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Differential allele-specific accumulation of bovine kappa-casein mRNA throughout lactation

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A differential allele-specific accumulation of κ-casein mRNA that is not linked to the κ-casein protein variants is described in Holstein cows. Actually, cows genotyped κ-casein AB were a mixed population. For the first group of κ-casein AB cows, allele A-specific κ-casein mRNA contents within mammary epithelial cells were lower than the allele B-specific ones (cows LH), suggesting that the allele A-specific κ-casein gene was expressed with lower efficiency in mRNA. For the other group of κ-casein AB cows, allele A- and B-specific κ-casein mRNA accumulated to a similar level within mammary epithelial cells (cows HH). The objective of this study was to determine whether the accumulation of allele-specific κ-casein mRNA remained constant throughout lactation for the two groups of cows. Quantitative RT-PCR was used to monitor Holstein cows κ-casein AB genotyped HH and LH throughout lactation for the proportion of allele B-specific mRNA accumulation relative to the total κ-casein encoded mRNA within mammary epithelial cells: RNA was extracted from milk somatic cells known to contain a small proportion of mammary epithelial cells. Mean values of allele B-specific mRNA content were 50·6±0·5 and 54·0±0·9%, for cows HH and cows LH, respectively, and did not vary during lactation ($P>0·10$). This suggests that the phenotypic expression of the genetic mutation that causes the differential allele-specific accumulation of κ-casein mRNA was not affected by physiological and environmental factors, which tend to vary considerably throughout lactation.

Keywords: Mammary epithelial cells, milk somatic cells, Holstein cows.

Kappa casein (κ-CN) plays a key role in the formation and maintenance of casein micelle structure (Kaminski, 1996). The two most common κ-CN protein variants in Holsteins are κ-CN A and κ-CN B. Several groups have reported associations between κ-CN protein variants and commercially important milk characteristics, such as protein, casein and κ-CN contents, by comparing the milk production traits of κ-CN*AA, κ-CN*AB, and κ-CN*AA cows (see Ng-Kwai-Hang & Grosclaude, 2002). Although interesting trends were shown, the associations were not consistent across studies and breeds. Consequently, the results are not convincing enough to justify a genetic selection programme based on κ-CN protein variants.

Van Eenennaam & Medrano (1991) reported that the allele-B encoding κ-CN (*CSN3*) gene has a propensity to produce a greater amount of κ-CN in milk than allele A-specific *CSN3* for κ-CN*AB cows, although variations in the ratios for κ-CN*A to κ-CN*B obtained in milk, between 1 : 1 and 1 : 14, reflect methodological problems and limit conclusions. Quantitative analysis of allele-specific accumulation of mRNA in heterozygous individuals is a good approach for studying differential allele-specific gene expression at transcriptional and post-transcriptional levels given the same physiological, environmental and genetic backgrounds. In the case of *CSN3* expression, we reported that a differential allele-specific accumulation of *CSN3* mRNA within mammary epithelial cells was detected in κ-CN*AB cows. Interestingly, this differential allele-specific accumulation of *CSN3* mRNA was not linked to the protein variants of κ-CN (κ-CN*A and κ-CN*B) (Robitaille & Petitclerc, 2000), as is the case for other milk proteins such as bovine α-lactoglobulin (Wilkins et al. 1995) and goat α_{s1}-CN (Brignon et al. 1990; Pérez et al. 1994). In fact, we detected a mixed population of κ-CN*AB cows. The first group included cows in which allele-A specific *CSN3* mRNA accumulated to a lesser extent than did allele B-specific *CSN3* ones and, in the second

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group of cows, allele-A and allele-B specific *CSN3* were expressed into mRNA at a similar level. These cows were considered heterozygous (cows LH) and homozygous (cows HH), respectively, in terms of allele-specific expression into *CSN3* mRNA. These results may explain why the findings on associations between protein variants and milk production traits were often inconclusive: cows homozygous for protein variant, κ-CN*AA and κ-CN*BB, may actually be heterozygous in terms of *CSN3* expression into mRNA because of a differential allele-specific accumulation of *CSN3* mRNA. A selection programme based on *CSN3* expression into mRNA would appear to be more appropriate than one using κ-CN protein variants.

The aim of the study was to evaluate the variation in the allele-specific κ-CN mRNA content in mammary epithelial cells isolated from cows HH and cows LH throughout lactation. To this end, we estimated the relative allele-specific *CSN3* mRNA content during lactation using the competitive RT-PCR-SSCP method on total RNA extracted from milk somatic cells instead of total RNA extracted from mammary gland biopsies.

