Theor Appl Genet (1996) 93: 887–893

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Variants within the 5′-flanking regions of bovine milk-protein-encoding genes. III. Genes encoding the Ca-sensitive caseins α_{s1}, α_{s2} and β

Received: 9 May 1995 / Accepted: 8 March 1996

Abstract The 5′-flanking regions of the Ca-sensitive casein-encoding gene family were analysed for DNA variants by automated DNA sequencing of 13 cows belonging to seven breeds. About 1 kbp of each 5′-flanking region, including non-coding exon I, was amplified by PCR and sequenced bidirectionally. A total number of 34 variable sites (17 for the α_{s1}, 10 for the α_{s2}, and 7 for the β casein encoding gene) was identified. Variants were computer-analysed for location in putative regulatory sites in order to predict potential influences on gene expression.

Key words α_{s1}, α_{s2} and β casein encoding genes · DNA sequencing · DNA variants · *Bovine* · Regulatory DNA sequences

Introduction

In 1977 Gaye et al. postulated a gene family of caseins on the basis of similarities in the signal peptide sequences. This so-called Ca-sensitive casein gene family is characterized by binding affinity to Ca ions. The precursor gene of the Ca-sensitive caseins was probably composed of four exons: (1) for the 5′-non-coding region, (2) for encoding the signal peptide, (3) for encoding the phosphorylating site(s), and (4) for encoding the hydrophobic domain(s) (Jones and Tjian 1985). It is postulated that the β-CN gene then evolved from this precursor by an alteration of splicing, in the duplication of the third exon and the incorporation of exons which code additional hydrophobic sites. The casein genes were then duplicated, providing a putative selective advantage in terms of fast-growing offspring. Thus the α_{s1}-CN and α_{s2}-CN genes evolved in ruminants from the β-CN gene (Bonsing and Mackinlay 1987). Analysis of the proteins and mRNAs of the casein-encoding genes, especially those for the Ca-sensitive caseins, has shown them to be one of the fastest-evolving gene families (Dayhoff 1976). Within these genes, typically, three areas are conserved (5′-non-coding, signal peptide-coding, and phosphorylating site-coding regions). Additionally, functional sites, important for the coordinated expression of the genes, are conserved in the 5′-flanking gene regions. Comparative analysis of casein-encoding genes in different species has detected conserved elements especially within the first 200 bp of the 5′-flanking regions (Laird et al. 1988; Vilotte et al. 1991). Analysis of transcription in cell lines (e.g. Hynes et al. 1990; Schmitt-Ney et al. 1991) and transgenic animals (e.g. Wright et al. 1991; Hennighausen 1992) has shown that regulatory sequence elements are located within about 1000 bp of the 5′-flanking regions of the casein-encoding genes. Associations have been described between alleles in coding regions of the milk protein-encoding genes and the level of expression of milk proteins in several cattle populations (e.g. van Eenennaam and Medrano 1991), possibly caused by linkage disequilibrium between variants of coding and regulatory regions. The objective of the present study was the analysis of variants within the 5′-flanking regions of milk protein-encoding genes. For this purpose, genomic DNA from cows of different breeds was comparatively sequenced at selected regions. Whilst the data of κ-CN- and β-LG-coding genes have been described already (Schild et al. 1994; Wagner et al. 1994), the present report deals with the three genes coding for the Ca-sensitive caseins.

Material and methods

The selection of animals and techniques for direct sequencing of PCR products have been described by Schild et al. (1994). Special conditions for the methods used in the analysis of the Ca-sensitive casein-coding genes are summarized in Tables 1 and 2. Sequence analysis of the different gene regions was performed with routines of the HUSAR programme package (German Cancer Research Center, Heidelberg) and further special computer programmes.

