosomal genes (*CSN1S1*, *CSN2*, *CSN1S2*, and *CSN3*, respectively). In the present paper, we report for the first time a quantitative characterization of buffalo casein transcripts and show that the 4 genes are not transcribed and translated with the same efficiency. In particular, the analysis of individual milk samples obtained from 9 Mediterranean river buffaloes showed that the most abundant casein fractions were β (53.45%) and α_{S1} (20.61%), followed by α_{S2} and κ, at 14.28 and 11.66%, respectively. Quantification of the corresponding mRNA showed that the percentage of transcripts of the 4 caseins was 16.48, 23.18, 55.87, and 4.47% for α_{S1}, β, α_{S2}, and κ, respectively. Translation efficiency was 0.25 for *CSN1S2*, 1.31 for *CSN1S1*, 2.39 for *CSN2*, and 2.69 for the *CSN3* transcripts, respectively. A comparison of nucleotide sequences with the Kozak consensus sequence was also carried out to investigate if the mRNA sequences might be responsible for the observed differences.

Key words: Mediterranean river buffalo, casein, mRNA, quantification

Short Communication

Buffalo milk is characterized by the presence of all 4 casein fractions (α_{S1}, β, α_{S2}, and κ) encoded by 4 tightly linked autosomal genes (*CSN1S1*, *CSN2*, *CSN1S2*, and *CSN3*, respectively) mapped on chromosome 7 (Iannuzzi et al., 2003). Today, the complete amino acid sequences of buffalo casein (D'Ambrosio et al., 2008) are available, as well as the complete sequence and the relative regulatory regions of genes encoding β- (Cosenza et al., 2009a) and κ-casein (Masina et al., 2007), the 5′ untranslated region (**UTR**), exon 1, and partial cDNA of the *CSN1S1* gene (EMBL no. GU593719, AF529305, AY948385, and AJ005430), and the sequences related to the cDNA as well as short intronic sequences of the *CSN1S2* gene (Cosenza et al., 2009b).

Recently, Feligini et al. (2009) developed a method for the quantification of α_{S1}-, β-, α_{S2}-, and κ-caseins in water buffalo milk using reverse-phase HPLC. Unlike what has been accomplished for cattle, sheep, and goat (Bevilacqua et al., 2006), no research has been carried out on the expression of casein genes in the buffalo species as well as on their translational efficiency.

The aim of this study was therefore to assess the translation efficiency of the casein-encoding genes in Mediterranean river buffalo through analysis of protein abundance and mRNA gene levels. For this purpose, individual raw milk samples from 9 Mediterranean river buffaloes at comparable age, in third calving, at 120 d in milking, belonging to the same farm located in province of Salerno (Italy), and free of clinical mastitis were collected together with the monthly SCC controls by the local breeder association (ANASB, Caserta, Italy). After collection, samples were immediately frozen and kept at −20°C until analysis to prevent any proteolytic reaction induced by possible high SCC.

Total RNA was extracted from somatic cells (SCC range from 10,000 to 12,000/mL) present in the fresh milk by using Nucleospin Blood and NucleoSpin Extract kits (Macherey-Nagel, Düren, Germany). The quantity, quality, purity, and integrity of RNA after DNase treatment were estimated by means of NanoDrop 2000c spectrophotometer (Thermo Scientific, Barrington, IL) and by electrophoresis on a denaturing agarose gel. The reverse transcription was performed using Improm-II Reverse Transcriptase (Promega, Madison, WI). By means of real-time quantitative PCR, the *CSN1S1*, *CSN2*, *CSN1S2*, and *CSN3* mRNA were quantified using standard curves, and the amount of each transcript occurring within each sample was expressed as relative to the amount of transcript measured for the single

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[1]Corresponding author: giacosen@unina.it

Table 1. Oligonucleotide primers sequence and positions

Gene	Position, nucleotides	Primer sequence (5′ to 3′)[1]		EMBL accession no.	Amplicon size, bp
CSN1S1	867–886	Forward	TCTTCTTGAGTTCTCTACTG	AY948385	114
	Complementary to 963–980	Reverse	ACTCAGTGGCCTTTATAC		
CSN2	126–144	Forward	AAGCCTTTCAAGCAGTGAG	FM946182	68
	Complementary to 174–193	Reverse	CCTCACTCTGAAACTTCTCA		
CSN1S2	574–591	Forward	AAAATCAGCCAGCATTAC	FM865618	111
	Complementary to 666–684	Reverse	GGGAATAACGTTTGTCTTA		
CSN3	14100–14117	Forward	TCAGTGAACAGAGAATAT	AM900443	106
	Complementary to 14189–14205	Reverse	GCTTTATTATGCAGGAA		
18S rRNA	1337–1352	Forward	CGTTCTTAGTTGGTGG	NR_036642	76
	Complementary to 1396–1412	Reverse	GTAACTAGTTAGCATGC		

