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HOW MOLECULAR GENETICS CAN CONTRIBUTE TO MODIFYING MILK COMPOSITION

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Summary

Structure and expression of caseins and milk whey protein-encoding genes have been throughly investigated in several species following the development of molecular biology, and nucleotide sequences of mRNAs (cDNAs) and genes have been reported (reviewed in Mercier & Vilotte, 1993). These genes, due to their high, developmentaly regulated and tissue-specific expression in mammary epithelial cells, could function as a paradigm to understand tissue-specific gene regulation. Furthermore, since several milk protein variants were known in ruminants, the identification at the molecular level of the relevant mutations was investigated both to allow the genotyping of these animals at any age, independently from the sex, but also the analysie the relationship between the structure of the gene and its expression level. Finaly, these genes were used in transgenic experiments to utilize mammary gland as a bioreactor or to produce milk with different nutritional or technological properties.

Introduction

Structure and expression of caseins and milk whey protein-encoding genes have been throughly investigated in several species following the development of molecular biology, and nucleotide sequences of mRNAs (cDNAs) and genes have been reported (reviewed in Mercier & Vilotte, 1993). These genes, due to their high, developmentaly regulated and tissue-specific expression in mammary epithelial cells, could function as a paradigm to understand tissue-specific gene regulation. Furthermore, since several milk protein variants were known in ruminants, the identification at the molecular level of the relevant mutations was investigated both to allow the genotyping of these animals at

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I - Molecular analysis of genetic variants: Polymorphism of goat caseins

The casein fraction of ruminants' milk consists in four caseins, namely αs1, αs2, β and κ, encoded by four clustered genes which in cattle reside in a 220/250-kb DNA fragment (Threadgill and Womack 1990; Ferretti et al., 1990) mapping on chromosome 4 of cattle, sheep and goats (Hayes et al., 1993). Rather to make only an exhaustive overview of the genetic polymorphism of goat caseins, emphasis will be first given to one of them (αs1-casein) which provides a meaningful example illustrating how our current knowledge of caseins polymorphism and the molecular biology of the relevant genes have contributed to improve goat milk protein quality and may be used to modify protein composition of milk.

1 - Polymorphism of the goat as1-casein

An unusual and complex polymorphism, was first discovered at the locus αs1-Cas, and shown to be responsible for a large individual variability in the casein content in milk (Boulanger et al., 1984; Grosclaude et al., 1987; Mahé and Grosclaude, 1989). Structural analyses performed at the protein (Brignon et al., 1989; 1990) as well as the genomic level (Leroux et al., 1990), definitely confirmed this genetic polymorphism and prompted the speculation that it might be exploited to improve, through selection, cheese-making properties of goat milk. The quantitative allelic variability which further adds to a high level of qualitative polymorphism, makes this system a strikingly original model, the molecular bases of which are today almost complately elucidated.

1.1 - Molecular bases

The most recent genomic data (Martin, 1993; Grosclaude et al., 1994) show the existence of at least 14 alleles at this locus, distributed in 7 different classes of protein variants (αs1-CasA to αs1-CasG), associated with 4 levels of expression ranging between 0 (αs 1 - Cas0) and 3.6 g/l (αs1-CasA, B and C), per allele. Whilst the αs1-casein E variant, which is 199 amino acid residues in length, only differs form variants A, B and C by amino acid substitutions (Brignon et al., 1989), variant F appears to be internally deleted by 37 residues (Brignon et al., 1990), leading to the loss of the multiple

phosphorylation site, a hydrophilic cluster of five contiguous phosphoseryl residues: SerP64-SerP-SerP-SerP-SerP-Glu-Glu70.

The establishment of the overall organization of the goat αs1-casein gene (19 exons scattered along 17 kb), the characterization of alleles F (Leroux et al., 1992) and E (Jansà Pérez et al., 1994) at the genomic level and a detailed analysis of their transcription products, have allowed to demonstrate that: i) deletion occurring within variant F arises from the outsplicing of three exons (9, 10 and 11) during the processing of primary transcripts, likely due to a single nucleotide deletion within the first unspliced exon (exon 9); ii) allele αs1-CasE contains a 457-bp insertion within the 19th and last untranslated exon, which corresponds to a truncated long interspersed repeated element (LINE) highly repeated in the goat genome. In both cases, as compared with the αs-1CasA allele, a reduction i the amount of mRNA, accounting for a lower content of as1-casein in milk, was observed. Such a reduction might be due either to a loss in the efficiency and accuracy of the splicing machinery (allele F) or to a decreased stability of the messengers (allele E).