Communicated by E. J. Eisen

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Table 1 PCR primers and conditions for amplification of the target regions

(a) Primers[a]

Gene	Sequence[b]	Location[c] (bp)
α_{S1}-Cn	5′-B-TCCTGTCATACAACTGTGAAT-3′	−1145/−1125
	5′-GGGGGTACCTCCTGTCATACAACTGTGAAT-3′	−1145/−1125
	5′-GGGAAGCTTTGAGACAGAGGAAAATCATAG-3′	+81/+101
α_{S2}-Cn	5′-B-TATGACATGTCGAGAAATGAG-3′	−1150/−1130
	5′-GGGGGTACCTATGACATGTCGAGAAATGAG-3′	−1150/−1130
	5′-GGGAAGCTTTTGGAACAATGCTATTAGGTT-3′	+97/+117
β-Cn	5′-B-GGTCTGGCTTAGACTCTATTG-3′	−1049/−1029
	5′-GGGGGTACCGGTCTGGCTTAGACTCTATTG-3′	−1049/−1029
	5′-GGGAAGCTTAGATGAGTAGCAGATACAATG-3′	+67/+87

[a] The biotin-labelled primers were used for solid phase sequence procedure according to Schild et al. (1994); the PCR products generated with the non-labelled primers were used for cycle sequencing
[b] B:Biotin. Each non-labelled primer with a restriction site of nine nucleotides at the 5′-end
[c] According to the sequences used in Figs. 1 to 3

(b) Conditions

Gene	Amplified fragment [bp][a]	Cycle number	Temperature [°C] and time [min]		
α_{S1}-Cn	1246		95°C	54°C	72°C
		1	3.0	2.0	4.0
		2–34	1.5	2.0	4.0
		35	1.5	2.0	7.0
α_{S2}-Cn	1267		95°C	52°C	72°C
		1	3.0	2.0	4.0
		2–34	1.5	2.0	4.0
		35	1.5	2.0	7.0
β-Cn	1136		95°C	54°C	72°C
		1	3.0	2.0	4.0
		2–34	1.5	2.0	4.0
		35	1.5	2.0	7.0

[a] According to the sequences used in Figs. 1 to 3

Table 2 Primers used for sequencing

Gene	No.	Sequence[a]	Location[b] (bp)
α_{s1}-Cn	1	5′-GGAAAATCATAGAAAT-3′	+77/+92
	2	5′-F-GGTTATTATGAGAACATGC-3′	−272/−254
	3	5′-ATAAGTTACATTATAT-3′	−487/−472
	4	5′-F-GTACTAGTTGTGGTAACTAG-3′	−607/−588
	5	5′-F-GCGAAAAATAAACCATAACTC-3′	−896/−876
	6	5′-GGGGGTACCTCCTGTCATACAACTGTGAAT-3′	−1145/−1125
α_{s2}-Cn	1	5′-GGGAAGCTTTTGGAACAATGCTATTAGGTT-3′	+97/+117
	2	5′-TGTTTGTGTAGCTCAGCTC-3′	−251/−233
	3	5′-GGGGGTACCTATGACATGTCGAGAAATGAG-3′	−1150/−1130
β-Cn[c]	1	5′-ATGAGTAGCAGATACAATG-3′	+67/+85
	2	5′-TGTCCAGTAGCTACAGACC-3′	−222/−204
	3	5′-TAGGAAACACAATCCACTC-3′	−562/−544
	4	5′-AGTGGGGATTTGTTGAGGG-3′	−815/−797
	5	5′-GGCTTAGACTCTATTGTAG-3′	−1044/−1026
	6	5′-ACATGACCCAGATTCACTG-3′	−683/−665
	7	5′-TACTAAAGAGACCTCATTG-3′	−292/−274

[a] F: Fluorescein
[b] According to sequences used in Figs. 1–3
[c] Primers nos 5–7 were used for eluted-strand sequencing

Results and discussion

By DNA sequencing of the 5′-flanking regions for 13 cows, 34 variable sites were found (Figs. 1 to 3; Table 3) within these regions of the Ca-sensitive casein encoding genes.

