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MILK PRODUCTION IN THE POST-GENOMIC ERA

Polona FRAJMAN [a)] and Peter DOVČ [b)]

[a)] Univ. of Ljubljana, Biotechnical Fac., Zootechnical Dept., Groblje3, SI-1230 Domžale, Slovenia.
[b)] Same address, Prof., Ph.D.

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ABSTRACT

Milk plays an important role in human nutrition. Nowadays, dairy industry is oriented in the production of increasing number of different milk products and technological properties of milk are gaining more and more attention. Introduction of recombinant DNA technology in the early 1970 and development of molecular genetics enabled studies of the organization of milk protein genes and mechanisms involved in their expression. Genome research in farm animals was oriented in production of low-density genetic maps with the emphasis on the genetic variation in some functionally important regions. In the public databases, 1598 cattle genes have already been mapped and partially sequenced by the end of 2003. In addition, numerous quantitative trait loci (QTL) were mapped for economically important traits. Typical examples include milk yield and milk composition in dairy cattle. The availability of genomic DNA sequences for a number of potential candidate genes with an impact on production traits allowed construction of cattle genome microarrays. Functional studies of milk protein genes revealed the impact of different genetic variants on technological properties of milk. Genomics approach thus offers an entirely new way to identify complex interactions among milk protein genes other genes involved in milk production and elucidation of the complex regulatory network allowing efficient milk production in the mammary gland.

Key words: milk production / technological properties / lactoproteins / molecular genetics / quantitative trait loci / QTL / genomics / micro array

PROIZVODNJA MLEKA V POST-GENOMSKI DOBI

IZVLEČEK

Sodobna mlekarska industrija se zaradi pomembnosti mleka v človeški prehrani usmerja v proizvodnjo vse večjega števila različnih mlečnih proizvodov, pri čemer v ospredje stopajo tehnološke lastnosti mleka. Uvajanje tehnik rekombinantne DNA v zgodnjih sedemdesetih in razvoj molekularno-genetskih tehnik sta omogočila raziskovanje organizacije mlečnoproteinskih genov ter mehanizmov, ki uravnavajo njihovo izražanje. Določanje nukleoticnega zaporedja celotnih genomov je postalo mogoče z razvojem zmogljivih orodij genomike. Raziskave genomov ekonomsko pomembnih domačih živali so bile usmerjene v proizvodnjo genskih kart z nizko gostoto in s poudarkom na genetskih variabilnosti funkcionalno pomembnih genov. V javno dostopnih podatkovnih zbirkah se je do konca leta 2003 nahajalo 1598 kartiranih in deloma sekvenciranih genov goveda. Kartiranih je bilo tudi mnogo kvantitativnih lokusiv (QTL) za ekonomsko pomembne lastnosti, kot so npr. količina in sestava mleka pri mlečnih pasmah goveda. Dostopnost genomskih zaporedij DNA za številne kandidatne gene z vplivom na proizvodne lastnosti je omogočila konstrukcijo genomskih mikromrež goveda. Funkcionalne študije mlečnoproteinskih genov so pokazale, da genetske variante vplivajo na tehnološke lastnosti mleka. Genomski pristop ponuja povsem novo pot pri proučevanju zapletenih interakcij

med mlečnoproteinskimi geni drugimi geni, ki so vključeni v kompleksno regulacijo učinkovite sinteze mleka v mlečni žlezi.