Materials and Methods

Animals

Thirty lactating Holstein cows κ-CN*AB from the herd of the Dairy and Swine Research and Development Centre, which had calved between September and February, were used. Genetic variants of milk proteins were identified by isoelectric focusing of a milk sample (Seibert et al. 1985). CN*AB cows were allocated to the group of cows HH ($n=9$) or the group of cows LH ($n=21$) based on a preliminary estimation of allele-B specific *CSN3* mRNA content as a percentage of total *CSN3* encoding mRNA (B-specific mRNA content). Farm management practices were identical for all of the cows.

Quantitative RT-PCR

Cows were milked four to eight times during the lactation period, for an average of six milkings and a total of 180 milk samples was taken. The milk, collected separately from each cow, was rapidly cooled and then processed for quantitative RT-PCR. Total RNA was extracted from milk somatic cells, as suggested by Lindquist et al. (1994). In summary, 20 ml of milk was diluted with one volume of cold phosphate buffer (10 mM-phosphate–150 mM-NaCl, pH 7·0) and centrifuged at 2400 ***g*** for 10 min. The pellet was frozen in liquid nitrogen and kept at −80 °C until use. Total RNA was extracted from somatic cell pellets and from mammary tissue biopsies with TRIZOL reagent in accordance with the manufacturer's instructions (Invitrogen Life Technologies, Burlington L7P 1A1, Canada) and stored at −80 °C. Quantification of the B-specific mRNA content by competitive RT-PCR-SSCP was carried out as described by Robitaille & Petitclerc (2000). To summarize, a combination of RT-PCR and single-strand conformational polymorphism gel analysis (SSCP) was used. The RT step, using ^{32}P-labelled forward primer (5′-TGTGCTGAGTAGGTATCCTAGTTATGG-3′), was carried out on 1 µl of total RNA in a 25-µl reaction mix (Titan^{TM} One tube RT-PCR kit; Roche, Laval H7V 4A2, Canada) in a PTC 200 thermocycler (MJ Research Inc., Watertown, MA 02172, USA). After an incubation of 30 min at 50 °C, reverse primer was added (5′-GTTTGAAGTAGTCATTTGGTTTCAGC-3′) and the PCR reaction was performed. PCR fragments were fractionated by gel electrophoresis (SSCP analysis), the gel was autoradiographed and the autoradiographic film was scanned and analysed using a computerized scanning densitometer and software. All the milk samples were analysed in triplicate each test-day and the mean values were used for statistical analysis.

Statistical analysis

Proc Mixed procedures of SAS (Littell et al. 1996) were used to verify the effects of lactation stage ($n=11$) on B-specific mRNA content for cows HH and LH, and to analyse the effects of the *CSN3* expression genotype (cows LH and cows HH) on milk production traits.

In the model to estimate the effects of the *CSN3* expression genotype on milk production traits over the first three lactations (305 d in milk), milk, protein and fat yields were included as variables. We used a completely randomized design in repeated measurements with the *CSN3* expression genotype as the main source of variability, the time effects, and the interaction between the main factor and time effects.

The following model was used:

$$Y_{ijk} = \mu + \text{genotype}_i + \text{cow}_{j(i)} + \text{time}_k + (\text{genotype} * \text{time})_{ik} + e_{ijk}$$

where:

μ = overall mean

genotype_i = fixed effects of the *CSN*3 expression genotype (cows HH and cows LH)

$\text{Cow}_{j(i)}$ = random cow effects

Time_k = time effect (stage of lactation, parity)

$(\text{genotype} * \text{time})_{ik}$ = interaction between genotype and time

e_{ijk} = residual random term

Results

A preliminary experiment was carried out to evaluate milk somatic cells as starting material for RNA extraction. As shown in Table 1, the means ± SD obtained for B-specific mRNA content starting with RNA preparations from milk somatic cells found in milk from individual cows LH, were close to the values obtained with RNA preparation from mammary tissue biopsies from the same individuals. For cows HH, values close to 50% were obtained for somatic

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Table 1. Comparison of allele B-specific mRNA content as a percentage of total κ-casein mRNA in RNA preparation from mammary gland biopsies and from milk somatic cells for individual cows homozygous for the allele-specific accumulation of κ-casein mRNA (cows HH) and for cows heterozygous for the allele-specific accumulation of κ-casein mRNA (cows LH)

Values are means ± SD for $n = 3$

		Total RNA	
	Cow No.	Biopsies	Somatic cells
Cows HH	463	51·6 ± 1·3	48·4 ± 0·6
	5008	50·2 ± 0·2	50·6 ± 1·6
	5060	50·4 ± 1·0	51·6 ± 1·3
	5090	49·0 ± 1·3	50·0 ± 1·0
Cows LH	5033	57·6 ± 0·3	57·0 ± 1·2
	5068	55·1 ± 0·4	53·7 ± 0·6
	5013	55·5 ± 1·7	54·2 ± 1·5
	5102	56·9 ± 0·8	56·3 ± 1·2

cells and for biopsies, as expected. This proved the validity of using milk somatic cells as starting material to evaluate the allele-specific *CSN3* mRNA accumulation within mammary epithelial cells.