A further internally deleted variant (αs1-CasG), also associated with a reudced αs1-casein content in milk, has been recently identified and characterized (Martin and Leroux, 1994). N-terinal amino acid sequence data and cDNA sequencing revealed that exon 4 (39 nt) is cleanly skipped during the course of pre-mRNA splicing. The mutational event responsible for outsplicing of exon 4 sequence, encoding amino acid residues 14 to 26 in the peptide chain, is a transition (G - > A) occurring in the 5' splice site consensus sequence of the following intron. In addition, one of the two null alleles detected so far (Leroux et al., 1990) is currently under investigation. Preliminary results obtained at the genomic level clearly demonstrate that αs1-CasO is severely deleted (over 8 kb) in its 3' region.

1.2 - Impact on technological properties and production traits

Deletion occurring in variant F removes a highly hydrophilic part of the molecule including the multiple phosphorylation site involved in calcium binding. This modifies the chromatographic behaviour of the protein (Jaubert and Martin, 1992) and the physico-chemical properties of the casein micelle, particularly in terms of siye and calcium content (Remeuf, 1993). The αs1-CasG variant, recently described in the goat species as internally deleted of 13 amino acid residues in its N-terminal part, has its counterpart in cattle. Indeed, the same peptide segment is also absent in the rare αs1-CasA variant detected in Holstein breed. One can therefore assume that the goat variant might lead to the same defects as the bovine, known to form a softer curd. In addition, proportions of the four caseins in milk of goats homozygous F/F at the αs1-

Cas locus are quite different from those of goat bearing two alleles associated with a high αs1-casein content (e.g., homozygous A/A). As expected, differences in the proportion of caseins, together with differences in the physico-chemical properties of αs1-casein variants, induce important differences in the tehnological properties of relevant milks.

In 1987, alleles αs1-Cas F and E, associated with a reduced amount of αs1-casein (0.6 and 1.6 g/l per allele, respectively), still accounted for 75 to 84%, depending on the breed, of the total αs1-Cas alleles in French milking flocks. Such a situation has been also observed in several european dairy goat breeds. Therefore an opportunity existed to increase the casein content of milk by selecting individuals for that trait. However, before committing to a selection programme in favour of "strong" alleles, it was necessary to assess possible effects of the polymorphism on production traits.

On-farm performance recording of the progeny of 5 bucks heterozygous at the αs1-Cas locus, has provided data indicating that the αs1-casein genetic polymorphism has no effect on the yield of milk but, unexpectedly, also revealed a higher fat content in A/A type milks (Mahé et al., 1994). As far as the physico-chemical and technological properties of milks are concerned, A/A type milks exhibit smaller micelles and provide significantly higher cheese yields than the F/F milks do, due to higher casein and fat contents (Remeuf, 1993). These results were confirmed in traditional processing of "Pélardon des Cévennes" type cheese whose goat flavour would be less pronounced with cheeses made form type A/A milks (Vassal et al., 1994).

1.3 - Typing procedure

Improvement of milk processing quality by selectin for interesting alleles was not easy to perform until recently, since milk production is a sex-limited trait and caseins are only expressed in lactating females. Therefore, expensive and lengthy progeny testing schemes were required to ascertain the genotypes of males at these loci. Developments and progress in DNA technology, as well as increasing knowledge in the molecular genetics of milk proteis, have made it possible genotyping of animals of any age and sex for milk proteins, thus providing and efficient tool for selection.

A procedure, based on the RFLP technique, which does not allow the determination of all possible phenotypes, was first proposed by Leroux et al. (1990). Characterization at the nucleotide level of the different alleles and the establishement of the structural organization of the goat αs1-casein transcription unit have provided information necessary for developing an allele-specific typing procedure, relying upon the PCR technique (Amigues, et al., 1995). This procedure, by which an early genotyping of animals, including he-

goats, can be achieved readily and automatically using the fluorescent DNA sizing technology, has allowed an increase in the efficiency of selection programmes since today allelic frequencies have reversed. Thereby, it has contributed to improving the cheese-making quality of goat milks.