Ključne besede: mleko / prireja / tehnološke lastnosti / laktoproteini / molekularna genetika / kvantitativni lokusi / QTL / genomika / mikromreže

INTRODUCTION

Milk is a major source of energy, proteins, minerals and vitamins for young mammals during their first period of life. Milk of some farm animals plays an important role in human nutrition and milk production is one of the most important branches of animal production. Dairy industry in developed countries is nowadays oriented in the production of increasing number of different milk products therefore technological properties of milk are gaining more and more attention. The increasing cheese production, for example, prefers milk with higher content of proteins with favorable cheese making properties (Lodes at al., 1996; Buchberger and Dovč, 2000). Study of milk proteins started with the determination of primary structure of four major caseins (α_{S1}-CN, α_{S2}-CN, β-CN and κ-CN) and two whey proteins, α-lactalbumin and β-lactoglobulin (α-LA and β-LG) (Aschaffenburg and Drewry, 1957, Grosclaude *et al*., 1973, Godovac-Zimmermann et al., 1985). Polymorphisms in amino acid sequence of α_{S1}-CN, β-CN and κ-CN allowed classical linkage studies, revealing clustering of casein loci on bovine chromosome 6 (Grosclaude *et al*., 1973, Gupta *et al*., 1982). In this period, the early studies on the impact of casein variants on technological properties of milk were published (Mariani *et al*., 1976). Introduction of recombinant DNA technology in the early 1970 allowed determining of cDNA sequences for all four major caseins and two whey proteins (Stewart *et al*., 1984; Gorodetsky *et al*., 1988; Alexander *et al*., 1988). Based on DNA polymorphisms, rapid genotyping of casein loci has been introduced. Further development of molecular genetics enabled study of the exon-intron organization of casein genes as well as study of DNA sequences of non-coding regions of the casein gene cluster (Ferretti *et al*., 1990, Threadgill and Womack, 1990). These studies revealed an insight into molecular mechanisms involved into the regulation of casein gene expression (Rijnkels *et al*., 1997). Application of genomic tools combined with advanced statistical methods introduced the concept of QTL (quantitative trait loci) affecting complex milk production traits (Bovenhuis and Spelman, 2000). It became clear, that a huge number of genes are involved in this complex regulatory pathway, which is also influenced by numerous environmental factors (Schrooten *et al*., 2004). New genomic tools allow us to analyze expression of thousands of genes in one experiment and to compare gene expression profiles among different stages of lactation and different environmental treatments. Gradually growing understanding of complex genetic machinery regulating the quantity as well as quality of produced milk, represents a basis for efficient marker assisted selection in dairy cattle.

THE COMPLEXITY OF ANIMAL GENOMES

Development of recombinant DNA technology allowed researchers to move from analysis of cDNA sequences to the studies revealing genomic organization of larger chromosomal regions and finally to decipher the whole genome sequence of an organism. The most appealing goal was certainly sequencing of a human genome, one of the most complex endeavors of the modern science, which was accomplished in 2001 (Bork and Copley, 2001). However, on the way to the sequence of human genome a number of less complex genomes, mainly from model organisms were sequenced, including microorganisms as *E.coli*, and *S. cerevisiae*, fruit fly *D. melanogaster* worm *C. elegans* and zebra fish. After the human genome, comprising 3.6 billion nucleotides, the mouse and rat genomes which are of similar complexity were released in 2002 (Anon. 2002)

and 2003 (Bromberg *et al*., 2003). Just recently, the chicken genome sequence was completed, representing a vertebrate genome of a bit lower complexity, containing 1.1 billion base pairs (McPherson *et al*., 2004). The avaiability of whole genome sequences for a number of more or less related species opens a whole new avenue of comparative genomic approach for the identification of gene function and regulation of complex metabolic pathways. The disappointing conclusion from the analysis of the first sequenced genomes is that from about 30.000 putative genes which were identified within the genome, to only 30–40% can be assigned a function, whereas physiological role for about 60% of the genes remains unknown.

The strategy of genome research in farm animals was a bit different from the strategy amployed by the human genome project. Since the available resources in farm animal genome research are incomparable with resources mobilized within the human genome project and large of species of interest further reduces the research inputs, the strategy had to adapt to this circumstances. Therefore, considerable effort has been spent in order to produce low density genetic maps of different species, which have enabled rough localization of selected loci into syntenic groups (Gellin *et al*., 2000). Synteny maps provided valuable information for practical animal breeding facilitating haplotype selection rather than simple selection for desired genotypes. For the practical animal breeding, the information about the genetic variation in some functionally important regions is far more important than entire nucleotide sequence from one animal. Therefore relative large population studies analyzing genetic polymorphisms within crucial genomic regions were performed.

