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Gene Technology and Milk Production

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Summary

The introduction of gene technology to the practice of animal breeding has opened new venues as we enter the 21st century. Using DNA based genotyping the new genetic variants of milk protein genes were identified and basic regulatory mechanisms of the lactoprotein gene expression were discovered. Genomic and cDNA sequences for all major lactoprotein genes were deposited in the GenBank and comprehensive analysis of these data revealed the molecular basis of some quantitative effects which have been reported to be associated with particular genetic variants. In addition, comprehensive analysis of the animal genome enabled chromosomal localization of candidate regions bearing quantitative trait loci with effects on milk traits. The possibility to assess maternal and paternal inheritance of desired lactoprotein alleles can be utilised for efficient selection of desired haplotypes. Beside the possibility to change the milk composition through selection of favourable lactoprotein alleles, there is also the chance to manipulate milk composition *via* metabolic pathways, which regulate fat and carbohydrate synthesis. Reduction of the amount of saturated fatty acids and lactose in bovine milk are two interesting tasks for the future in order to adapt bovine milk to the requirements of modern human nutrition and to make bovine milk acceptable for special groups of consumers. Finally, the availability of recombinant growth hormone induced new technologies of milk production based on prolonged lactation with high persistence of milk yield.

Key words: DNA technology, milk composition, regulation of gene expression, growth hormone, QTL (quantitative trait loci)

Introduction

Milk is an essential source of energy, proteins, minerals and vitamins for young mammals in the first period of their life. In human nutrition, the consumption of ruminant milk and milk products is extended throughout life for a vast majority of the population. The high nutritional value of milk stimulated production of ruminant milk as one of the most important branches of animal production since domestication. In the modern dairy industry technological properties of milk are becoming more and more important due to the permanently increasing proportion of milk which is subjected to various manufacturing processes after production. The extensive research work in the 1970s revealed the primary structure of casein components *(1)* and led to the identification of four types of casein (α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN) and two major types of whey proteins (α-LA and β-LG). The genetic variants of caseins were used as markers for Mendelian segregation analysis and

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revealed tight linkage between three casein loci in a postulated relative gene order α_{s1}-CN-β-CN-κ-CN *(2)*. Numerous genetic variants were identified for all lactoprotein loci *(3)* and their influence on milk composition and technological behaviour of milk was demonstrated *(4,5)*. Lactoprotein variants have impact on quantitative *(6,7)* and qualitative milk traits *(8,9)*. The distribution of genetic variants among cattle breeds varies considerably *(10,11)* and despite some controversial results *(12)* lactoprotein haplotypes were considered as an additional selection criterion *(13)*. Introduction of molecular methods to practical animal breeding enabled direct genotyping of lactoprotein loci in both sexes and made selection for desired haplotypes possible. Genetics and biotechnology offered enormous potential for increasing the productivity and improving the quality of milk.

