MAKING MORE OUT OF MILK: A CHALLENGE TO THE BIOTECHNOLOGIST

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Summary

The development of the technology to incorporate genes encoding for proteins that enhance the yield and quality of agricultural commodities into the genome of domestic animals has provided the geneticist with a powerful means of altering milk characteristics. Once this technology has been refined to the point where the level of transgene expression in particular tissues or cell types can be controlled, the dairy industry will be well placed to establish lines of transgenic animals fulfilling specific functions. The synthesis of 'designer milks' to meet the specific dietary and therapeutic requirements of particular sectors of the community will be commonplace while the range of dairy products will be widened. Similarly the cost of particular rare pharmaceuticals will be decimated as transgenic mammary specific expression systems are developed. The dairy industry is well placed to reap the rewards of the molecular revolution.

I. INTRODUCTION

Milk is an important biological resource used extensively to meet human dietary needs for protein, carbohydrate, fat, vitamins and minerals quite apart from its role in providing the neonate with the appropriate balance of these constituents to support early growth and development. Advances in dairy technology have ensured that this rich source of essential nutrients acts as the precursor of a diverse range of dairy products for the consumer. Its importance is demonstrated by the fact that milk comprises 25% of the total protein consumed by the populace of the USA (Swaisgood 1973). Despite this popularity, the increasingly discerning consumer has demanded products containing more protein and less fat as the awareness of the health risks associated with a high dietary intake of saturated fat diets grows.

This trend has provided the dairy industry with the problem of boosting the protein concentration of milk while at the same time suppressing the fat content. Other properties of milk detract from the universal acceptance of dairy products since up to 70% of the world population is deficient in the lactase activity required for lactose digestion (NDC 1985), with adverse side effects such as cramps, diarrhoea and even malnutrition in infants being apparent (Saavedra and Perlman 1989).

II. THE REGULATION OF MILK COMPOSITION

(a) Major nutrients

Why do we have to resort to the artificial manipulation of milk protein genes to alter the nature of the protein component of milk? It is no secret to the dairy farmer that the concentration of protein in milk does not respond to nutritional manipulation. Although significant gains have

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been achieved through the milk yield through the optimization of the protein and energy components of the cow's diet, changes in protein concentration have been small and highly variable (Sutton 1992). Thus the infusion of rate-limiting essential amino acids such as methionine, whose uptake is correlated closely with output in milk protein or the use of diets high in rumen non-degradable protein has failed to stimulate milk protein concentration, although the total protein output can be increased significantly (Metcalf et al. 1991; Rajczyk et al. 1994). Thus the cow is extremely effective at stabilising protein intake for its calf. In contrast both milk fat concentration and degree of unsaturation can be manipulated by dietary means (Sutton 1992; Ashes et al. 1992).

(b) The formulation of human milk substitutes

There can be little doubt that the best balanced nutrient source for the newborn child is maternal milk, however there are many circumstances in which this is either not available or is produced in insufficient quantities. The traditional substitute for human milk has been cow's milk and although its composition is similar to that of human milk, there are important differences in the pattern of milk proteins, while there are also differences in their amino acid sequences which dictate the rate and extent of enzymatic degradation in the gastro-intestinal tract of the newborn (Jennes 1974).

Cow's milk, for example, contains β–lactoglobulin (BLG) which is not found in human milk and is thought to be responsible for some of the human allergic reactions to cow's milk. Similarly a 17 amino acid sequence of bovine serum albumin is thought to induce antibodies that bind to a specific pancreatic β-cell surface antigen which marks these cells for destruction by the immune system (Sheard 1993) thereby inducing diabetes. Bovine milk is also low in lactoferrin which, in addition to its role in the transport of iron, has important functions as a bacteriostat and an anti-inflammatory agent (Platenburg et al. 1994). It is not surprising that adverse immune reactions occur in the neonate, since infant formulae provide the first wave of foreign antigens that their under-developed immune system is exposed to.