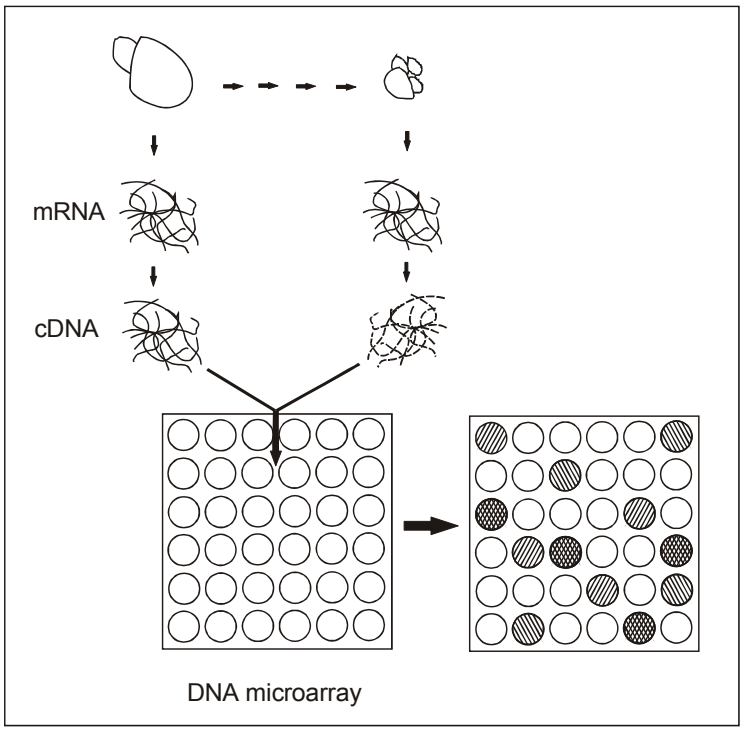

Figure 1. Developoment of microarrays enabled large-scale analysis of gene expression.
Slika 1. Razvoj mikromrež je omogočil sočasno analizo izražanja velikega števila genov.

GENOMIC TOOLS

DNA sequence analysis started with establishment of cDNA libraries, where relatively short DNA fragments (up to 2-3 kb) were cloned in the plasmid vectors. However, prerequisite for genomic research was the ability to handle large genomic sequences in the range of 0.1 – 1.0

Mb. The discovery of the new generation of vectors as yeast and bacterial artificial chromosomes (YAC, BAC) enabled researchers to clone large stretches of genomic DNA and to produce genomic libraries (Eggen *et al*. 2001), covering the entire genome in a reasonable number of overlapping clones. Further development and automation of DNA sequencing procedures was another important milestone, making genomic research feasible. Organization of DNA sequences in public databases, allowing searching for DNA sequences from different species and bioinformatics tools for sequence analysis made analysis of complex genomic data accessible for a wider scientific community. The number of DNA sequence entries in the public databases was growing exponentially during the last 20 years. Finally, in addition to powerful technology which allowed sequencing of entire genomes, microarray technology enabled analysis of gene expression in the whole genome in a single experiment. A new term, transcriptomics, was coined describing a high throughput analytical approach for the study of transcriptional activity of the genome. This approach can provide information about differential gene expression in different developmental and physiological stages as well as reaction to different environmental stimuli.