Molecular Characterisation of Lactoprotein Genes

In the 1980s, as the cDNA- and genomic sequences for major bovine lactoproteins became available *(14–18)*, the new era of DNA based typing of lactoprotein loci began. Previously described lactoprotein variants were first identified by RFLP analysis using DNA/DNA hybridisation *(19)*. The introduction of PCR enabled amplification of polymorphic fragments of the coding region followed by RFLP analysis *(20)*. More recently, application of allele specific primers and allele discrimination by primer length combined with automated detection of fragments with a DNA sequencing instrument has become a useful tool for genotyping of lactoprotein loci *(21)*. In some cases silent mutations, which could not be detected at the protein level, were used for discrimination of already known alleles: in the κ-CN B gene one *Pst*I restriction site is abolished due to a point mutation at the third position of the codon for amino acid 168 *(22)*. Application of DNA technology also enabled molecular characterisation of two new κ-CN variants (κ-CN F and κ-CN G) which were previously characterised only by their electrophoretic mobility *(23)*. Direct sequencing of PCR products made possible the identification of appropriate restriction sites for discrimination between κ-CN alleles A, B and E *(24)*. Application of polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) enabled fast and cost effective identification of four κ-CN alleles A, B, C and E *(25)* and identification of a rare bovine α_{s1}-CN allele D *(26)*. An interesting modification of PCR, based on amplification created restriction site (ACRS), has been developed for identification of a rare bovine β-LG variant I using *Sma*I *(27)*. In addition to the polymorphisms in the coding region, extended molecular analysis revealed also a polymorphic microsatellite in the third intron of the κ-CN gene, related to the previously described protein variants. This example proved that evolution of the polymorphism in the non-coding region was not independent from the evolution of polymorphisms at the DNA and protein level *(28)*. A new genetic variant at the α_{s1}-CN locus (α_{s1}-CN G) is characterised by an insertion of 371 bp relict of a long interspersed element (LINE) into the last noncoding exon (19th). Milk from heterozygous carriers of the α_{s1}-CN G allele contains about 6 % less true protein and total casein and has a lower casein number compared to control animals. The lower amount of α_{s1}-CN in milk obtained from heterozygous animals can be explained by a reduced mRNA stability of the α_{s1}-CN G allele *(29)*.

Occurrence of lactoprotein pseudogenes

In addition to the transcriptional active copies of α-LA and β-LG genes, a second inactive copy of both genes is present in the bovine genome. From a bovine genomic library a fragment beginning downstream of exon 2 and ending in the 3'-untranslated region of exon 4 of the bovine α-LA gene was isolated. This fragment showed 78 % DNA sequence similarity with the α-LA gene and was flanked by two directly repeated LINE sequences *(30)*. Similarly, the 4.8 kb fragment representing bovine β-LG pseudogene was found 14 kb apart from the β-LG gene *(31)*. The introns I–V are extremely divergent but exons I–V show sequence similarity in the range of 60–87 %. However, the sequence similarity with the β-LG gene in the last two exons (VI and VII) together with the last intron is about 92 %. It has been suggested that this is the result of a recent gene conversion event involving conversion of the pseudogene by the authentic β-LG gene *(31)*. β-LG pseudogenes have also been identified in goat and sheep *(32)*.

Exon skipping

Bovine α_{s1}-CN A is characterized by the deletion of the amino acid residues 14 to 26 of the mature protein. Comparison of the mRNA- and genomic DNA sequences of the α_{s1}-CN A revealed exon 4 skipping as the molecular basis for observed polymorphism *(33)* rather than deletion of the exon 4 sequence from the genomic DNA. Exon skipping is related to the allele specific mutation at position +6 in the splice donor site distal to exon 4. Partial skipping of exon 16 in the bovine α_{s1}-CN is also caused by a nucleotide substitution within the donor splice site *(34)*. Differential splicing of α_{s1}-CN pre-mRNA in goat causes occurrence of multiple forms of mature caprine α_{s1}-CN. Analysis of the α_{s1}-CN mRNA species demonstrated that shorter forms of the protein originate from alternative skipping of exons 13 and 16 and from the presence of the criptic splice site within the exon 11. Since these splicing abnormalities are present in alleles A, B and C, it has been suggested that alternative exon splicing is a general feature of caprine α_{s1}-CN *(35)*.

Organisation of the bovine casein gene cluster

Based on traditional linkage analysis clustering and relative gene order of four casein loci, α_{s1}-CN-β-CN-α_{s2}--CN-κ-CN was proposed *(36)*. *In situ* hybridisation studies revealed localisation of the casein gene cluster on bovine chromosome 6 *(37)*. Molecular proof of linkage and gene order was provided using long-range restriction analysis of the casein gene cluster *(38,39)*. Close linkage among casein gene loci is an important fact, which has to be considered in selection for favourable casein alleles. Pulse-field gel electrophoresis analysis showed that the length of the entire casein gene cluster is about 250 kb. The transcriptional orientation of the β-CN gene