Fig. 1 Analysed region of the α_{S1} casein-encoding gene. The search for DNA elements was performed by using the computer routines MULTALIGN and FIND of the HUSAR programme package, German Cancer Research Center, Heidelberg. Variants are indicated by *asterisks* (*capital letters* represent base substitutions, *small letters* base deletions). CAAT-, TATA-boxes and potential protein-binding sites are displayed by *shaded letters*; regions of congruent factors are *underlined*; regions shared by two factors are *double underlined*; regions shared by three factors are written in *italics*. PCR primer sequences are *hatched*. End of exon I is marked by a *triangle* (▽). Ambiguities: *W* A/T; *Y* C/T; *H* A/C/T; *R* A/G; *M* A/C; *S* G/C. *t*: deletion/insertion. *RC* Reverse Complement. Abbreviations of factors: *AP* Activator Protein (Lee et al. 1987; Mitchell et al. 1987; Chiu et al. 1987; Mermod et al. 1988; Mitchell and Tjian 1989); *CREB* cAMP-Responsive Element-Binding Protein (Mitchell and Tjian 1989), *GCF* GC-binding Factor (Kageyama and Pastan 1989), *GR* Glucocorticoid-Receptor (Beato 1989); *HNF* Hepatocyte Nuclear Factor (Raymondjean et al. 1991; Rossi et al. 1992); *ISGF* Interferon-Stimulated Gene Factor (Fairsst and Meyer 1992); *MAF* Mammary Cell-Activating Factor (Mink et al. 1992); *MGF* Mammary Gland Factor (Schmitt-Ney et al 1991); *NF* Nuclear Factor (Watson et al. 1991); *NF-κB* Nuclear Factor kappa B (Lenardo et al. 1987); *OCT* Octamer-Binding Factor (Groenen et al. 1992); *PMF* Pregnancy Specific Mammary Nuclear Factor (Lee and Oka 1992); *PR* Progesteron-Receptor (Bailly et al. 1986, Dean et al. 1983), *SP* Promoter-Specific Transcription Factor (Mitchell and Tjian 1989); *TR* Thyroid-Receptor (Glass et al. 1988: Näar et al. 1991: Umesono et al. 1991; Adan et al. 1992); *YY1* Common Factor 1 (Seto et al. 1991)

In the 5′-flanking region of the α_{S1}-CN gene, 17 variable sites were observed (Fig. 1, Table 3), one of them (R1.1) was in the non-coding exon I. Fifteen of the sites are base substitutions with 12 transitions (5 A↔T; 7 T↔C) and three transversions (1 A↔C; 2 A↔T). The remaining two sites are deletions/insertions. In comparison to the genomic sequence published by Koczan et al. (1991), the poly(T) region –223 to –230 bp was found to be reduced by one T in all individuals. Ten variable sites were detected for the 5′-flanking region of the α_{S2}-CN gene (Fig. 2, Table 3). All of them are substitutions, with six transitions (2 A↔G; 4 T↔C) and four transversions (3 A↔C; 1 T↔C). The site R2.1 was found in the non-coding exon I, and the site R2.2 in the first intron. In comparison to the genomic sequence analysed by Groenen et al. (1993) for the α_{S2}-CN gene, the sequence is different in position –15 bp (G instead of C). Seven variable sites occurred in the 5′-flanking region of the β-CN gene (Fig. 3; Table 3). Six are substitutions with four transitions (1 A↔G; 3 T↔C) and two transversions (1 A↔T; 1 G↔C). One site is of the deletion/insertion type. The genomic sequence of the β-CN gene described by Bonsing et al. (1988) has an additional T in position –849 and –833 bp, and a C in position –893 bp.