GENOMICS APPROACH IN FARM ANIMALS

Historically, pedigree analyses and establishing of suitable mapping populations was an important goal of animal genetic research. In farm animals creation of special mapping populations is often too costly, therefore suitable statistical models (e.g. daughter design) were developed in order to extract genetic information from already available population structure (Mosig *et al*., 2001) . An important tool in genomic research in farm animals were radiation hybrid cell panels, which allowed physical assignment of gene loci to genetic map. The fluorescent in situ hybridization was also successfully applied for physical mapping. Further development of animal gene maps was reached by the introduction of highly polymorphic genetic markers as RFLPs, microsatellites and single nucleotide polymorphisms (SNPs) for the fine mapping using reference populations. Recombination studies enabled narrowing of the mapping interval for the localization of candidate genes to the interval shorter than 1 cM, and introduction of large-capacity vectors (BAC, YAC), made physical cloning of candidate gene regions feasible. Genomic libraries containing ordered collection of large genomic fragments (mostly BAC clones) represent one of the most important genomic resources for genomic research in every species. At present BAC libraries with several fold coverage of the genome are available for all farm animal species. However, some genomic regions are still poorly covered and significant gaps are present in most of such libraries. More recently, expression sequence tag (EST) libraries from different tissues were established, serving as an excellent tool for identification of expressed genes. Information from EST libraries has been also used for assignment of gene ontology, shedding a new light into the functional organization of the genome. Since polygenic traits are of crucial importance in animal breeding, statistical methods for identification of genomic regions with significant phenotypic impact on quantitative traits have been developed. The concept of quantitative trait loci (QTL) overruled the old infinitesimal model of gene action. Using different strategies, genomic regions explaining 10–15% of the phenotypical variance were identified.

One of the most frequently used strategies based on genetic analysis of phenotypic tail of the population is presented on figure 2.

Two approaches are mainly used in mapping of production trait genes in farm animals:

1. *Candidate gene approach***:** the target gene can be identified via clinical symptoms or physiological changes from human or mouse. This approach is successful for monogenic traits as some inherited disorders and some simple traits as coat color etc.

2. *Positional cloning approach*: there is no clear candidate gene evident. Linkage analysis pinpoints chromosomal region with unknown gene and subsequent fine mapping of the region can identify candidate genes by positional cloning. Using this approach the inconsistency of QTLs across breeds is a serious problem, hampering reliable localisation of the target region.

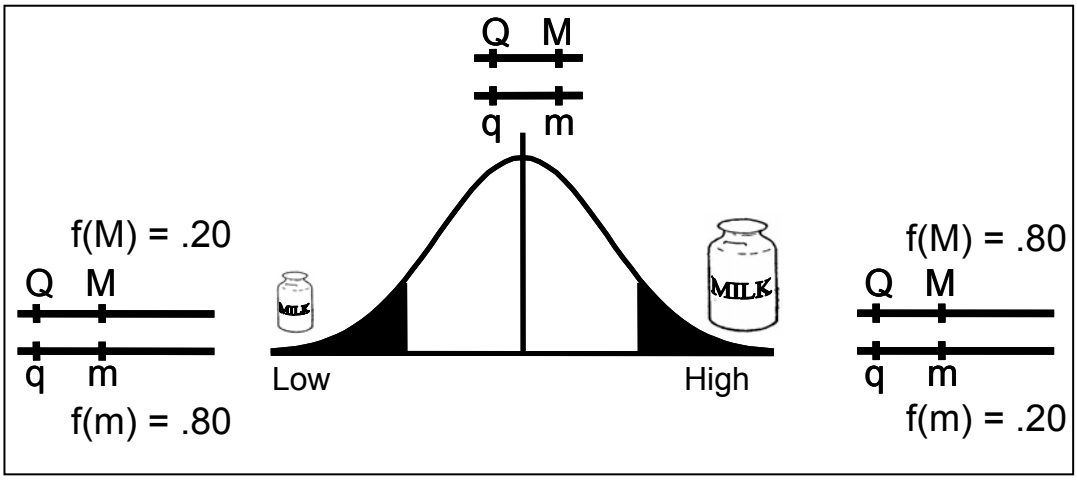

Figure 2. Association of marker gene polymorphisms and linked QTL locus with quantitative trais enables identification of genomic regions with QTL loci.
Slika 2. Asociacija polimorfizmov genskega markerja in vezanega kvantitativnega lokusa (QTL) s fenotipskimi lastnostmi, omogoča identifikacijo regij genoma, kjer se nahajajo QTL.

