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Author(s)	Keating, A. F.; Davoren, P.; Smith, T. J.; Cairns, Michael T.
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INTERPRETIVE SUMMARY

Bovine *Kappa-Casein* Promoter Haplotypes With Potential Implications For Milk Protein Expression by A.F. Keating, P. Davoren, T.J. Smith, R.P. Ross and M.T. Cairns.

Bovine kappa (κ)-casein protein comprises approximately 8% of total milk protein and is important in maintaining the structure of the casein protein complex. Different genetic haplotypes of κ -casein have been associated with varying levels of κ -casein protein in milk. This study investigated the regulatory region of the κ -casein gene and found two haplotypes in this area, which differed at three locations. A screen of animals representing dairy, dual-purpose and beef breeds found that one haplotype was more common in animals bred for dairy purposes. The transcriptional activity of each haplotype was investigated in a mammary cell model.

**RUNNING HEAD: GENETIC VARIATION IN MILK PROTEIN
EXPRESSING GENES**

**Bovine *Kappa-Casein* Promoter Haplotypes with Potential Implications For Milk
Protein Expression.**

A.F. Keating^{*†}, P. Davoren^{*}, T.J. Smith[‡], R.P. Ross[†] and M.T. Cairns.^{*}

^{*} National Diagnostics Centre, National University of Ireland, Galway, Ireland.

[‡] National Centre for Biomedical Engineering Science, National University of
Ireland, Galway.

[†] Teagasc, Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.

^{*} The first two named authors contributed equally to this paper and should be considered joint first
authors.

Corresponding Author: Dr. Michael Cairns, National Diagnostics Centre, National University of
Ireland, Galway. Telephone: 0035391492094, Fax no. 0035391586570. Email:
michael.cairns@nuigalway.ie

Abstract

Genetic analysis of the *kappa-casein* (*CSN3*) promoter regions of 42 cattle representing nine different breeds revealed that two distinct haplotypes (A and B) exist at this locus, differing from each other by single base changes at positions –514 (T,G), –426 (T,C) and –384 (T,C), where haplotype A has bases T, T and T and haplotype B has bases G, C and C. The AA and AB haplotypes were found to occur at a higher frequency in the animals tested with 69.0% and 21.4% being homozygous and heterozygous respectively. The sequences that include these polymorphisms are potentially important in transcriptional regulation of the *κ-casein* gene as they contain putative sites for binding of a number of transcription factors. Linkage disequilibrium between the *κ-casein* promoter haplotype and either one of the two major *κ-casein* coding sequence haplotypes was not evident. The A allele is dominant in all groups (dairy, beef and dual purpose) with an allele frequency of 80% and is higher among high yielding dairy animals (88.9%) than among beef animals (75%). The AB haplotype is comparatively rare in the dairy cattle (11.1%) compared to both dairy and dual purpose animals. The BB haplotype, though rare overall (9.5%), is much higher in dual purpose animals (18.8%) than dairy (5.6%) animals. In contrast the B allele is much more representative of the *κ-casein* promoters from other ruminants.

(Key words: *Kappa-casein*, promoter haplotype, expression studies)

INTRODUCTION

The bovine κ -casein gene (*CSN3*) consists of five exons distributed over approximately 13kb of the bovine genome (Alexander et al., 1988; Martin et al., 2002). Eleven genetic haplotypes of κ -casein have been identified to date that differ in the coding region (Farrell et al., 2004). The two most common genetic haplotypes, designated A and B, differ in two positions at codons 136 (A-Thr: B-Ile) and 148 (A-Asp: B-Ala) of exon 4 (Mercier et al., 1973; Ron et al., 1994) and can be distinguished by an RFLP-PCR reaction (Nierop Groot et al., 1995). Milk from BB cows reportedly contains higher amounts of protein (Ng-Kwai-Hang et al., 1984; Ron et al., 1994; Bobe et al., 1999) and is associated with shorter rennet coagulation time, firmer curd, and higher cheese-yield than milk from AA animals (Marziali and Ng-Kwai-Hang, 1986; Schaar, 1984; Martin et al., 2002).