is opposite to the orientation of the other three genes in the cluster *(40)*. From the evolutionary point of view the three related calcium sensitive casein genes (α_{s1}-CN-β--CN-α_{s2}-CN) arose from a common ancestral sequence through intra- and intergenic duplication and exon shuffling. They also share regulatory motifs in the proximal 5′-flanking region *(41)*. The last gene in the casein cluster (κ-CN) is not evolutionary related to the other casein genes, although it follows a similar expression pattern and its protein product is essential for micelle formation and stability *(18)*. However, the regulation of transcription of the bovine κ-CN gene might differ from transcriptional regulation of the other casein loci considerably.

Regulation of Lactoprotein Gene Expression

High level of tissue specific expression stimulated study of lactoprotein gene promoters. In addition to the binding sites for ubiquitous transcription factors (Oct-1, NF1, AP-2, YY1) binding sites for mammary gland specific transcription factor (mammary gland factor, MGF) were found in the promoter regions of the milk protein genes. MGF was identified as a mediator of prolactin response and belongs to the Stat (signal transducer and activator of transcription) family of transcription factors (Stat5) *(42)*. In the mammary epithelial cells Stat5 is present in two closely related variants, Stat5a and Stat5b, which are encoded by two distinct genes *(43)*. Putative binding sites for MGF were found in all proximal promoters of the lactoprotein genes. In order to elucidate the regulatory role of chromatin structure the DNase I hypersensitive sites were located in the region of β-LG gene *(44)*. The effect of methylation was studied at the 5' end of the α_{s1}-CN gene where hypomethylation at two *Hpa*II sites was observed *(45)*.

Association of different genetic variants with quantitative differences in expression stimulated detailed studies of allele specific polymorphisms in the promoter region of lactoprotein genes. Allelic variants of κ-CN and β-LG have the biggest effect on milk composition and technological properties of milk. In the proximal promoter region of the β-LG gene from different breeds, 14 polymorphic sites were identified *(46)* but only twelve are allele specific and five of them are situated within the potential transcription factor binding sites *(47)*. Mobility shift assay and DNaseI footprinting confirmed differential binding affinity of both promoters for activator protein 2 (AP-2), probably due to an allele specific mutation within the binding site for AP-2. Based on these results, we proposed a modulator role of AP-2 in differential allelic expression of bovine β-LG gene. In a subsequent cell experiment it has been shown that two promoter variants also differ in the expression of the reporter gene *(48)*. However, quantitative effect could not be restored after introduction of reciprocal mutations in the AP-2 binding site.

Sequence analysis of the κ-CN gene promoter of 13 animals from seven breeds revealed 15 polymorphisms within the proximal promoter region *(49)*. The functionality of the MGF binding site within the proximal promoter has been confirmed *in vitro (50)*. However, the detailed analysis of allele specific differences within 1 kb of the proximal promoter region in the Holstein-Friesian breed showed no allele specific polymorphisms between κ-CN variants A, B and E *(51)*. In order to find molecular background for differential allelic expression of the κ-CN gene, which has been reported at the protein level *(52)*, the additional 1 kb of the κ-CN promoter was sequenced. Although one allele specific polymorphism was found, it was not within a potential transcription factor binding site. Relative positions of putative transcription factor binding sites in the κ-CN promoter are shown in Fig. 1. Further experiments were focused on quantification of allele specific κ-CN mRNA in lactating mammary gland. Direct sequencing of RT-PCR and quantification of heterozygous bands were performed revealing similar A:B ratio (47:53) at the mRNA level as previously reported at the protein level (Fig. 2). Our current results allow the assumption that differential allelic expression of the κ-CN gene may rather be a consequence of differences in mRNA stability than differential transcription rate.