STATE OF THE ART IN CATTLE GENOME RESEARCH

Improvement of farm animal's production traits started immediately after domestication, when people begun to breed animals for a certain purpose in adapted environment. Classic selection is based on phenotypic performance which can be recorded either directly on the individuum (performance test) or its relatives, mainly offspring (progeny test). However, in both cases the impact of genes affecting particular trait is blurred by a number of environmental factors. The development of recombinant DNA techniques in the last few decades enabled identification of genes that underlie genetic variation of production traits observed in livestock species. Identification of these genes is expected to contribute considerably to the development of more efficient selection procedures, which will employ genetic markers. This strategy, called marker assisted selection (MAS) will allow also better insight in the physiological background of corresponding traits (Davis and DeNise, 1998).

In cattle, several national and international projects were focused on production of molecular markers and improvement of existing genetic maps. In addition to that, interest in whole genome sequencing was growing with the improvement of technical tools for such project. At present the main initiative for whole genome sequencing is shared between research institutes at Baylor, NHGRI/NIH and A&M in Texas, as well as Canadian and New Zealand groups. The plan was to achieve 5–7 fold genome coverage using the shot-gun sequencing by the end of 2004. In the public databases 1598 cattle genes have already been mapped and partially sequenced by the end of 2003. More than 322.000 EST sequences were determined and deposited in public databases. The most comprehensive information regarding cattle genome organization is available from genome databases such as ArkDB (http://www.thearkdb.org/browser?species=cow) and BOVMAP. Cattle linkage maps contained in 2003 more than 2000 mapped loci, which is

comparable with about 2000 mapped loci on radiation hybrid maps. In addition, numerous QTLs were mapped for economically important traits. Typical examples include milk yield and composition in dairy cattle and growth and carcass characteristics in beef cattle. Two main strategies have been employed in order to identify genes underlying QTLs:

- experimental crosses between two strains, breeds or subspecies were performed to identify the genes contributing to the differences observed for a trait of interest between these two strains (breeds, subspecies).
- mapping of QTLs that are underlying the genetic variance, observed for a trait of interest in a commercial population, was carried out with a help of the outbred pedigrees.

Mapping of QTL is in general not very straightforward procedure because of the large genome regions occupied by them. QTLs normally comprise about 20 to 40 millions of base pairs containing several hundreds of genes, representing 1/50 to 1/100 of the whole genome. In addition, quantitative traits are affected by different breeding methods, interactions between environment and genotype, epistatic effects and by genetic imprinting. The precision of QTL mapping is therefore significantly reduced compared with a single gene locus. Another problem, associated with QTL identification is, that even they are determined in one population (breed) their consistency between populations is often low. Projects attempting to map genes affecting milk production traits in dairy cattle populations demonstrate different experimental designs. The Bov MAS project is one of the largest initiatives dealing with QTLs affecting milk production. The aim of this EU funded project is mapping of genes, which have an impact on milk production in order to provide tools for successful marker assisted selection.

Conservation of genome organization between cattle, sheep and goat was already demonstrated comparing mapping data from these species. The most advanced genome map in cattle can therefore serve as a model for sheep, goat and even deer genome mapping.

The availability genomic DNA sequences for a number of potential candidate genes with an impact on different production traits allowed construction of cattle genome micro arrays, which can be applied for large scale expression profiling in different physiological states, during infection and at different production levels. Complex expression profiles can help by identification of co-expressed genes and genes being involved in the same physiological pathways.