A number of studies have been carried out to analyze the promoter region of the κ -casein gene. Coding sequence associated allele-specific polymorphisms have been identified in the distal promoter regions at positions -2035 (G/T), -1651 (A/T) and -999 (-/T) (Robitaille et al., 2005). Fifteen single nucleotide changes were identified in thirteen cows from seven different breeds in a study by Schild et al. (1994) and a *DdeI* RFLP polymorphism within a 214bp fragment of the κ -casein promoter has also been identified (Kaminski, 1996). While these polymorphisms have been noted in the κ -casein gene promoter, it is unclear whether they are characteristic of a distinct haplotype or if they play a functional role by modulating gene expression.

In this study, polymorphism occurrence in the κ -casein gene promoter and the potential effect on transcriptional activity of the promoter were investigated. The

bovine breeds chosen were considered representative of the Irish herd and included dairy, dual-purpose and non-dairy (beef) breeds. Potential links between promoter polymorphisms and the two most common coding sequence genetic polymorphisms were also investigated.

MATERIALS AND METHODS

Blood sampling and DNA extraction

Approximately 15ml of blood was collected into heparinized tubes from the coccygeal vein of 42 cattle representing nine breeds. Breeds sampled were high genetic merit Holstein-Friesian, low genetic merit Holstein-Friesian (Kennedy et al., 2003), Irish-Friesian, Dutch-Friesian, Limousin, Montbeliarde, Charlois, Normande, Norwegian Red and Kerry. With the exception of the Limousin, Charlois, and Kerry animals, these animals were all part of the extended herd at Teagasc, Moorepark, Fermoy, Co. Cork. Limousin and Charlois samples were from Teagasc, Grange, Co. Meath, and Kerry samples were obtained from Duchas, Muckross House, Killarney, Co. Kerry. Blood samples were obtained for all animals and DNA extractions were carried out using the Gentra Capture Column™ (Gentra, UK) system for approximately 200µl of whole blood per animal. Blood was stored at –80°C and DNA was stored at –20°C until further use.

Primer design

Primers were designed as in Table 1 and synthesized by MWG Biotech.

Polymerase Chain Reaction (PCR)

PCR was carried out from a starting template of approximately 200ng of genomic DNA in a final volume of 50µl containing 1X *Taq* DNA polymerase buffer (Invitrogen), 1.5mM MgCl₂, 200µM dNTPs (Promega), 0.3µM each primer, and 1U

Taq polymerase (Invitrogen). The reaction was amplified for 35 cycles. An initial incubation at 95°C for 2min was followed by 35 cycles of 95°C for 1min, Ta for 1min and 72°C for 1min. (Annealing was carried out at 2°C below the melting temperature (T_m) specific for each primer pair). Cycling was performed in a DNA Engine thermal cycler (MJ Research). Two constructs were designed and the corresponding fragments for both the A and B haplotypes were amplified from the genomic DNA of homozygous animals by PCR using a proof-reading *Taq* polymerase. The primers pAK20 and pAK21 incorporated restriction enzyme sites for *Pst*I and *Xho*I respectively allowing cloning into the plasmid pCMVtkLUC. Six AK constructs (AK1 to AK3 for each allele) were subsequently amplified from the two purified plasmid templates and cloned into a luciferase reporter vector pGL3-Basic. Each fragment used the same downstream primer (pAK21, *Xho*I site) and one of the three upstream primers (pGLAK1-pGLAK3) incorporating a *Mlu*I restriction site. All plasmids were sequenced on construction and again after a number of transfections. Primers pAK06, pAK08, pAK20 – pAK24 were used to amplify κ -casein promoter fragments for sequencing. Primers pAKp1; pAKp4 were used in PCR-RFLP to genotype κ -casein coding sequence variants.

Restriction digests

Restriction digests were carried out in a final volume of 20 μ l containing 10 μ l of PCR product, 1X reaction buffer and 1U restriction enzyme. Restriction enzymes used for screening purposes were *Bcl*II and *Hind*III and for cloning purposes were *Pst*I, *Mlu*I and *Xho*I. Reactions were incubated at temperatures recommended by the manufacturer (Roche Diagnostics).

Sequencing and Bioinformatics

Sequencing of PCR products was carried out by MWG Biotech. The resulting sequences were analysed using the Vector NTI® Suite of software (Invitrogen). Alignment of sequences for all 42 animals was carried out, and potential polymorphic sites identified. Examination of chromatogram sequence files to confirm homozygotic and heterozygotic animals was also performed. Vector NTI® was also used for promoter multi-species alignments: sequences aligned (with accession no.) were bovine (M75887), yak (AF194988), goat (Z33882), horse (AY579426), mouse (AJ309571), rabbit (AJ309572), sheep (L31372) and water buffalo (AJ628346). MatInspector (Genomatix Software GmbH) was used to identify potential transcription factor binding sites in the bovine promoter sequences (Rel. Professional 7.4.3 Jul 2006) (Cartharius et al., 2005).