Transgenic animals and lactoprotein gene expression

In vitro expression systems allow analysis of regulatory mechanisms and study of the lactoprotein gene expression under simplified and standardised conditions *(53)*. However, the study of complex spatial and temporal expression patterns of lactoprotein genes in a hormone dependent manner requires the application of experimental animals. The possibility of modifying the animal genome in order to study regulatory mechanisms of lactoprotein expression make transgenic animals a very suitable tool for expression studies. Different combinations of promoters and coding regions for lactoproteins have been tested in numerous experiments. The bovine α_{s1}-CN and β-CN genes were successfully expressed in transgenic mice but α_{s2}-CN and κ-CN genes failed to express detectable amounts of proteins *(40)*. A number of experiments which failed to express the κ-CN gene under transcriptional control of the endogenous promoter suggest the presence of the cis--acting regulatory element, which might be a part of the locus control region (LCR), required for κ-CN expression. However, the rabbit κ-CN gene has been successfully expressed, albeit at low level, under its homologous promoter in transgenic mice *(54)*. Bovine and caprine β-CN promoters induced the expression of bovine β-CN *(55)* and bovine κ-CN *(56)* in transgenic mice, respectively. High level of rabbit κ-CN was also produced in transgenic mice under the transcriptional control of the rabbit whey acidic protein promoter *(57)*. Similarly, the bovine lactoglobulin gene was expressed successfully in the milk of transgenic mice *(58)*. The function of β-CN was studied using the β-CN deficient mice produced by gene targeting technology. Heterozygous and homozygous animals had less protein in milk and casein micelles with reduced diameter. It seems that β-CN has no essential function and that the casein micelle is quite tolerant to changes in milk composition *(59)*. Disruption of the α-LA gene in mouse using homologous recombination in embryonic stem cells caused α-LA defficient phenotype in homozygous mutant females *(60)*. They produced highly viscous milk, rich in