[1]Primers were designed by means of Oligo 5.0 software (National Biosciences Inc., Plymouth, MN).

internal control (*18S rRNA*) used for normalization. Primer sequences for *CSN1S1*, *CSN2*, *CSN1S2*, *CSN3*, and *18S rRNA* amplification are reported in Table 1.

The results show that the transcript percentages were 16.48 (SD 4.99), 23.18 (SD 5.41), 55.87 (SD 8.22), and 4.47% (SD 0.96) for α_{S1}, β, α_{S2}, and κ, respectively. These values are significantly different from those characterizing the transcripts of the same genes in cattle, sheep, and goats. In fact, for these species, each casein transcript represents nearly 25% of the whole casein transcript population (Bevilacqua et al., 2006). Although the starting material is different (milk somatic cells in the present work and mammary gland cells in Bevilacqua et al., 2006), the results obtained by different authors so far are comparable. In fact, different studies on goat and beef cows showed that the relative amount of milk protein mRNA in milk somatic cells and mammary tissue samples is highly correlated (Boutinaud et al., 2002; Murrieta et al., 2006).

The quantitative determination of the 4 casein fractions of the 9 individual buffalo milk samples was carried out according to the method of Feligini et al. (2009). In the examined samples, β- (19.81 g/L, SD 5.14) and α_{S1}-casein (7.62 g/L, SD 2.52) were the major caseins, accounting for 53.45% (SD 6.63) and 20.61% (SD 4.29) of the whole casein fraction, respectively. On the contrary, α_{S2}- (4.99 g/L, SD 0.92) and κ-casein (4.25 g/L, SD 1.05) were less abundant, at 14.28% (SD 4.88) and 11.66% (SD 2.26), respectively.

These results are in agreement with those found for buffalo by Feligini et al. (2009), but quite different from those obtained for cattle, sheep, and goat, which show a percentage distribution of β- and α_{S1}-casein fractions of about 38% each (Bevilacqua et al., 2006). The observed differences in the percentage distributions of the 4 casein fractions in buffalo milk and, particularly, of the β-casein fraction, could account for its peculiar technological properties.

The ratio between the percentage of single milk protein fractions and the percentage of transcripts produced in the mammary gland was estimated in order to evaluate the translation efficiency of the buffalo gene casein transcripts. The values obtained show low translation efficiency (0.25, SD 0.07) for the *CSN1S2* transcripts, whereas the efficiency was higher for the *CSN3*, *CSN2*, and *CSN1S1* transcripts: 2.69 (SD 0.74), 2.39 (SD 0.49), and 1.31 (SD 0.30), respectively. These results disagree from those obtained by Bevilacqua et al. (2006) in cattle, goat, and sheep, in which the *CSN2* and *CSN1S1* mRNA showed higher translational efficiency.

A comparison of nucleotide sequences with the Kozak consensus sequence was accomplished to investigate if the mRNA sequences might be responsible for the observed differences. The context of the translation initiation codon (AUG) plays an important role in determining the translation rate (Kozak, 1991a,b). The Kozak consensus sequence occurs in eukaryotic mRNA and has the sequence GCCGCCRCCAUGG, where R is a purine (adenine or guanine) 3 bases upstream of the start codon (AUG), which is followed by another G (Kozak, 1987). The A nucleotide of the AUG codon is referred to as number 1 and, although nucleotides −6, −3, and +4 are the most conserved positions in natural mRNA sequences, it seems likely that other nearby nucleotides also contribute to the translation process (Kozak, 1984; De Angioletti et al., 2004). In general, the more the sequence around the initiation codon is homologous to the Kozak sequence (i.e., "strong" consensus), the higher the efficiency of mRNA translation (Kozak, 1984).