Generation of truncated promoter luciferase reporter constructs

Three promoter fragments of decreasing length were amplified for each haplotype using primers that incorporated restriction sites at their 5' ends. Promoter fragments AK1, AK2 and AK3 were amplified with primers pGLAK1, pGLAK2 and pGLAK3 respectively paired with pAK21 (Table 1) from a common plasmid for each haplotype. Fragment sizes were 505bp, 752bp and 1.3kb and were designated pGLAK1, pGLAK2 and pGLAK3 respectively. Digestion was carried out to create compatible ends for sub-cloning into the pGL3-Basic (Promega) promoter-less expression vector. Ligation reactions were transformed into TAM Ultracomp chemically competent *E. coli* cells (Active Motif Europe) as described by the manufacturer. All constructs were verified by sequencing using the pGL3for and pGL3rev sequencing primers (Table 1)

Cell culture

Human mammary MCF7 cells (ATCC – HTB22) were cultured in Eagles Minimum Essential Media (Invitrogen) containing 10% (v/v) foetal bovine serum (Invitrogen) and 1% (v/v) non-essential amino acids (Invitrogen). Cells were routinely passaged every 3-4 days by washing with phosphate buffered saline (PBS) and treating with 3ml EDTA-trypsin (Sigma) to remove adherent cells. Cells were maintained in a humidified incubator at 37°C and 5% CO₂. Cells were grown to 80% confluency and transiently co-transfected with luciferase reporter vectors using Fugene 6 transfection reagent (Roche Diagnostics). Four independent transfections were carried out. Six-well transfection plates were seeded at a concentration of 3 x 10⁵ cells per well and incubated overnight at 37°C and 5% CO₂. Experimental constructs (1µg) were co-transfected with 100ng of pRL-SV40 plasmid (Promega) to control for transfection efficiency. Forty-eight hours after transfection, media was removed from the wells and 300µl Passive Lysis Buffer (Promega) added. Following incubation at room temperature for 10min, samples were either assayed immediately as described below or stored at -20°C for later analysis.

Luciferase assay

The Dual Luciferase Assay Kit (Promega) was used to measure both Renilla and Firefly luciferase expression by the reporter vectors on a Tecan Spectrafluor Plus luminometer using the Magellan software (Tecan). Mean Firefly luciferase activity values were corrected for variations in transfection efficiency using the corresponding mean Renilla luciferase figures.

Statistical analysis

Linkage disequilibrium of the promoter haplotype and coding sequence haplotype was analysed by χ^2 -test: the null hypothesis was that no association existed between haplotypes. Statistical analysis of the reporter construct luciferase values was carried

out using either the ANOVA function or individual t-test function of SPSS for Windows (Rel. 12.0.1).

RESULTS

Amplification and sequence analysis of the κ -casein gene promoter

Nine bovine breeds were chosen that were considered representative of the Irish herd.

Amplification and sequencing of the κ -casein promoter region (2072bp) identified

three polymorphic sites at positions -514, -426 and -384 (T/G, T/C and T/C). The

three SNPs were found to be linked: animals were either homozygous or heterozygous

for all three of the base variations, creating three promoter haplotypes - AA, AB or

BB. The base change from T to C at -426 destroys a recognition site for the

restriction enzyme *BclI*, allowing a rapid screen for this base change and hence for the

κ -casein promoter haplotypes (Figure 1). All animals were also genotyped for the

SNPs present in exon 4 of the κ -casein gene to determine whether a relationship

exists between promoter haplotypes and the A and B major coding sequence

haplotypes. The two major coding region haplotypes were again identified by

restriction digestion: a 443bp fragment was amplified and digested with restriction

enzyme *HindIII* (Nierop Groot et al., 1995). Statistical analysis of these results using