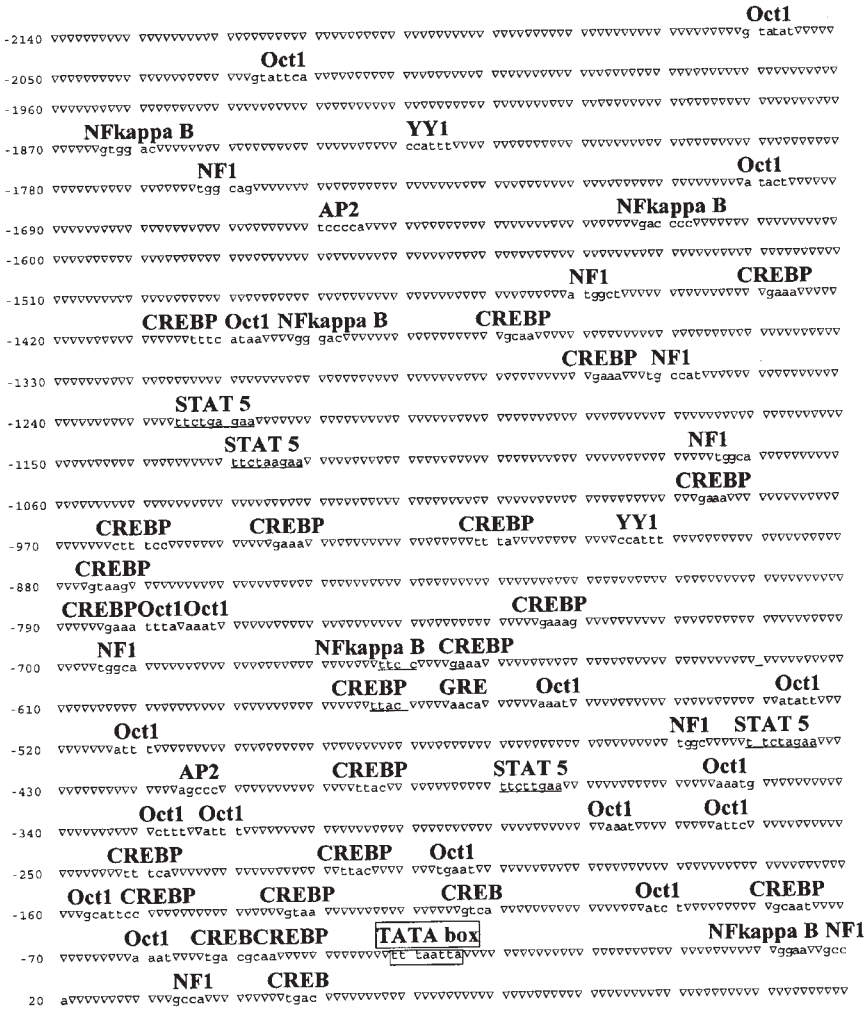

Fig. 1. Relative position of putative transcription factor binding sites within the 2140 bp of the κ-CN gene promoter

fat and protein but devoid of α-LA and lactose that pups were unable to remove from the mammary gland.

Recently developed technology enabled targeted deletion of genes from the mouse genome and allowed dissection of genetic components of mammary gland development. The knockout mice are the model of choice for functional and developmental studies in the mammary gland. A number of mutants lacking genes for hormones, growth regulators, receptors, transcription factors and proteins involved in the control of the cell cycle have been produced *(61)*. The study of these knockout phenotypes revealed new insights in development and function of the mammary gland. The important role of mammary gland specific transcription factors Stat-5a and Stat-5b was confirmed during development of the mammary gland as well as during lactation.

Manipulation of Milk Composition

The production of recombinant human proteins in the milk of transgenic farm animals offers a safe and renewable source of pharmaceutically important proteins. The capacity of the mammary gland to produce relatively high amounts of protein in milk and availability of efficient protein purification methods make production of biologically active proteins for pharmaceutical use also economically important. Anti-thrombin III from transgenic goats, α1-antitrypsin from sheep and α-glucosidase from transgenic rabbits, are already in clinical trials *(62)*. More than 20 recombinant proteins have been produced using transgenic technology in five species (cow, goat, pig, rabbit and sheep). The high efficacy of producing recombinant proteins in the mammary gland of transgenic animals can be illustrated by the estimate that only four transgenic pigs producing factor IX could produce 2 kg of this protein per year. This amount would cover the yearly demand for this protein world-wide *(62)*.

Another interesting topic is manipulation of milk composition in order to improve technological and dietary properties of milk. Transgenic animals, which produce intestinal lactase – phlorizin hydrolase in the mammary gland, are an attractive model for production of low-lactose milk, suitable for people with pronounced lactose intolerance *(63)*. Insertion of additional copies of lactoprotein genes under transcriptional control of different mammary gland specific promoters could alter protein concentration and influence micelle size and stability. Such modified milks could have improved cheese

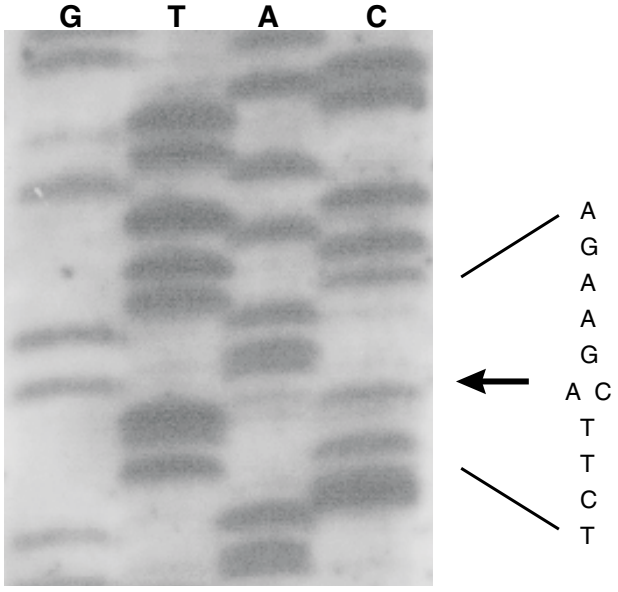

Fig. 2. Quantification of the allele specific mRNA from a κ-CN heterozygous animal using direct sequencing of the RT-PCR product and chemiluminescent labeling of the sequencing primer; the arrow indicates position of the heterozygous nucleotide (A/C). The photodensitometric quantification of both bands revealed relative ratio 47:53 between the κ-CN alleles A and B, respectively