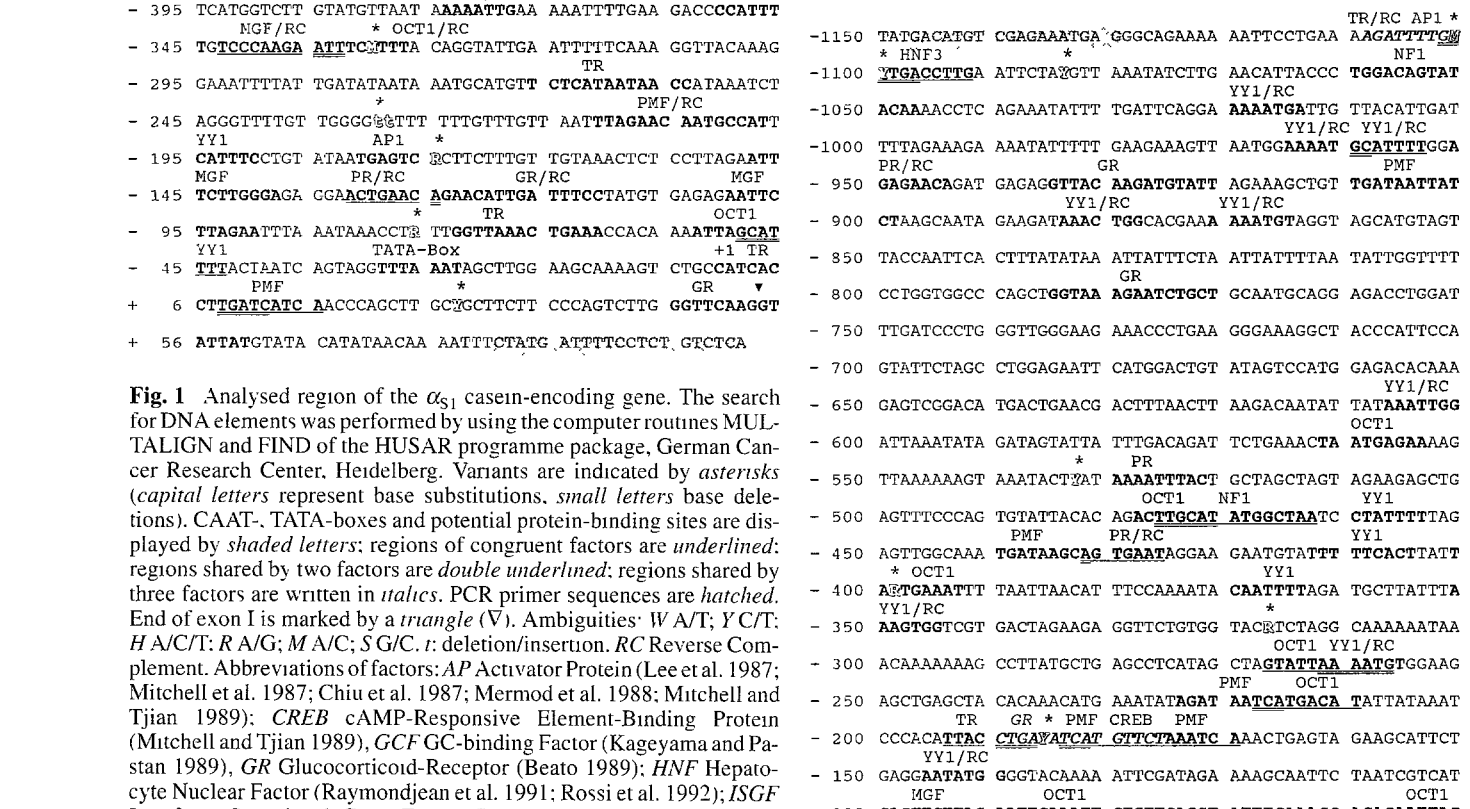

Fig. 2 Analysed region of the α_{S2} casein-encoding gene. For legend see Fig. 1

Table 3 Genotypes of polymorphic positions in single animals

Gene locus	Animal No.	Breed[a]	Polymorphic position (bp)																
			R.1.1 +28	R1.2 −76	R1.3 −175	R1.4[b] −230	R1.5 −330	R1.6 −521	R1.7 −536	R1 8 −621	R1.9 −722	R1.10[c] −728	R1.11 −733	R1.12 −741	R1.13 −774	R1.14 −796	R.15 −820	R.16 −876	R.17 −1016
α_{S1}-CN	1	BV	CC	AA	GG	1	CC	GG	GG	CC	AA	2	TT	TT	CC	CC	GG	TT	TT
	2	BV	TT	GG	AA	1	AA	AA	AA	CC	AA	1	TT	CC	CC	TT	GG	CC	AA
	3	FV	TC	GG	AG	2	AC	AG	AG	CC	AA	1	TT	CC	CC	TT	GG	CC	AA
	4	FV	TT	GG	AA	1	AA	AA	AA	CC	AA	1	TT	CC	CC	TT	GG	CC	AA
	5	JE	CC	AA	GG	1	CC	GG	GG	CC	AA	2	TT	TT	CC	CC	GG	TT	TT
	6	JE	TT	GG	AA	3	AA	AA	AA	CC	AA	1	TT	CC	CC	TT	GG	CC	AA
	7	SB	TC	GA	AG	1	AC	AG	AG	CC	AA	1	TC	CT	CC	TT	GG	CT	AT
	8	SB	TT	GG	AA	3	AA	AA	AA	CC	AT	1	TT	CC	CT	TT	GA	CC	AA
	9	GW	TT	GG	AA	1	AA	AA	AA	CC	AA	1	TT	CC	CC	TT	GG	CC	AA
	10	GW	TT	GA	AG	2	AC	AA	GG	CC	AA	1	TC	CT	CC	TC	GG	CC	AA
	11	SH	TT	GG	AA	3	AA	AA	AA	CT	AT	1	TT	CC	CT	TT	GA	CC	AA
	12	SH	TT	GG	AA	3	AA	AA	AA	CC	AA	1	TT	CC	CC	TT	GG	CC	AA
	13	ZB	TT	GA	AA	2	AC	AG	AA	CT	AT	1	TT	CC	CC	TT	GG	CC	AT