χ^2 -testing indicated that there was no apparent link between κ -casein promoter and

coding sequence haplotypes A and B ($P = 0.89$). These results, however, do not

preclude the possibility that the promoter haplotypes are linked to other κ -casein

coding sequence genetic haplotypes. The frequency of the BB coding region

haplotype is known to be low and this was confirmed here where a frequency of 4.7%

among all animals was found. The B allele frequency was 19.4% in dairy breeds,

37.5% in dual-purpose breeds and 31.3% in beef breeds. Although the A allele is the

dominant allele in all groups of animals (dairy: 71.4%; dual purpose: 62.5%; beef:

68.8%), the AA homozygote is only more frequent than the AB heterozygote in the dairy animals (66.7% against 62.5%) whereas in the non-dairy animals the AB heterozygote is nearly twice as frequent as the AA homozygote (62.5% against 33.3%). This probably reflects the hybrid vigour of the dual purpose animals compared to those specifically bred as high-yielding dairy animals.

The genetic haplotypes for the κ -casein gene promoter for all forty two animals screened are listed in Table 2. The frequencies of each haplotype of the κ -casein promoter were as follows: AA (69%), AB (21.5%) and BB (9.5%). The BB haplotype was therefore quite rare in comparison to the AA haplotype. In fact the A allele frequency (80%) was again dominant over the B allele frequency (20%). When the breed differences were examined the frequency of the A allele in dairy animals was 88.9% compared with 71.9% in the dual-purpose breeds and 75% in the beef breeds. It was interesting to note that while the allele frequency was similar for the beef and dual purpose animals, the beef animals showed the greater heterozygosity (50% as compared to 18.8%).

Functional analysis of the κ -casein promoter alleles

Two constructs were designed and the corresponding fragments for both the A and B haplotypes were amplified from the genomic DNA of homozygous animals by PCR using a proof-reading Taq polymerase. Six AK constructs (AK1 to AK3 for each allele) were subsequently amplified from the two purified plasmid templates and cloned into the luciferase reporter vector pGL3-Basic. All plasmids were sequenced on construction and again after a number of transfections.

MCF7 cells were transfected (using Eugene 6) with purified plasmid DNA preparations (endotoxin-free) of the six constructs together with a Renilla transfection efficiency control. Forty eight hours after transfection luciferase activity was measured using the Dual Luciferase Assay Kit. Activities were background corrected and adjusted for transfection efficiency. Four independent transfections were carried out and all values were presented as a percentage of the AK1-A value (Figure 2). The AK3 promoter fragment (1.3kb), irrespective of haplotype, generally resulted in the highest luciferase expression of the three constructs. Statistically both the AK3-A and AK3-B alleles were significantly higher than their AK1 counterparts ($P < 0.05$). In most instances (7 of 8) luciferase expression levels under the control of the AK3 promoter fragment were also higher than their corresponding AK2 allele in the same experiment but statistical confirmation was lacking.

There were no significant differences detected between the two haplotypes in MCF7 cells but both AK3-B and AK1-B were consistently higher (7 of 8 within experiment comparisons) than their AK3-A and AK1-A counterparts. The closest significant difference was between the AK1-B allele and the AK1-A allele ($P = 0.10$).

DISCUSSION

Two haplotypes of the κ -casein promoter, differing in three nucleotide positions, have been identified. These nucleotide changes are present in regions of proposed importance for the regulation of the κ -casein gene expression. One of these base changes destroyed a recognition site for the restriction enzyme *BclI* allowing a rapid screen by a PCR/RFLP method. Different frequency patterns for the A allele in dairy (88.8%), dual-purpose (71.9%) and beef (75%) breeds were revealed. Animals were also screened for known genetic haplotypes in exon 4 of the coding sequence of the κ -casein gene, however statistical analysis of both promoter and coding sequence haplotypes suggested that no linkage disequilibrium existed between the promoter and coding regions.

Putative transcription factor binding sites were identified in the κ -casein promoter sequence from both the literature and a search of transcription factor databases. The three polymorphic sites identified were in regions predicted to be of importance in the regulation of milk protein gene expression (Figure 1). Functional studies of the κ -casein promoter have been carried out previously in mouse cell lines (Adachi et al., 1996; Rijnkels et al., 1995) and have provided evidence of pregnancy-specific and lactation-specific transcription factor binding sites in a segment of the promoter that includes the -384 and -426 polymorphic sites investigated in this study (Adachi et al., 1996). AK3 expression (approximately 1.3kb of promoter) was found to be higher in MCF7 cells than AK1 expression (approximately 500bp of promoter) irrespective of haplotype. This would be consistent with the reported presence of two crucial MGF/STAT5 binding sites highly conserved in six species in the additional stretch of promoter (Gerencser et al., 2002). Similar experiments were carried out in triplicate in

CHO cells (data not shown): although there were no significant differences between the different sized fragments of each allele, AK1 tended to be stronger than AK3 (opposite that of MCF7 cells) and AK1-A was significantly greater than AK2-B. The tissue-specific nature of the activation factor in the longer AK3 fragment is therefore borne out by the absence of activation in CHO-K1 cells. It is also in agreement with recent findings that a 2040bp κ -casein promoter fragment was stronger than a 925bp promoter fragment (Debeljak et al., 2005).