(c) The endocrine function of the mammary gland

Although milk is traditionally thought of as a rich source of nutrients, it also contains a complex mixture of hormones and growth factors (Koldovsky and Thornburg 1987) which are presumably provided to regulate developmental processes and behaviour in the neonate. The presence of gonadal steroids in milk was realised more than 60 years ago and since then, numerous hormones have been identified in milk including a range of brain and pituitary hormones such as prolactin, somatostatin, oxytocin, growth hormone releasing hormone, gonadotropin releasing hormone, calcitonin, glucocorticoids and all of the thyroid axis hormones, TRH, TSH, T3 and T4 (see Koldovsky and Thornburg 1987).

Thus the maternal influence over the metabolism of the newborn is extended beyond birth. Evidence is available, albeit controversial, to show that premature babies maintained solely on maternal milk develop higher intelligence quotients than those fed substitute formulae, an effect that is most likely induced by the finely tuned balance of hormones and growth factors found in maternal milk (Lucas et al. 1992) . This is supported by the significant body of evidence that shows that the level of hormones that tissues, including the brain, are exposed to during the neonatal stage, can influence the pattern of tissue development subsequently through to and beyond puberty (Csaba 1980). Certainly in the first few days post-partum there appears to be an intimate relationship between mammary function and the development of digestive function through the release of trophic and maturation factors as the gastro-intestinal tract undergoes the transition from intrauterine to extrauterine life (Weaver 1992).

(d) Where do the hormones in milk originate?

The growing recognition that a number of hormones traditionally thought to be synthesized in specialised endocrine glands are also expressed in many other peripheral tissues,

has altered our thinking on the endocrine regulation biosynthetic activity. Although there is direct evidence that many of these are sequestered from the circulation there is an increasing realisation that the mammary epithelial cells are capable of synthesizing and secreting some hormones and growth factors into milk. One such group of peptides that we have been investigating is the stress-responsive proopiomelanocortin derived peptides including bendorphin and α-MSH, which have been localised to secretory cells in lactating ovine mammary tissue (Bolouri Oskoui and Wynn, unpublished results) and which have been detected in bovine and ovine milk although at lower concentrations than in the circulation. When circulating levels of these peptides in the systemic circulation were increased up to 10-fold by the induction of chronic immunity to adrenocorticotropin (ACTH), which is a product of the same pro-hormone, there was no change in the expression of these peptides in milk. Thus systemic hormone levels have no influence on the concentration of these peptides in milk and therefore they most likely emanate from the mammary epithelium. Although the mechanism for the regulation of their synthesis is unknown, the stimulus of milking may exert an influence.

III. AUTOREGULATION OF MILK COMPOSITION

(a) The significance of our native marsupials

The autoregulatory capacity of the mammary gland is no better demonstrated than in our native marsupials. Whereas the milk of eutherian species varies little throughout the course of lactation, the relatively immature neonate of the marsupial requires milk for a much longer period of development. To accommodate this need the quantity and composition of this milk varies markedly from a relatively low fat, high carbohydrate milk in phase two of lactation (one to 200 days post partum) to one that is high in fat, lower in carbohydrate and higher in protein later in lactation (phase three: 200-330 days post partum). Of major interest is the marked increase in the concentration and change in the composition of the protein fraction during this transition: whereas there are small but significant changes in the casein fraction, there are marked changes in the composition of the whey proteins whereby the expression of whey acidic protein is lost and is replaced with the high expression of two novel related proteins, late lactation proteins A and B (Nicholas et al. 1987; Collet et al. 1989; Nicholas et al. 1995).

The most striking feature of the tammar wallaby maintained in the wild is its ability to produce milks from glands at different stages of lactation varying in composition concurrently. Thus the regulatory mechanisms for milk composition must reside within the mammary epithelia as each gland is exposed to the same endocrine environment in the circulation.

Although it is well recognised that a number of hormones play an integral role in the expression of the individual proteins, the major ones of which are prolactin, cortisol and insulin, it would appear that the specificity of their actions in different glands is determined by the responsiveness of their receptor-effector mechanisms within the gland (Nicholas et al. 1995).

However this mechanism may be insufficient to provide the exquisite level of control required to simultaneously orchestrate so many changes in milk composition. Therefore it was not surprising that Wilde and colleagues identified specific inhibitors of milk secretion in both goat's and human milk, the amino acid sequences of which did not resemble any other sequenced protein (Wilde et al. 1987; Wilde et al. 1995). A similar mechanism has been identified in the tammar wallaby (Nicholas et al. 1995) and is currently being investigated in other species. It is an understanding of the control of lactation in our native fauna that may unravel the mysteries of the control of milk protein synthesis in the ruminant.