FUNCTIONAL STUDIES IN MILK PROTEIN GENES

Linkage analysis revealed clustering of all four casein loci in the relative gene order αs1-CN-β-CN-αs2-CN-κ-CN (Grosclaude *et al*., 1973). In the 1980s as the cDNA- and genomic sequences for major bovine lactoproteins became available (Stewart *et al*., 1984; Stewart *et al*., 1987; Vilotte *et al*., 1991; Gorodetsky *et al*., 1988; Alexander *et al*., 1988; Bonsing and Mackinlay, 1987) the new era of casein research began. Exon intron organization and nucleotide sequences of regulatory regions were determined. In situ hybridisation studies revealed localisation of the casein gene cluster on the bovine chromosome 6 (Gupta *et al*., 1982). However, the molecular proof of linkage and gene order was provided later using the long-range restriction analysis of the casein gene cluster DNA (Ferretti *et al*., 1990; Threadgill and Womack, 1990), which occupies about 250 kb of genomic DNA. The transcriptional orientation of the β-CN gene is opposite to the orientation of the other three genes in the cluster (Rijnkels *et al*., 1997). From the evolutionary point of view the three related calcium sensitive casein genes (αs1-CN-β-CN-αs2-CN) arose from the common ancestor through intra- and intergenic duplication and exon shuffling. They also share regulatory motifs in the proximal 5' flanking region (Groenen and Van der Poel, 1994). However, the kappa casein gene (k-CN), the last member of the casein gene cluster is not evolutionary related to the other casein genes, although

it follows the similar expression pattern and its protein product is essential for micelle formation and stability (Alexander *et al*., 1988).

All casein genes are present in numerous genetic variants (β-CN: 7, κ-CN: 6, α_{s1} –CN: 6, α_{s2}-CN: 4). Frequencies of casein genetic variants are breed-specific and, with exception of α_{s2}-CN, have an impact on milk composition and technological properties of milk. Their expression is hormonally regulated by lactogenic hormones prolactin, glucocorticoid and insulin. They act either directly by binding to DNA (like glucocorticoid hormone) or via different signal transduction pathways and transcription factors (TF) as activators of lactoprotein gene transcription by binding of TF on their binding sites in promoter regions of lactoprotein genes. Prolactin activates STAT5, which is a most important activator of lactoprotein gene expression (Doppler *et al*., 2001). Considering consensus sequences of different TFs, their potential binding sites within the promoter regions could be identified and than further analyzed for their functionality using functional studies on cell cultures or transgenic animals.