Three linked polymorphisms have been identified in the promoter of the bovine κ -casein gene. If these SNPs occur within transcription factor binding sites they may alter the expression of the *cis*-related transcript and ultimately could have effects on milk yield or milk fat/protein composition. All three polymorphisms were noted in a previous study (Schild et al., 1994) but there was no suggestion that these were linked in any of the breeds they investigated. The promoter constructs were all functional in MCF7 cells (and CHO cells), but although the promoter haplotypes did not reveal a significant difference between promoter haplotypes there was some indication that expression from the haplotypes differed and that haplotype B was expressed more strongly than haplotype A in MCF7 cells.

At positions 1 (-514) and 2 (-426) there are reported (Schild et al., 1994) potential sites for binding of the transcription factor PMF (TGATN₁₋₂ATCA) although the sequences differ from the consensus with an A or a C instead of the T (marked in bold). In both positions the B haplotype with a G (position 1) or a C (position 2) substitution at the (underlined) T position is likely to be less favourable for PMF binding than the A haplotype (Figure 1). Surprisingly the A haplotype is unique at

position 1 (T) amongst the published κ -casein promoter sequences (goat, sheep, horse, mouse, water buffalo and rabbit) which without exception have the B haplotype-like G at this position. Of the published sequences there is no consensus at position 2 though all the ruminants in common with the B haplotype share the C at this position – the T is only seen in the A haplotype and the mouse sequence. It should be stated that although the literature on κ -casein suggests that the polymorphisms at positions 1 and 2 overlap with sites for the binding of transcription factor PMF the evidence for this factor binding site comes from studies of the beta casein gene in the non-ruminant mouse (Lee and Oka, 1992). Information on the sequence variations that bind this transcription factor is very scant, nor is it included in the major transcription factor databases.

At position 3 there is a reported potential AP-2 site (CCCCAGGC) as originally described by Mitchell et al. (1987) and mentioned in relation to kappa-casein by Schild et al. (1994); however, only the inner six nucleotides of the eight match (see figure 1). In this case haplotype B (ACCCAGGT) is expected to bind more tightly than haplotype A (ACTCAGGT). Alignment of the sequences across species suggests that the C found in the B haplotype is the more common nucleotide with only the horse and the yak (like *Bos taurus* in the Bovidae family) sharing the T with haplotype A. Although CREB, OCT1 and CEBP surround this site, MatInspector does not identify a transcription factor binding site that might be affected by the polymorphism.

It appears therefore that haplotype A, even though it is much more common in the breeds analysed in this study, is quite unique across species and that haplotype B is

336 more consistent with other species, especially the ruminants. Indeed haplotype B
337 (G/C/C) is shared by goat, sheep and water buffalo.

338

339 Analysis using the MatInspector program (Cartharius et al., 2005) suggests that the
340 major transcription factor binding sites present in the regions around the three
341 polymorphic sites, before selecting for factors known only to be expressed in
342 mammary tissue, are HNF6 and OCTB (position 1), HAML and HOXF, (position 2)
343 and CREB, HOXF, HOMF, PARF, CEBP and OCTB (position 3). Haplotype A also
344 presents a number of unique transcription factor binding sites not identified in
345 haplotype B at one of the polymorphic positions (GFI1, HOXC, HOXF, EVI1)
346 whereas haplotype B has only one unique site not identified in haplotype A (SRF).
347 The identification of a novel site only reflects that the polymorphism has made the
348 sequence more homologous to the consensus matrix for that site where one version
349 (absent) falls below a threshold score (0.75/Optimized) and the other version is above
350 it (present). Of these transcription factor binding sites, analysis suggests (Malewski &
351 Zwierzchowski, 2002) that the most relevant to the expression of milk proteins are
352 HOXF, OCTB, CEBP and EVI1.