One of the unique features of the regulation of tammar milk whey proteins is that maximal induction of mRNA expression for both BLG and α-lactalbumin is achieved with the addition of prolactin alone to mammary explants (Collet et al. 1990; Collet et al. 1991). The simplicity of this regulatory mechanism may have advantages for the design of expression vectors for transgenic cows.

(b) Naturally occurring milk protein variants

Before embarking on any program of biotechnological manipulation of milk protein synthesis, it is important to note that naturally occurring genetic variants of milk proteins are associated with differential expression of the milk proteins, milk fat, calcium, phosphorus and citrate (McLean et al. 1984). These subtle changes result in a decrease in the time for rennet-treated milk to coagulate resulting in a much firmer (50%) curd, an increase in cheese yield and in the thermal stability of the skim milk (McLean et al. 1987). Yet the change in the casein components between genotypes does not always result in an increase in the yield of casein: for example cows expressing the β–casein B variant produce milk of a higher concentration of κ–casein and a lower level of αs1 casein which counter-balance each other (McLean et al. 1984) thereby providing yet further evidence for the presence of an auto-regulatory mechanism on total protein synthesis.

There are some important guidelines for the biotechnologist within these observations: firstly the expression of a foreign transgenic milk protein is likely to lead to changes in the expression of other proteins and that if the right combination of gene expression is achieved, there are likely to be major gains in the quality of milk for manufacturing purposes.

IV. THE INTRODUCTION OF FOREIGN GENES INTO THE BOVINE GENOME

The most significant advance that has occurred in the genetic alteration of domestic livestock in the past 15 years has been the development of the technology to introduce exogenous DNA into the germline of animals (Pursel and Rexroad 1993; Cameron et al. 1994) Although pro-nuclear microinjection, in which multiple copies of the gene construct are physically deposited within the single cell embryo has been the method of choice for the development of transgenic domestic farm animals, the methods with the greatest potential have been the use of viral vectors and embryonic stem cells to introduce the foreign DNA. The major problem with the injection technique has been that exogenous DNA integrates stably into about 20% of these cells and that the incorporation is randomly distributed throughout the chromosomal sequences (Capecchi 1980). However if the foreign DNA sequence bears homology with endogenous sequences, it is possible to target a transgene to sites within chromosomes through the recombination of this foreign gene sequence with complementary stretches of endogenous DNA (homologous recombination). The introduction of this material into pluripotent embryonic stem cells provides the means for the introduction of this specific genetic mutation into embryos (Capecchi 1989).

In addition to the introduction of transgenes that promote production, a range of approaches have been developed to suppress the expression of a gene which may, for example, encode a product that inhibits milk secretion. The recognition of the presence of naturally-occurring antisense RNAs that modulate gene expression has lead to the use of genes that encode for RNA species that are complementary to the mRNA that is to be targeted, under the control, for example, of a milk protein promoter, thereby preventing translation of the targeted gene product. Alternatively enzymes that cleave specific RNA sequences through their endonuclease activity (ribozymes) may be expressed at specific sites determined by antisense RNA adjacent to the catalytic site. Gene constructs encoding for toxin genes associated with cell specific promoters may also be used to ablate specific cell lineages (Palmiter et al. 1987).

Irrespective of the means of transgene introduction, genes normally expressed within a particular tissue in one species generally retain the same tissue specificity when introduced into another mammalian species. Thus the potential for the introduction and expression of milk protein transgenes into the bovine genome to alter milk characteristics has been recognised.

(a) Manipulating milk composition

The first major success was achieved by Simons et al. (1987) who incorporated the ovine

BLG gene into transgenic mice, resulting in high levels of expression of this protein in mouse milk. Despite this expression the most disappointing aspect of this exciting finding was that the mammary gland retained its ability to regulate total protein concentration, as the increase in BLG expression was compensated for by proportionately decreasing the level of expression of the mouse milk proteins. Thus, as with the naturally occurring milk protein variants, the expression of the transgene is inextricably associated with the expression of the other milk protein genes. This may be expected since the four major bovine caseins, for example, are closely associated within a 200 kb region of chromosome 6 (Threadgill and Womack 1990). There are also obvious functional reasons for this association as well in that the seryl phosphate groups of αs1, αs2 and β-caseins associate with calcium phosphate complexes in milk to form loosely ordered aggregates or micelles which are maintained within a colloidal suspension by a surface coating of κ-casein (Jimenez-Flores and Richardson 1988).