2 - Polymorphisms at the other casein loci

β-casein, which is the major protein component in goat's milk, has been for a long time considered to be monomorphic, until Dall'Olio et al. (1989) first find, in the Italian Garganica breed, a milk sample in which β-casein was lacking. This phenotype has been detected later in island goat populations (Corsica and Creole of Guadeloupe) and very recently in the Pyrenean French breed (Mahé, 1991, unpublished; Mahé and Grosclaude, 1993; Ricordeau, 1995, unpublished). The genetic bases for this null phenotype has been investigated in the Creole goat population and the existence of 2 different null alleles has been suspected (Mahé and Grosclaude, 1993). In addition to the common β-CasA allele, these authors also report the existence of a further rare allele (β-CasB). From these data one can expect that at least 4 alleles exist at the β-Cas locus. The most frequently encountered null allele (β-CasO) has been characterized at the nucleotide level and shown to exihibit a single nucleotide deletion in the seventh exon of the gene (Persuy et al., 1995, unpubished). As previously mentioned for the αs1-CasF allele, such a mutational event provokes a frame shift, leading to the occurrence of a prematured stop codon, and likely induces a dysfunction of the splicing machinary whose efficiency is then dramatically reduced. This phenomenon, is probably responsible for the observed very low amount of unfunctional messengers and, thus, the absence of β-casein in milk.

As far as technological properties of β-null type milks are concerned, Chianese et al. (1993) have shown that individual milks from several italian breeds which did not contaitn β-casein give longer renneting times and weaker curd firmness.

αs2-casein is also polymorphic in the goat. Beside the two variants (αs2-CasA and B) initially described by Boulanger et al. (1984) in Alpine and Saanen breeds and later biochemically characterized by Bouniol et al. (1993), a third variant (αs2-CasC) hs been identified (Bouniol et al., 1994). The three variants only differ by amino acid substitutions. The existence of a fourth putative αs2-casein variant, only characterized by its highers anodic mobility amongst the casein components, has been reported (Chianese et al., 1992).

The existence of a polymorphism at the κ-Cas locus has been first suggested by Russo et al. (1986) and later confirmed by Di Luccia et al.

(1990) who identified two κ-casein variants (A and B) that possibly differ for an Arg (variant B) residue presumably located in the N-terminal part (para-κ-casein) of the protein.

3 - General impact of molecular biology

As highlighted above, the application of molecular biology has allowed the characterization of already known and the identification of new alleles. Thus, these observations are of some help in the understanding of spliced tissue-specific gene expression. Furthermore, it has ultimately resulted in the development of genotyping procedures based on PCR analyses that allow the selection process to take place at birth, independently of the sex of the animal. However, these studies are obviously limitated by the naturally existing polymorphism. An alternative is to create new genetic variation by transgenesis.

II - Creation of new genotypes by transgenesis

Transgenesis allows the obtention of new genotypes that do not naturally exist and cannot be obtained through natural selection. Furthermore, it enables the species barrier to be transversed. Since 1986 the potential of this technology for modifying milk composition, either altering the nutritional and/or tehnological properties of milk (Mercier, 1986) or by using the mammary gland as a bioreactor to produce pharmaceutical proteins (Lathe et al., 1986) received attention. Although the aim of these studies is the obtention of transgenic farm animals, it remains that for technical and economical reasons most experiments hafe been first performed in mice. Two mains techniques are currently used in mammals to obtain transgenics; pronuclear micro-injection into fertilised eggs, or embryonic stem (ES) cell manipulatin which is presently restricted to mice.

1 - Production and analysis of transgenic mammals

Pronuclear micro-injection has been successfully applied to all mammal species studied so far. This methodology implies the use of large number of embryos and, subsequently, of pseudo-pregnant recipients. To lowe costs of production, when applied to rage animals, embryos from these species are now obtained through *in vitro* maturation and fertilisation of oocytes derived from ovaries collected in slaughterhouses (Krimpenfort et al., 1991). Also, *in vitro* culture systems have been developed to mature embryos to morulae or blastocysts, allowing the selection of viable eggs. Attempts to identify embryos that are transgenic by PCR alalysis have not been reliably successful

to date (Hyttinen etl al., 1994, Bowen et al., 1994). Currently, the analysis of amniotic fluid in eyrly gestation (F.R. Pieper, unpublished data, quoted in Vilotte and L'Huillier, 1995) shows more promise than PCR analysis. Despite a technical challenge, DNA fragments as large as YAC DNA have been successfully transferred by micro-injection (Gaensler et al., 1993, Schedl et al., 1993). However, the integration site and the number of copies integrated are not controlled, and in mice insertional mutations have been estimated to occur in between 5-10% of transgenic lines (Meisler, 1992).