making properties. The high proportion of saturated fatty acids in bovine milk fat raised nutritional concerns related to development of arteriosclerosis. Recent research has opened the prospects that selection of more effective desaturases could increase the proportion of unsaturated fatty acids in bovine milk. In addition, increased activity of stearoyl-CoA-desaturase could lead to higher proportion of conjugated linoleic acid in milk which would considerably improve the dietary value of bovine milk fat *(64)*.

Application of Bovine Growth Hormone (bST) in Milk Production

Availability of recombinant bovine somatotropin (bST) enabled stimulation of milk production through the application of bST. Administration of bST for four consecutive lactations caused an average increase of milk yield (3.7 kg milk/day) and 37 % more body weight compared to the control animals *(65)*. No considerable changes in milk composition and animal health were observed. The other aspect of bST application in milk production is the possibility of lactation prolongation. Extension of calving interval from 12 to 18 months had positive effects on conception rate and increased life milking performance in experimental animals *(66)*.

Relevance for Animal Breeding

Estimation of quantitative effects of allelic variants on milk yield and milk composition are often controversial. The reason might be that quantitative effects are not only caused by lactoprotein loci but rather by closely linked quantitative trait loci (QTL) *(67)*. Crossing over between lactoprotein loci and associated QTL would complicate estimation of the quantitative effect. For future breeding, we can expect to obtain a number of informative markers associated with the QTLs, which would facilitate direct selection of desired genotype. At present, β-LG B and κ-CN B in cattle and α_{s1}-CN A in goat are the only lactoprotein loci with clearly positive effects on milk composition and cheese making ability of the milk *(68)*. In our opinion, selection on desired lactoprotein haplotypes, as additional selection criterion, could be recommended especially in herds where large proportion of milk is subjected to manufacturing.

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Genska tehnologija i proizvodnja mlijeka

Sažetak

Uvođenje genske tehnologije u praksu uzgoja životinja otvorilo je nove mogućnosti pri prijelazu u 21. stoljeće. Koristeći tipizaciju gena na osnovi DNA, identificirane su nove genetičke varijante gena za proteine mlijeka te otkriveni osnovni regulacijski mehanizmi za ekspresiju laktoproteinskih gena. Genomske i cDNA sekvencije svih glavnih laktoproteinskih gena pohranjeni su u banci gena(GenBank), a opsežnom analizom tih podataka otkrivena je molekularna baza nekih kvantitativnih učinaka, opisanih u literaturi, koji su povezani sa specifičnim genetičkim varijantama. Osim toga, iscrpna analiza životinjskoga genoma omogućila je kromosomsku lokalizaciju u određenim područjima što sadržavaju bitne kvantitativne lokuse s utjecajem na značajke mlijeka. Mogućnost postizanja majčinskog i očinskog naslijeđa za određene laktoproteinske alele može se koristiti za djelotvornu selekciju poželjnih haplotipova. Osim mogućnosti promjene sastava mlijeka, selekcijom

poželjih laktoproteinskih alela, postoji i mogućnost mijenjanja sastava mlijeka metaboličkim putovima koji reguliraju sintezu masti i ugljikohidrata. Dva interesantna cilja u budućnosti su smanjenje količine masnih kiseina i laktoze u kravljem mlijeku, kako bi se ono prilagodilo zahtjevima suvremene prehrane i omogućilo da bude prihvatljivo specijalnoj skupini potrošača. Konačno, postojanje rekombinantnog hormona rasta omogućuje nove tehnologije u proizvodnji mlijeka, čime se postiže produljena laktacija uz trajno održavanje količine mlijeka.