The comparison of the sequence of the 4 transcripts in river buffalo with the Kozak sequence showed that *CSN1S1* had 4 positions out of 6 conserved in the GCCRCC consensus motif, whereas *CSN2*, *CSN3*, and *CSN1S2* had 3 homologous nucleotides (Table 2). In particular, 3 residues directly upstream of the initiation codon were consecutive in *CSN1S1* and *CSN2* (−3, −2, and −1) and 2 in *CSN3* (−3 and −2). On the contrary, no conserved nucleotides were consecutive in *CSN1S2*

Table 2. Comparison of start codon flanking sequences of the 4 casein transcripts in the Mediterranean river buffalo

	Position[2]									
Sequence[1]	−6	−5	−4	−3	−2	−1	+1	+2	+3	+4
Kozak consensus sequence	G	C	C	R	C	C	A	U	G	G
CSN2	A	G	A	**G**	**C**	**C**	**A**	**U**	**G**	A
CSN1S2	**G**	U	A	**A**	A	**C**	**A**	**U**	**G**	A
CSN1S1	A	**C**	A	**A**	**C**	**C**	**A**	**U**	**G**	A
CSN3	**G**	G	U	**A**	**C**	A	**A**	**U**	**G**	A

[1]Kozak consensus sequence = the optimal context for initiation of translation in mammals. *CSN2*, *CSN1S2*, *CSN1S1*, and *CSN3* are the genes encoding β-, α_{S2}-, α_{S1}-, and κ-casein, respectively.

[2]The start codon (AUG) in the 4 casein transcripts is underlined; conserved nucleotides are shown in boldface.

(−6, −3, and −1; Table 2), which could account for the lower translational efficiency recorded for the α_{S2}-casein transcripts.

Concerning the higher translation efficiency of the κ-casein transcript compared with that observed by Bevilacqua et al. (2006) for cattle, sheep, and goat, an explanation could be found through comparison of the homologous messenger sequence among these species. The *CSN3* mRNA in buffalo, cattle, sheep, goat, mouse, rabbit, and pig have 2 consecutive AUG and, with the exception of buffalo, in all these species, the first start codon shows a guanine in position −3 (Table 3).

It was proven (Kozak, 1984) that a start codon flanked by A in position −3 compared with G works considerably better; that is, has higher translational activity. Therefore, the initiation sites may also be designated as "strong" or "weak" based on this position. Ribosomes will initiate at the first AUG codon to a limited extent even when the context is weak, but the poor context allows some ribosomes to bypass the first AUG and reach a start codon further downstream, thus increasing the complexity level of an organism by alternative gene expression pathways. This is called leaky scanning (Kozak, 1984, 2005). Therefore, the presence of A in position −3 in the first start codon could represent the optimal situation to ensure a more accurate and efficient translation of the buffalo *CSN3* transcript compared with that in other ruminants.

In conclusion, we report here for the first time a quantitative characterization of the buffalo casein transcripts and show that the 4 mRNA are not transcribed and translated with the same efficiency. Although the analysis of the sequences flanking the start codon can help to formulate hypotheses concerning some of the observed differences in translation efficiency of the transcripts of the investigated genes, other elements need to be analyzed to fully understand the mechanisms of regulation of their expression. Based on results of different studies in higher eukaryotes and yeast, it is generally accepted that not only the context of the translation initiation codon (AUG), but also the length or alteration of the 5′ UTR, secondary structure, GC content, upstream AUG codons or upstream open reading frames, and the length of the 3′ UTR play important roles in determining the translation rate (Kozak 1991a,b; Tanguay and Gallie, 1996; Morris and Geballe, 2000; Koda et al., 2004).

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Table 3. Comparison of start codon flanking sequences of the κ-casein transcripts in different species

		Position[1]											
Species	EMBL accession no.	−6	−5	−4	−3	−2	−1	+1	+2	+3	+4	+5	+6	+7
Buffalo	AM900443	G	G	U	A	C	A	A	U	G	A	U	G	A
Cattle	AY380229	G	G	U	G	C	A	A	U	G	A	U	G	A
Sheep	NM_001009378	G	G	U	G	C	A	A	U	G	A	U	G	A
Goat	X60763	G	G	U	G	C	A	A	U	G	A	U	G	A
Pig	NM_001004026	G	G	U	G	C	A	A	U	G	A	U	G	A
Rabbit	Z18243	G	G	U	G	C	A	A	U	G	A	U	G	A
Rat	NM_007786	G	G	U	G	C	A	A	U	G	A	U	G	A

[1]The first of 2 consecutive AUG codons is underlined.