ES cells are totipotent cells derived from the embryonic inner cell mass that can be propagated and manipulated *in vitro* while retaining their ability to fully participate in embryonic development when re-introduced into blastocysts. So far, ES cells have only been established in mice while ES-like cells have been described in hamsters, rat, pigs, cow and even humans. This technology allows the introduction of subtle mutations in the genome by means of homologous recombination. After gene transfer and selection, ES cells are reintroduced into blastocysts leading to chimeric animals in which a contribution to the germ line might be derived from the ES cells. Calves and lambs have already been successfully obtained following transfer of nuclei derived from the inner mass of cells to enucleated oocytes (Wilmut et al., 1990, Sims and First, 1994). This strategy should avoid the chimeric generation when true ES cells become available for these species.

2 - Production of pharmaceuticals in the milk

Following the observation that some native milk protein genes can be efficiently and tissue-specifically expressed in transgenics, their regulatory sequences have been used to target expression of foreign cDNAs or genes in the mammary gland of various mammals. Several designs of constructs involving or resembling milk protein encoding genes have been tested (Vilotte and L'Huillier, 1995 for review). From these numerous experiments, it appears that intronless transgenes are often only poorly expressed. However, it is still not possible to derive general guidelines for the design of constructs. The levels of expression observed are almost always dependent on the integration site and independent from the number of copies integrated. Furthermore, although such comparison are difficult to make, some constructs have been better expressed both in terms of their levels of expression and the tissue-specificity in some species compared to other. Finally, such association of sequences from different origins can sometimes lead to unexpected splicing event or ectopic expression. Unexpected splicing results in the absence of synthesis of the expected protein while ectopic expression may be detrimental for the animals health. However desite these complications, some pharmaceutical proteins have successfully been produced

at high concentrations (>1 mg/ml) in the milk of transgenic mice, pig, goat and sheep. Generally, the expression pattern observed is consistent from one lactation to the next one and transmitted.

Since the aim of these experiments is to produce pharmaceuticals for human therapy, the recombinant proteins have to be highly purified and characterised. Human protein C, al-antitrypsin or tissue-plasminogen activator have successfully been purified to an apparent high homogeneity from pig sheep and goat milk, respectively (Drohan et al., 1994, Wright et al., 1991 and unpublished results, Denman et al., 1991). All recombinant proteins studied so far were found to be biologically active. However, some of these proteins differ biologically from their human counterparts in terms of co- and/or post-translational modifications and thus specific activity. They can also differ from each other when expressed in different species. Furthermore, some recombinant proteins have been found to be heterogeneous in the milk, have influenced the lactation process, but often only when expressed at high levels. At the present time, it is unclear whether these differences are genuine or are only related to the extremely high levels of expression achieved.

All these observations suggest that the mammary gland can be used as a bio reactor but that the properties of each recombinant protein will have to be tested both in terms of biological and antigenic properties. For some of those that need complex co- and/or post-translational modifications, the choice of the species, the design of the construct aiming to limit its level of expression, even the possibility to modify by transgenesis the enzymatic activity of the mammary epithelial cells may need to be closely examined.

3 - Modification of the nutritional and/or technological properties of milk

Many recent reviews have focussed on potential beneficial modifications of nutritional and/or technological properties of milk by transgenesis. In general, two types of modifications have been described; the over-expression of native or mutated milk protein genes, and decreased expression or even knock-out of endogenous genes. For example, humanisation of ruminant milk could be achieved by altering the ratio between caseins and when proteins, increasig the ratio of κ-casein/Ca^{2+}-sensitive caseins to affect to size of the micelles, expressing human lactoferrin and/or lysozyme and removing β-lactoglobulin. To decrease the lactose content of the milk is also an important goal not only for the consumption of milk by lactose-intolerant people but also to reduce milk volume. The identification and utilisation of animals possessing natural null or 'very low expressing' alleles for milk proteins such as b-lactoglobulin as the starting genetic material for the production of transgenic animals would considerably expedate 'humanisation' of ruminant milk.