353

354 Of course computer-aided analysis can only suggest potential transcription factor
355 binding sites. Possibly the most relevant is HOXF which has potential sites in 2 of the
356 3 positions but has a more optimal site (at position 1) in haplotype A. Similarly
357 haplotype A might favour binding of EVI1. In addition it has been suggested that
358 OCT1 (ATTTGCAT) may also bind to position 1 (Schild et al., 1994) although the C
359 (bold) is replaced by G in both bovine haplotypes A and B: haplotype B may bind
360 OCT1 preferentially as the G (underlined) in haplotype A is replaced by a T.

361

362

CONCLUSIONS

363 In summary, haplotype A is the more common form seen in the bovine breeds studied
364 and may potentially be favoured from the standpoint of stronger potential
365 transcription factor binding. However, haplotype B is much more consistent with
366 other known κ -casein genes and the results suggest that, at least in one mammary cell
367 line, gene expression is stronger from this promoter form. It could be argued that the
368 expression pattern may be different in bovine mammary cells, that the effects may
369 have been more pronounced upon hormonal induction, that gene expression might not
370 reflect protein expression, or that stronger expression of κ -casein may not be
371 beneficial to overall milk characteristics, but this initial study does suggest that under
372 these particular *in vitro* conditions expression is favoured by one allele over the other
373 and that the haplotype should be investigated further, with a larger number of animals,
374 to determine whether there is an association of haplotype with milk quality
375 characteristics. The promoter A and B haplotypes do not appear to be associated with
376 the coding region haplotypes A and B, but this does not exclude the possibility that
377 they are linked to another factor that is key to milk quality.

378

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462 regions of bovine milk protein genes. I. κ -casein-encoding gene. Theor. Appl. Genet.
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464

FIGURES

Table 1. Primers used in this study

Primer	Sequence	Tm
pAK06	CGGAATTCTGGGCATATAAAAGATGG	69.2
pAK08	AACTGCAGCTAAAAGCCAAAAGGAAGAAAAT	69.2
pAK20	CGCGCTGCAGTATTTGATATGGGGAATG TTCAGGCTCA	72.7
pAK21	CGCGCTCGAGTCCACTGTAAGGAACACCCAGGTAAAT	73.8
pAK22	CGCGCTGCAGCTCGTGTTAGCAGTTTGGAAATTCT	71.8
pAK23	CGCGCTCGAGTGGAATGCAGAGGAGGAAATAGAGTTGT	73.8
pAKp1	TGAGCAGGTATCCTAGTTATGGACT	62.8
pAKp4	GTCTTCTTTGATGTCTCCTTAGAG	59.9
pGLAK1	GCGCACGCGTGCATATAAAAGATGGTCAG	68.1
pGLAK2	GCGCACGCGTGAAGAAAGGGGAATCCTCC	72.3
pGLAK3	GCGCACGCGTCTCGTGTTAGCAGTTTGGAAATTC	71.9
pGL3for	CTAGCAAAATAGGCTGTCCC	57.3
pGL3rev	TATGTTTTTGGCGTCTTCC	52.4

1. Restriction site sequences: CTGCAG=*Pst*I, CTCGAG=*Xho*I, ACGCGT=*Mlu*I,

Table 2. *κ*-casein coding sequence and promoter haplotypes

Dairy	CDS haplotype	Promoter haplotype	Dual purpose	CDS haplotype	Promoter haplotype	Beef	CDS haplotype	Promoter haplotype
HF	AB	AA	N	AB	AA	C	AA	AB
HF	AA	AB	N	BB	AA	C	AB	AA
HF	AA	AA	N	AB	AA	L	AA	AA
HF	AB	AA	N	AB	AA	L	AB	AB
HF	BB	AA	M	AA	BB	L	AB	AA
HF	AA	AA	M	AB	AA	L	AB	AB
HF	AA	AA	M	AB	AA	L	AB	AA
HF	AA	AA	M	AA	BB	L	AA	AB
IF	AA	AA	K	AB	BB			
IF	AA	AA	K	AB	AA			
IF	AB	AA	K	AB	AB			
IF	AB	BB	K	AB	AB			
NR	AA	AB	K	AB	AA			
NR	AA	AA	K	AA	AA			
DF	AA	AA	K	AA	AB			
DF	AA	AA	K	AA	AA			
DF	AA	AA						
DF	AB	AA						
Allele frequency								
A	80.6	88.8		62.5	71.9		68.8	75.0
B	19.4	11.1		37.5	28.1		31.3	25.0
Haplotype frequency								
AA	66.7	83.3		31.3	62.5		37.5	50
AB	27.8	11.1		62.5	18.8		62.5	50
BB	5.6	5.6		6.3	18.8		0	0