The incorporation of more copies of any of the casein genes into the bovine genome remains a primary target for the dairy industry. In view of the close interaction of the proteins in the development of the casein micelle, it may be desirable to increase the level of expression of all of these proteins simultaneously as an imbalance may alter the processing efficiency of the resulting milk. The development of vectors that may be used to incorporate larger sequences of DNA into transgenic animals make this a more realistic aim. Increased expression of the caseins may be more readily achieved by associating the coding regions for the caseins with whey protein gene regulatory elements (Henninghausen et al. 1990).

There are a number of reasons for wanting to alter the composition of bovine milk for human consumption. The development of techniques for suppressing the casein locus (or BLG) while at the same time increasing the expression of lactoferrin and lysozyme will increase the use of bovine milk for feeding infants. Given that this market is worth $4 billion annually (Spalding 1992), the commercial incentive to develop such a product is apparent. A transgenic cow expressing the human lactoferrin gene under the control of a bovine αs1 promoter has already been developed (Krimpenfort et al. 1992), while human lysozyme has been expressed in transgenic mice (Maga et al. 1994).

In view of the allergenic nature of BLG it is conceivable that the expression of this gene may be suppressed by antisense mRNA or ribozyme expression, however this protein is also a vitamin A binding protein and therefore the loss of this function would have to be evaluated. In contrast evidence is accumulating to show that certain components of the whey fraction play a role in enhancing the bodyís protective mechanisms against colon cancer (Regester 1993).

The supply of certain amino acids provided by bovine milk is inadequate to meet the dietary requirements of infants (Boland et al. 1992). The most notable deficiency is in the sulphur-containing-amino acids and already a bovine κ–casein molecule has been expressed in E. coli in which three additional methionine residues have been incorporated at a single site in the gene sequence (Oh and Richardson 1991). The next step is to incorporate the gene into a transgenic animal and determine the effect of the mutation on the expression of this and the other milk proteins.

The down-regulation of the concentration of α-lactalbumin may prove advantageous to produce more concentrated milk, as this protein regulates the activity of the enzyme that catalyses the conversion of UDP-galactose and glucose to the major osmole for milk, lactose (Wilkins 1991). Such an effect has already been demonstrated in transgenic mice in which the gene for α-lactalbumin was knocked out by homologous recombination in embryonic stem cells. The milk was highly viscous, rich in fat and protein and devoid of α-lactalbumin and lactose: thus the principle clearly works (Stinnakre et al. 1994).

The heat stability of milk protein is an important attribute in view of the routine use of pasteurization and ultra heat treatment of the milk supply. Again the naturally occurring variants provide important clues with higher levels of β- and κ-casein and lower levels of αs1- and β-casein being correlated with greater heat stability of skim milk (McLean et al. 1987). Again transgenic approaches could be used to further improve this property of milk.

In view of the very tight control that the mammary gland exerts over the uptake of amino acids, it is possible that the overexpression of the family of amino acid transporters in the mammary epithelial cell membrane would increase the rate of protein synthesis. Their cloning

and incorporation in mammary-specific expression elements may boost milk protein synthesis. However the key to actually increasing milk protein concentration and total production will await the identification of some of the mammary-specific autocrine regulatory molecules that either inhibit or stimulate the rate of milk synthesis or regulate blood flow to the gland. Their coding sequences will be incorporated into expression vectors designed either to suppress or promote their synthesis.

(b) Changing the manufacturing capabilities of milk

Many factors contribute to the efficiency of conversion of milk into cheese. These include the genotype of the cow, stage of lactation, lactation number, seasonal and nutritional effects and the health status of the herd (Marziali and Ng-Kwai-Hang 1986). The relative importance of different milk proteins to cheese manufacture have been provided by comparative studies using milk from cows displaying different variants. For example milk from the κ-casein B variant has higher concentrations of casein, fat, calcium and phosphorus levels and a lower citrate concentration. This milk requires a lower renneting time, displays faster syneresis and forms a firmer curd and yields more of the final product. With our increasing ability to fine-tune gene expression it is likely that subtle changes in milk protein composition similar to these may be achieved through transgenic approaches.