REFERENCES

Bevilacqua, C., J. C. Helbling, G. Miranda, and P. Martin. 2006. Translational efficiency of casein transcripts in the mammary tissue of lactating ruminants. Reprod. Nutr. Dev. 46:567–578.

Boutinaud, M., H. Rulquin, D. H. Keisler, J. Djiane, and H. Jammes. 2002. Use of somatic cells from goat milk for dynamic studies of gene expression in the mammary gland. J. Anim. Sci. 80:1258–1269.

Cosenza, G., M. Feligini, A. Mancusi, A. D'Avino, A. Coletta, D. Di Berardino, and L. Ramunno. 2009a. Italian Mediterranean river buffalo *CSN2* gene structure and promoter analysis. Ital. J. Anim. Sci. 8:57–59.

Cosenza, G., A. Pauciullo, M. Feligini, A. Coletta, L. Colimoro, D. Di Berardino, and L. Ramunno. 2009b. A point mutation in the splice donor site of intron 7 in the αs2-casein encoding gene of the Mediterranean River buffalo results in an allele-specific exon skipping. Anim. Genet. 40:791.

D'Ambrosio, C., S. Arena, A. M. Salzano, G. Renzone, L. Ledda, and A. Scaloni. 2008. A proteomic characterization of water buffalo milk fractions describing PTM of major species and the identification of minor components involved in nutrient delivery and defense against pathogens. Proteomics 8:3657–3666.

De Angioletti, M., G. Lacerra, V. Sabato, and C. Carestia. 2004. Beta+45 G --> C: A novel silent β-thalassaemia mutation, the first in the Kozak sequence. Br. J. Haematol. 124:224–231.

Feligini, M., I. Bonizzi, J. N. Buffoni, G. Cosenza, and L. Ramunno. 2009. Identification and quantification of α_{S1}, α_{S2}, β, and κ-caseins in water buffalo milk by reverse phase-high performance liquid chromatography and mass spectrometry. J. Agric. Food Chem. 57:2988–2992.

Iannuzzi, L., G. P. Di Meo, A. Perucatti, L. Schibler, D. Incarnato, D. Gallagher, A. Eggen, L. Ferretti, E. P. Cribiu, and J. Womack. 2003. The river buffalo (*Bubalus bubalis*, 2n = 50) cytogenetic map: Assignment of 64 loci by fluorescence in situ hybridization and R-banding. Cytogenet. Genome Res. 102:65–75.

Koda, A., T. Minetoki, K. Kenji Ozeki, and M. Hirotsune. 2004. Translation efficiency mediated by the 5′ untranslated region greatly affects protein production in *Aspergillus oryzae*. Appl. Microbiol. Biotechnol. 66:291–296.

Kozak, M. 1984. Point mutations close to the AUG initiator codon affect the efficiency of translation of rat preproinsulin in vivo. Nature 308:241–246.

Kozak, M. 1987. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. 15:8125–8148.

Kozak, M. 1991a. Effects of long 5′ leader sequences on initiation by eukaryotic ribosomes in vitro. Gene Expr. 1:117–125.

Kozak, M. 1991b. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. 266:19867–19870.

Kozak, M. 2005. Regulation of translation via mRNA structure in prokaryotes and eukaryotes. Gene 361:13–37.

Masina, P., A. Rando, P. Di Gregorio, G. Cosenza, and A. Mancusi. 2007. Water buffalo kappa-casein gene sequence. Ital. J. Anim. Sci. 6(Suppl. 2):353–355.

Morris, D. R., and A. P. Geballe. 2000. Upstream open reading frames as regulators of mRNA translation. Mol. Cell. Biol. 20:8635–8642.

Murrieta, C. M., B. W. Hess, E. J. Scholljegerdes, T. E. Engle, K. L. Hossner, and G. E. Moss. 2006. Evaluation of milk somatic cells as a source of mRNA for study of lipogenesis in the mammary gland of lactating beef cows supplemented with dietary high-linoleate safflower seeds. J. Anim. Sci. 84:2399–2405.

Tanguay, R. L., and D. R. Gallie. 1996. Translational efficiency is regulated by the length of the 3′ untranslated region. Mol. Cell. Biol. 16:146–156.