1. Breed Abbreviations. HF = Holstein Friesian, IF = Irish Friesian, NR = Norwegian Red, DF = Dutch Friesian, N = Normande, M = Montbeliarde, K = Kerry, C = Charlois, L = Limousin.

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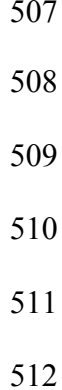


Figure 2. *κ*-casein promoter activity in MCF7 cells

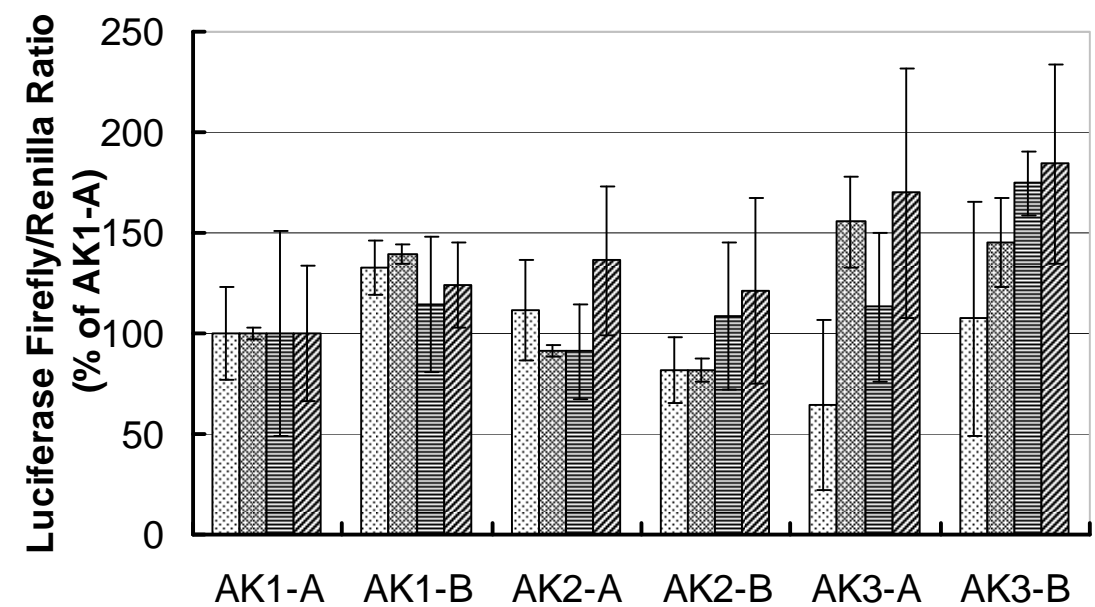


FIGURE LEGENDS

Table 1: Primers used in this study.

Table 2. Bovine breeds and κ -casein coding sequence and promoter haplotypes.

Figure 1. Promoter haplotypes of κ -casein in relation to potential transcription factor binding sites

A. Haplotypes (A & B) in the bovine κ -casein promoter sequence are aligned across the three linked polymorphic sites (SNPs highlighted). Transcription factor binding sites potentially affected by the polymorphism are given below the sequence and the nucleotide position is indicated about sequence. The *BclI* site used for the RFLP screen (see C) is marked. B. Alignment across the three linked polymorphic sites of κ -casein promoter sequences from other species (for accessions nos. see Materials and Methods). The neighbouring MGF binding site is also marked in bold.

Figure 2. κ -casein promoter activity in MCF7 cells

Plasmids AK1 (505bp promoter), AK2 (752bp promoter) and AK3 (1.3kb promoter) of both haplotypes (A & B) carrying the Firefly luciferase reporter under the control of the various κ -casein promoter fragments were transfected into MCF7 cells together with a Renilla luciferase control vector. Luciferase values were expressed as a ratio of Firefly to Renilla and corrected to the AK1-A value (100%). Results are from 4 independent experiments. Error bars show standard deviations for triplicates assayed in each experiment.