Gene locus	Animal No.	Breed[a]	Polymorphic position (bp)									
			R2 1 +57	R2.2 +7	R2.3 −7	R2.4 −186	R2.5 −317	R2.6 −399	R2.7 −433	R2.8 −1048	R2.9 −1100	R2.10 −1101
α_{S2}-CN	1	BV	TT	CC	CC	TT	GG	AA	TT	CC	AA	AA
	2	BV	TT	CC	AA	TT	GG	AG	TT	CC	AA	AA
	3	FV	TT	CC	CA	TT	GG	AA	TT	CC	CC	AA
	4	FV	TT	CT	AA	TC	GG	AA	TT	CT	AA	AC
	5	JE	TT	CC	CC	TT	GG	AA	TT	CC	AA	AA
	6	JE	TT	CC	CA	TT	GG	AA	TT	CC	AA	AA
	7	SB	TT	CC	CA	TT	GA	AA	TT	CC	AA	AA
	8	SB	TT	CC	AA	TT	AA	AA	TT	CC	AA	AA
	9	GW	TT	CC	CA	TT	GA	AA	TT	CC	AA	AA
	10	GW	TT	CC	CC	TT	GG	AA	TT	CC	AA	AA
	11	SH	TT	CC	CC	TT	AA	AA	TT	CC	AA	AC
	12	SH	GG	CC	CA	TT	GA	AA	TT	CC	AA	AC
	13	ZB	TT	CT	AA	TC	GG	AA	CC	CT	AA	AA

Table 3 Continued

Gene locus	Animal No.	Breed[a]	Polymorphic position (bp)						
			R3.1 −109	R3.2 −264	R3.3 −352	R3.4 −474	R3.5[d] −520	R3.6 −851	R3.7 −928
β-CN	1	BV	CC	TT	CC	TT	1	AA	AA
	2	BV	CC	TC	TT	TT	1	TT	AA
	3	FV	CC	TT	TC	TT	1	TA	AA
	4	FV	CG	TT	TT	TT	2	TT	AA
	5	JE	CC	TT	CC	TT	1	AA	AA
	6	JE	CC	TT	TT	TT	1	TT	AA
	7	SB	CC	TT	TC	TT	1	TA	AA
	8	SB	CC	TT	TT	TT	1	TT	AA
	9	GW	CC	TT	TT	TT	1	TA	AA
	10	GW	CC	TT	TT	TT	1	TT	AG
	11	SH	CC	TT	TT	TT	1	TT	AA
	12	SH	CC	TT	TT	TC	1	TA	AA
	13	ZB	CC	TT	TC	TT	1	TA	AA