More important information has been generated, however, by the food chemist as the structural requirements for a protein for maximum efficiency of conversion to food protein is identified. This not only entails a knowledge of the primary amino acid sequence but also physico-chemical and functional observations on enzymatically or chemically modified proteins. For example the impact of changes in the phosphorylation status of β–casein on cheese curd formation, calcium binding, surface hydrophobicity and solubility can be deduced by enzymatically treating the protein with a phosphatase (van Hekken and Strange 1993).

The cloning of specific cleavage sites into or out of nucleotide sequences of milk protein genes is now common practice and may involve single base up to whole domain substitutions, deletions or additions. Thus an increase in the expression of chymosin-sensitive Phe-Met residues within the caseins may be important in increasing the removal of glycopeptides from relatively insoluble calcium paracaseinate, which in turn precipitates to form the desired curd for cheese production (Yu 1994).

Cheese maturation is dependent on the activity of residual chymosin within the curd, which hydrolyses various peptide bonds in proteins within the curd at different rates. An increase in the number of more readily hydrolizable bonds may shorten the time for cheese ripening (Bawden et al. 1994).

These are some examples with potential for commercial exploitation, however, in view of the complexity of the structure of the casein micelle, the possibilities for the improvement of the functionality of the milk proteins by using recombinant DNA technology is endless.

(c) Synthesis of high value therapeutic agents by transgenesis

Since milk protein genes are expressed effectively and for the most part specifically in the mammary gland, it has been recognised that the promoter and related regulatory elements should be useful for the expression of other sequences in this tissue. As many human proteins require post-translational modifications such as phosphorylation, glycosylation, amidation and proteolytic cleavage to acquire full biological activity, they are unsuited for expression in bacterial systems because of the lack of some of these modificational mechanisms. Furthermore some of these proteins are not correctly folded as a result of the failure to form the correct disulphide bridges and therefore lack the appropriate secondary and tertiary structure. Expression in mammalian cell culture systems solves many of these problems, although the cost and low levels of expression currently limit the use of this technology (Bawden et al. 1994).

The initial success of Clark and colleagues in the expression in ovine milk of human factor IX, a serine protease essential for the normal clotting of blood, and alpha-1-antitrypsin, a serine protease that regulates the expression of elastase and therefore prevents emphysema in the lung

(Clark et al. 1991) has paved the way for many other transgenic animals. Up until this time both of these products of the liver had been purified at great expense from human plasma. Although the level of expression of these factors was insufficient to be commercially viable, subsequent refinements of the expression systems have improved the yield of a range of products from the milk of mice, sheep, goats and pigs.

It is likely that the development of transgenic cows will be focussed on both the introduction of transgenes designed to yield highly valued pharmaceuticals and to increase the casein content of milk. It is not surprising that there is a focus on the former given the cost involved in establishing a bovine transgenic program.

(d) Manipulation of hormone levels in milk

In view of the broad spectrum of hormones that have been detected in milk, there is little doubt that milk has pharmacological effects on the newborn. Therefore it is conceivable that these properties may be altered by transgenic expression of hormones in the mammary gland. Of these peptides, derivatives of β-casein, the β-casomorphins are of particular interest as they convey analgesic properties to milk presumably to sedate the neonate (Brantl and Teschemacher 1983). In addition to these peptides milk also contains β–endorphin at physiological levels which are increased in response to stress (Bruckmaier et al. 1993). The increased expression of the pro-hormone from which this peptide is derived, proopiomelanocortin in the mammary gland by means of a transgenic approach will result in a milk with unique therapeutic properties. The up-regulation of the expression of other hormones and growth factors found in milk may convey to the product other properties that may for example boost the immune response or increase the maturation of the gastro-intestinal tract of the newborn.

V. THE FUTURE

There is little doubt that transgenesis will revolutionise the dairy industry to the point where specialised herds will be retained for the production of particular dairy products or for the synthesis of specific therapeutic agents. The challenge for our dairy industry will be to hasten the adoption of these novel approaches to ensure that our economy benefits in a tangible way rather than resorting to the expensive practice of importing technology from others.

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