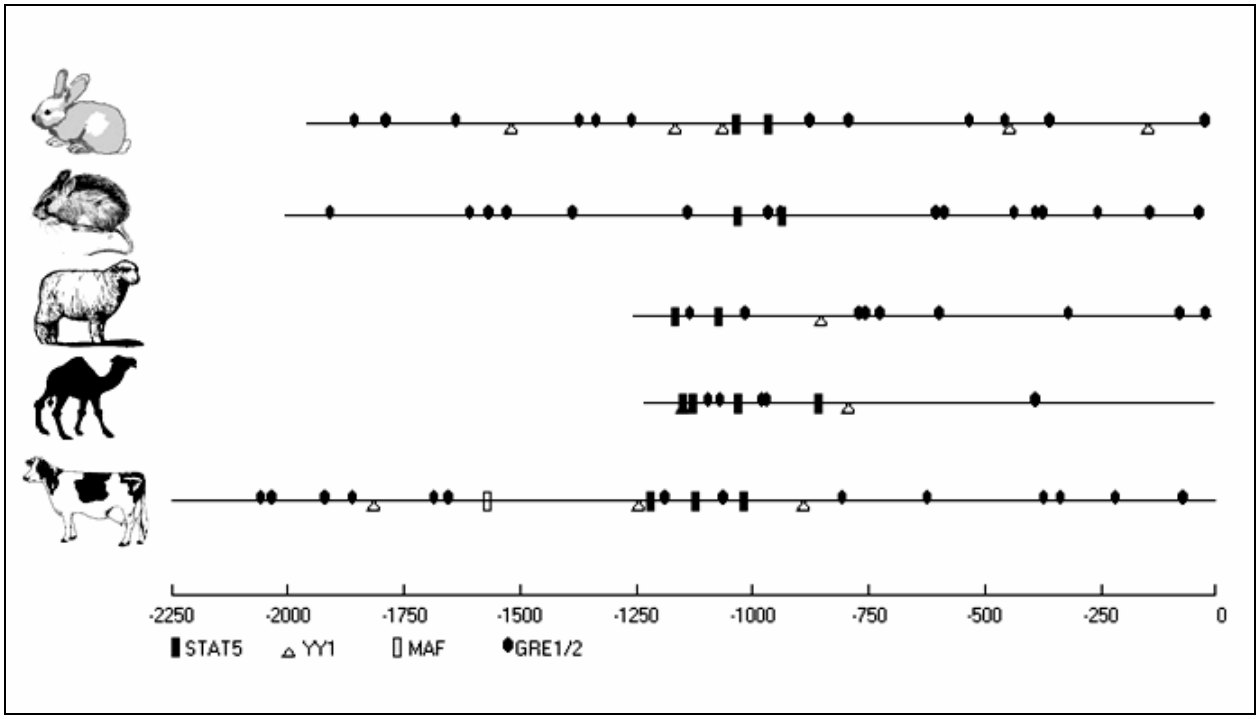

Figure 3. Diagrammatic representation of putative transcription factor binding sites in 2250 bp of the bovine κ-casein promoter compared to distribution of the putative TF binding sites in the κ-casein gene promoters of some mammalian species. Putative transcription factor binding sites were identified using consensus transcription factor binding sequences.

Slika 3. Shematska ponazoritev domnevnih vezavnih mest transkripcijskih faktorjev (TF) v 2250 bp κ-kazeinskem promotorju goveda, v primerjavi z razporeditvijo domnevnih vezavnih mest TF v κ-kazeinskih promotorjih pri nekaterih drugih živalskih vrstah. Ugotavljanje domnevnih vezavnih mest je potekalo s pomočjo konsenzus zaporedij transkripcijskih faktorjev.

In the case of the bovine κ-CN promoter, potential TF binding sites were determined on the basis of sequence similarity with consensus sequences (Adachi *et al*.; 1996; Debeljak *et al*., 2000,). Four potential STAT5 binding sites were selected for further functional analysis using EMSA. Two promoter fragments of different length (925bp and 2064bp) were used for

luciferase reporter gene expression study in bovine mammary epithelial cell line BME-UV1. The expression experiment revealed important positive regulatory elements in the distal part of the bovine k-CN promoter (Debeljak *et al*., 2004, in press).

The 3' region of the mRNA can also have an impact on gene expression level, mostly by its effect on the length of polyA tail. It has been shown, that the length of polyA tail in the β-CN is affected by lactogenic hormones prolactin and glucocorticoid and is therefore changing during the different stages of lactation. In the fourth and fifth exon of the κ-CN gene, several polymorphisms were found, which could potentially influence the length of the polyA tail in different genetic variants of κ-casein (Debeljak, 2000).

Studies on the bovine β-lactoglobulin gene showed the importance of a transcription factor AP-2 binding site for a high level of β-lactoglobulin expression (Lum *et al*., 1997). A single point mutation in the promoter region, binding AP-2 caused significant reduction of β-lactoglobulin/luciferase reporter gene in the HC11 cell line (Folch *et al*., 1999). Recently this polymorphism has been confirmed as a first known genetic polymorphism in lactoprotein genes with clear quantitative effect (Kuss *et al.*, 2003).