[a] BV: Braunvieh (German Brown Swiss); FV: Fleckvieh (German Simmental); JE: Jersey; SB: Schwarzbunt (German Friesian); GW: Galloway; SH: Scottish Highland; ZB: Zebu
[b] 1:T_6/T_6; 2:T_6/T_8; 3:T_8/T_8
[c] 1:T; 2:TT
[d] 1:T_5/T_5; 2:T_4/T_5

Nine (26.5%) of the variable sites (Table 3) were found in one individual only. This result may be a consequence of the selection of a genetically heterogeneous group of cows (see Schild et al. 1994) and the small number of sequenced animals. In order to exclude false variants because of sequencing errors, multiple as well as bidirectional sequencing strategies were used. Moreover, some of the variants were verified by RFLPs in populations (see Table 4).

We have found similar combinations of variants in distinct breeds, perhaps reflecting the separate history of breeds. However, distinct combinations of allelic variants at different sites were also found in animals from different breeds (Table 4). These specific polymorphic types of sequences ("intragenic haplotypes") were found for the 5′-flanking regions of all casein genes and previously have been reported for the β-lactoglobulin- (Wagner et al. 1994) and κ-casein-encoding genes (Schild et al. 1994). They may indicate specific patterns of DNA recognition sites which reflect the evolution of functionally significant variants for gene expression. Analysis of these haplotype combinations will require a larger number of animals and should be performed along with studies of gene expression.

The 5′-flanking regions of the Ca-sensitive casein-encoding genes were analysed for sequence homologies with consensus sequences for mammary gland-specific factors, hormone receptor sites and ubiquitory transcription factors. Some of the potential regulatory sequences are indicated in Figs. 1 to 3. As shown, 17 (50%) of the variants are located within known potential regulatory sequences, suggesting an influence of the variants on the binding of transcription factors. Thus, the variants may affect the expression of the Ca-sensitive casein-coding genes. The influence of 5′-flanking DNA variants on gene expression has been reported by Hayashi et al. (1991) for the human cytochrome P450 IIEI gene and by Grant and Maeda (1993) for the human haptoglobin-coding gene. Furthermore, Ehrmann et al. (1996) have detected large differences in milk