Expression of human lactoferrin and lysozyme has been achieved in mice (Platenburg et al., 1994; Maga et al., 1994) and transgenic cows were

obtained for a human lactoferrin-based transgene (Krimpenfort et al., 1991). High level expression of a goat hybrid κ-casein gene in mice was recently described (Persuy et al., 1995) and its effect on the size of the micelles is being investigated. The knock-out of the endogenous mouse β-casein gene was shown to decrease the total protein content of the milk but also to reduce the size of the micelles (Kumar et al., 1994).

The knock-out of the mouse α-lactalbumin gene has demontrated that this protein is solely responsible for the induction of lactose synthesis in the mammary gland (Stinnakre et al., 1994). The absence of lactose results in the production of milk that is too viscous to allow its natural removal by pups. Therefore, it appears that lower expression of the α-lactalbumin gene should result in the synthesis of a more concentrated milk with less lactose in it. To achieve this goal, ribozymes targeted against the bovine α-lactalbumin mRNA were designed and preliminary results obtained in mice transgenic for both the bovine α-lactalbumin and ribozyme transgenes are very encouraging (L'Huillier et al., 1995).

4 - Present drawbacks and future developments

Control of both the level of expressin and the tissue-specificity of a transgene is still a major obstacle limiting the utility of transgenic technology in larger animals, both for the modification of milk composition and in the production of pharmaceuticals. Prediction of the functionality of a transgene is still not possible, with the exception that some milk protein promoter sequences achieve reasonably reliable levels of tissue- and physiological state-specific expression. No geneal rules or guidelines are available for the design of highly efficacy transgenes, and in vitro systems for the rapid analysis of multiple designs still do not exist.

It is generally accepted that the inclusion of introns enhances the performance of most constructs. The complex nature, and lack of understanding of the transcription and translation processes, limit full exploitation of genetic elements regulating transcription rate, splicing, mRNA stability, and translation. In addition, the expression of exogenous genes for the production of pharmaceuticals has uncovered problems associated with protein processing and stability. The correct utilisation of new genetic elements in construct design will require considerably more research in mice. With the exception of β-lactoglobulin and whey acidic protein, expression is site integration dependent and copy number independent. The isolation and characterisation of yet unknown regulatory elements of milk protein genes is probably necessary to overcome this problem.

The application of transgenesis to large animals is thus very costly, limited on the one hand by the unreliability of transgene expression, and on the other

hand by low efficiencies of production of transgenic animals and long generation intervals. The 'random' nature of integration of transgenes presents possibly an undefinable hazard in farm animals, should insertin interfere with the physiology or health of the animal.

The possibility to manipulate YAC constructs, and to insert them into mice using homologous recombination might, in terms of expression and site of integration, be an alternative to ES cells manipulation. The development of the double gene replacement strategy in ES cells offers a wide range of new potential manipulations (Stacey et al., 1994). Similarly, ES cells could be used to insert cre-lox recombination site in chromatin domains that are active in lactating mammary epithelial cells in order to subsequently target transgenes integration (Araki et al., 1995). Obtention of ES cells from large animals will be a major break-through. The recently observed potentiality of primordial (Matsui et al., 1992) germ cells and spermatogonia (Brisnter et al., 1994 a, b) manipulations to obtain transgenic mice could also lead to the development of alternative aproaches.

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KAKO MOLEKULARNA GENETIKA MOŽE DOPRINIJETI MODIFICIRANJU SASTAVA MLIJEKA

Sažetak

Struktura izrađenog kazeina i gena koji kodiraju protein mliječne sirutke temeljito su istraživani u nekoliko vrsta razvoja molekularne biologije a prikazani su i nukleotidni nizovi mRNA (cDNA) i gena. Ti geni zbog svoje visoke izaženosti regulirane razvojem i specifične za tkivo u epitelnim stanicama mliječne žlijezde mogli bi djelovati kao primjer za razumijevanje reguliranja gena specifičnog za tkivo. Osim toga, budući da je poznato nekoliko varijanata proteina mlijeka u preživača, prepoznavanje relevantnih mutacija na razini molekula istraživano je radi određivanja genotipa tih životinja u bilo kojoj dobi, bez obzira na spol, ali isto tako radi analize odnosa između struktura gena i razine njegove izraženosti. Na kraju, ti su geni upotrebljeni u transgenetskim pokusima da se iskoristi mliječna žlijezda kao bioreaktor ili za proizvodnju mlijeka raznih hranidbenih i tehnoloških svojstava.

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