DESIGNER MILK

The tissue specific expression of milk proteins offers a safe and renewable source for production of recombinant human proteins, useful for pharmaceutical industry, in the milk of transgenic farm animals. 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 attractive. The human transferin from transgenic cattle, anti-thrombin III from transgenic goats, a1-antitripsin from transgenic sheep and α-glucosidase from transgenic rabbits are examples of successful introduction and expression of human genes in the farm animal mammary gland (Rudolph *et al*., 1999). More than 20 recombinant proteins have been produced using transgenic technology in five species (cow, goat, pig, rabbit and sheep). The efficacy of recombinant protein production in the mammary gland of transgenic animals is best illustrated by the fact that only four transgenic pigs producing factor IX could produce 2 kg of this protein which represent yearly demand for this protein worldwide.

Another attractive field of research represent manipulation of milk composition in order to improve technological and dietary properties of milk. An example for a model for production of milk with reduced level of lactose are transgenic animals, which produce intestinal lactase - phlorizin hydrolase in the mammary gland, producing milk, suitable for people with pronounced lactose intolerance (Jost *et al*., 1999). 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 (Baranyi *et al*., 2004). Such modified milk could have interesting cheese making properties. The high proportion of saturated fatty acids in bovine milk fat raised nutritional concerns related with development of arteriosclerosis. Selection of dairy cattle for 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 (CLA) in milk, which would considerably improve dietary value of bovine milk fat (Bauman and Perfield, 2002). The content of CLA in milk is mostly influenced by nutrition and also by physiological factors such as breed, fertility, stage of lactation, level of the CLA desaturase. With changing of nutrition the level of CLA in milk could be up to five folds higher. In cattle, breed differences in CLA content in milk were observed, influenced by index of CLA-desaturase. Differences in CLA content in the milk fat could be up to three fold among individuals with the same nutrition with no remarkable

influence of the breed, parity or stage of lactation. Physiological and genetic background of individual differences in CLA content in milk still remains to be defined.

MASTITIS

Mastitis is most prevalent disease in dairy cattle, affecting around 40% of dairy cow population. Mastitis is a disease with low heritability and therefore selection attempts has little success. In sheep, but not in cows, genetic correlation between somatic cell count in milk and quantity of milk, was observed (Barillet *et al.*, 2001). Clinical forms of mastitis in sheep are rare but it seems that it would be possible to lower even subclinical forms of mastitis with selection for mastitis resistance using somatic cell count.

Recent research is oriented in identification of relevant genes and mechanisms controlling the pathogen specific immune defence in the mammary gland of ruminant dairy species. State-of-the-art functional genomics techniques are used to analyse specimens from udders of cows and goats, which have been experimentally infected with different pathogens. Genes with relevance for pathogen-specific immune defence can be characterized by comparative transcriptome analyses. Hierarchical clustering of the data is the next step in identification of relevant genes involved in immune response. Characterization of their genetic variants in relationship to relevant QTL will help dairy cattle breeders to improve dairy cows' genetics for mastitis resistance.

CONCLUSION

Genomics approach to identification of important genes with an impact on the milk quantity, quality and composition offers an entirely new way to identify complex interactions among genes and their physiological role for milk production. For the first time we can anaylse complex relationship among genes within the entire genome and perform complex expression experiments, which were not feasible without high throughput genomic tools. As a consequence, the faster and more efficient selection for higher productivity, and even more interesting, for milk characteristics, influencing technological properties of milk or having better impact on human health will be possible. For example, selection on certain favorable haplotypes (κ-CN B and β-lactoglobulin B) will contribute to better technological properties of milk, especially cheese making properties. In the future, more robust QTLs have to be defined and search for appropriate markers for MAS needs to be continued. Functional polymorphisms within genes influencing production traits have to be evaluated *in vitro* and *in vivo* in order to enable selection for alleles with positive effects. And finally, gene expression patterns, revealed in micro array experiments will allow identification of new candidate genes involved in the expression of production traits.

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