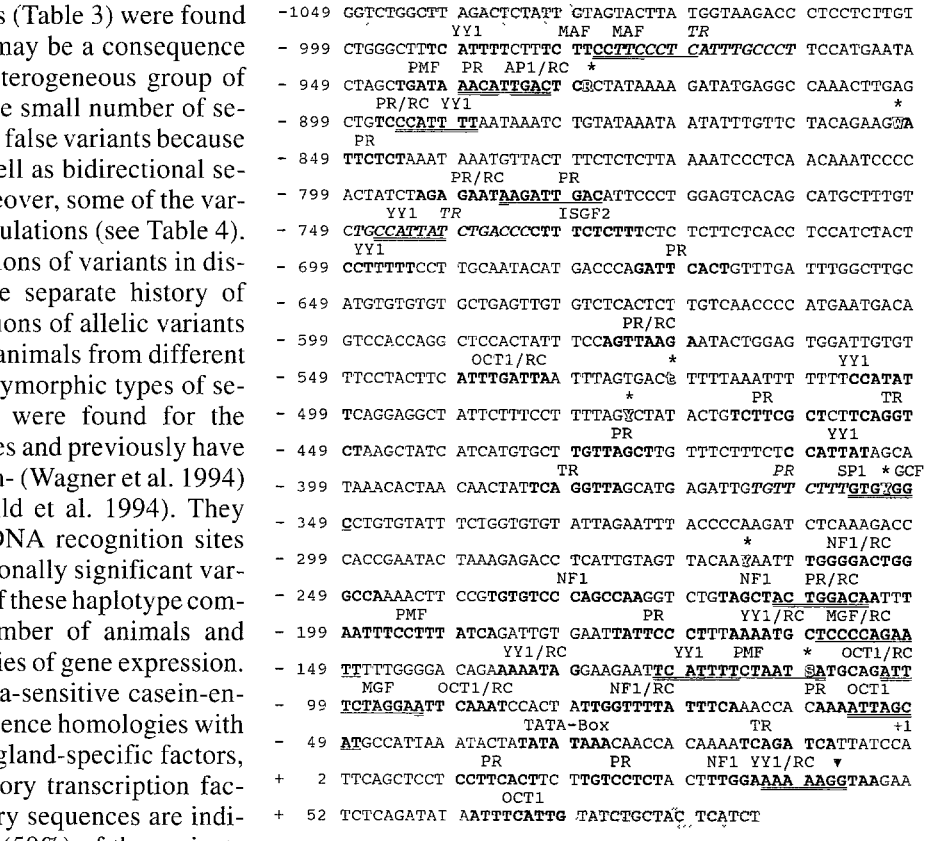

Fig. 3 Analysed region of the β casein-encoding gene. For legend see Fig. 1

protein expression for cows with different variants in the 5′-flanking regions of the β-lactoglobulin-coding gene. We are now applying cell culture and transfection techniques to directly determine the effect on gene expression of sequence variants. So far, our results indicate that differences in expression of the Ca-sensitive casein-coding genes can

Table 4 DNA variants within the 5′-flanking regions of the α_{S1}, α_{S2} and β casein encoding genes which can be detected by RFLPs

Gene	Variant[a]	Position (bp)	Base	Enzyme	RFLP[b] (bp)
α_{S1}-Cn	R1.1	+28	T	*Bbc*I	711, 447, 88
			C	*Bbv*I	799, 447
α_{S1}-Cn	R1.3	−175	G	*Mae*III	475, 279, 176, 126, 124, 66
			A	*Mae*III	475, 405, 176, 124, 66
α_{S1}-Cn	R1.7	−536	A	*Apo*I	400, 208, 166, 144, 134, 49, 39, 28, 26, 23, 20, 9
			G	*Apo*I	534, 208, 166, 144, 49, 39, 28, 26, 23, 20, 9
α_{S1}-Cn	R1.10	−728	–	*Ssp*I	828, 145, 143, 130
			T	*Ssp*I	972, 145, 130
α_{S1}-Cn	R1.13	−774	C	*Ava*I	876, 370
			T	*Ava*I	–
			C	*Hpa*II	875, 371
			T	*Hpa*II	–
α_{S2}-Cn	R2.3	−7	C	*Dde*I	397, 220, 143, 123, 108, 88, 81, 57, 35, 15
			A	*Dde*I	397, 220, 211, 143, 108, 81, 57, 35, 15
α_{S2}-Cn	R2.4	−186	T	*Eco*RV	965, 302
			C	*Eco*RV	–
α_{S2}-Cn	R2.5	−317	G	*Mae*II	766, 435, 66
			A	*Mae*II	1201, 66
α_{S2}-Cn	R2.6	−399	A	*Mse*I	380, 187, 158, 148, 110, 89, 69, 49, 34, 23, 11, 5, 4
			G	*Mse*I	380, 187, 159, 158, 110, 89, 69, 49, 34, 23, 5, 4
α_{S2}-Cn	R2.8	−1084	C	*Mae*II	766, 435, 66
			T	*Mae*II	832, 435
β-Cn	R3.1	−109	C	*Nla*III	264, 198, 173, 133, 96, 77, 64, 59, 40, 32
			G	*Nla*III	323, 198, 173, 133, 96, 77, 64, 40, 32
β-Cn	R3.3	−352	T	*Hae*I	561, 436, 139
			C	*Hae*I	997, 139

[a] Number of variants according the position within the sequenced fragments, see Figs. 1–3
[b] –: No restriction site. The RFLPs 1.7, 1.10, 1.13, 2.3, 2.4 and 3.1 have been verified in the sequenced animals and in a sample of 25 additional animals

not be attributed to the influence of single sites, but rather to the sum effect of intragenic haplotypes within the 5′-flanking regions. Clearly, the regulation of expression of a single eukaryotic gene is a multifactorial process.

Acknowledgements The study was supported by a grant from the Deutsche Forschungsgemeinschaft. We gratefully appreciate the technical support of Pharmacia Biotechnology, Freiburg, and Applied Biosystems, Weiterstadt.

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