Mean values of B-specific mRNA content were 50·6 ± 0·5% and 54·0 ± 0·9% of the total *CSN3* RNA for cows HH and cows LH, respectively. Throughout lactation, the B-specific mRNA content ranged from 47·3 to 51·6% of total *CSN3* RNA and from 53·2 to 56·4% of total *CSN3* RNA for individual cows HH and individual cows LH, respectively. There was no difference ($P > 0·10$) in B-specific mRNA contents during the course of lactation within each group of cows.

The impacts of the *CSN3* expression genotypes on production traits per lactation, as evidenced by the data accumulated during the first three lactations are shown in Table 2. No significant association ($P > 0·10$) was found between the κ-CN genotype and milk, protein and fat yields.

Discussion

As the phenotype of a character depends on the cow's genotype modulated by various environmental factors such as physiological and health status, it is important to analyse phenotypic expression throughout lactation for the cows HH and LH, given that milk yield and composition vary greatly during the lactation period. Accordingly, we conducted a longitudinal study to estimate the variations of B-specific *CSN3* mRNA content during lactation for cows HH and LH.

A non-invasive approach was selected to minimize trauma caused by repetitive mammary gland biopsies. We used the cellular fraction of milk (the somatic cells) as a surrogate for lactating mammary tissue for RNA extraction. Somatic cells are known to contain mammary epithelial

Table 2. Milk, fat and protein yields per lactation over three parities for cows homozygous for allele-specific accumulation of CSN3 mRNA (cows HH) and for cows heterozygous for allele-specific accumulation of CSN3 mRNA (cows LH)

Values are means with SEM

	CSN3 expression genotype			
	Cows HH	Cows LH	SEM	*P* values
Mean of milk production traits				
Milk yield (kg)	8606	7948	479	0·24
Fat yield (kg)	334	305	23	0·28
Protein yield (kg)	282	257	15	0·16

cells that are shed into milk during the milk secretion process and milking. These cells have been reported to be viable and to exhibit the characteristics of differentiated alveolar cells (Boutinaud & Jammes, 2002). Results presented in Table 1 clearly show that B-specific mRNA content as measured in milk somatic cells and in biopsies from individual cows was similar for cows HH and for those genotyped LH. RNA preparations obtained from milk somatic cells are of sufficient quality for studying the regulation of milk protein encoding gene expression at transcriptional and post-transcriptional levels in terms of mRNA content. To our knowledge, it is the first time a study has demonstrated the effectiveness of using milk somatic cells to detect and estimate differences in mRNA accumulation within bovine mammary epithelial cells.

Using this approach, we were able to demonstrate that B-specific mRNA contents did not vary during lactation in cows HH and cows LH. This suggests that the phenotypic expression of the genetic mutation that causes the differential allele-specific accumulation of *CSN3* mRNA is not triggered by physiological factors, such as the fluctuation in the level of lactogenic hormones (Koprowski & Tucker, 1973), or by environmental factors that vary during lactation.

We also analysed mean production traits per lactation for the first three lactations, which can be considered indicative of lifetime performance (Ng-Kwai-Hang, 1990). We were unable to find any effect of *CSN3* expression genotype on production traits. The absence of significance can be explained by the limited number of cows included in the study. This occurred firstly because cows from one herd only were selected to eliminate herd effects in the statistical analysis. Secondly, cows κ-CN*AA and κ-CN*BB were excluded because genotyping can be carried out only on cows κ-CN*AB. Consequently, we could not include in the statistical analysis cows κ-CN*AA for which the two *CSN3* alleles express the gene into mRNA with relatively low efficiencies compared with cows κ-CN*AB genotyped LH and HH for *CSN3* expression genotype. This situation decreases the power of the statistical analysis

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to evaluate effects of *CSN3* expression genotype on milk production traits. Hence, it is important to locate the causal genetic alteration and to validate a genotyping methodology at DNA level that can replace quantitative RT-PCR in population studies.

In conclusion, the present study definitively settled that, in cows, allele-specific *CSN3* mRNA accumulation genotype is not linked to the genotype defining the protein variant of κ-CN found within milk (κ-CN*A and κ-CN*B) and that differential allele-specific *CSN3* mRNA accumulation was not modulated by hormonal status or other environmental factors during the course of lactation. This study also demonstrated the usefulness of milk somatic cells as a source of mammary epithelial cells for investigating gene expression of milk proteins at transcriptional and post-transcriptional levels under different physiological conditions.

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