

# Alteration of Milk Composition Using Molecular Genetics

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## ABSTRACT

Advances in genetic technology have made it possible to consider making substantial changes either in the composition of milk or in the production of entirely new products in milk. The technological capabilities that have given rise to the introduction and expression of new genes in animals are discussed. Examples are given of transgenic animals that express foreign proteins in their milk. Advantages of the mammary synthesis of proteins are discussed and potential alterations of milk composition and scenarios for introduction of new proteins are considered. Technological capabilities that either currently exist or are being developed are discussed along with the requirements for making it feasible to utilize the technology on a broad scale in dairy cattle.

## INTRODUCTION

Through genetic engineering it is now possible to make substantial changes either in the composition of milk or to produce entirely new products in milk. Although there are at present few examples of transgenic animals in which foreign milk proteins have been expressed, it is possible to put genes for virtually any protein of interest under the control of the genetic controlling elements of a milk protein. For example, it is possible to express sheep  $\beta$ -lactoglobulin and other proteins of pharmaceutical value in the milk of mice and sheep (17, 40, 41). Advantages of the mammary synthesis of proteins include the ability of the secretory cells to phosphorylate, glycosylate, and secrete a protein-containing fluid (milk) in large quantities. The purification of proteins from milk is

apt to be simpler than purification from fermentation systems. In addition, the animals reproduce, feed, and water themselves. Many of the disadvantages of fermentation systems can be eliminated.

The genes for these foreign proteins are transmitted as Mendelian dominant characteristics and the animals produce the proteins only in their milk during lactation. New genes could be propagated in the dairy cattle population by use of embryo transfer and artificial insemination. This article focuses on types of technological capabilities that currently exist and are being developed, what hurdles must be overcome to make it economically feasible to utilize the technology on a broad scale in dairy cattle, what implications this technology has for animal breeding as it is currently practiced, and what other considerations might have potential ramifications for the dairy industry.

## TECHNOLOGY

### Genetic engineering

It is not the purpose of this paper to discuss the various technical aspects of genetic engineering technology. Readers wishing to become familiar with the technology in general are directed to several textbooks that offer good discussions in this area [e.g., (3, 30)].

### Transgenic Animals

It has become relatively routine to introduce cloned genes into mice via microinjection (9, 15, 21). Genes injected into the mice by these means can be stably incorporated into the animals' genome and are transmitted to their offspring as Mendelian dominant genetic characteristics. Those interested are directed to the review papers (14, 23, 34, 36). Because of the obvious potential agricultural utility, similar attempts have been made with farm animal species. In particular, transgenic pigs (7, 20), sheep (41), and cattle (4) have been produced, and the

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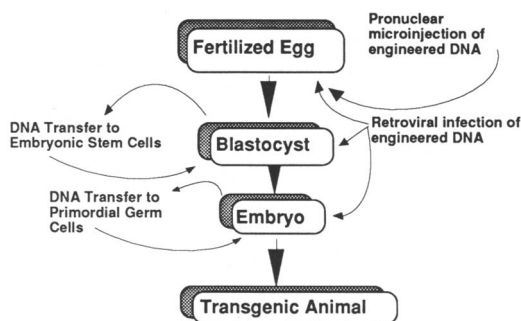


Figure 1. Methods of gene introduction into fertilized eggs.

frequencies of generation of transgenic farm animals have been comparable with those seen in the production of transgenic mice. Between .5 and 2% of the microinjected embryos result in positive transgenic animals. Of particular relevance to dairy production and manufacturing was the mammary-specific expression of two different genes in the milk of lactating sheep (41). These experiments have therefore demonstrated the technical feasibility of introduction of cloned genes (or combinations of genes) into the germ lines of domestic animals and their expression in mammary gland as milk proteins.

The various methods by which transgenic animals can be produced are shown in Figure 1. The cloned genes can be inserted at various stages of embryonic development. The first and most common way of making transgenic animals is microinjection of a foreign gene into the male pronucleus of a fertilized egg (16). The fertilized eggs are obtained using embryo transfer techniques (21). When the genes are injected at the one-cell stage, they most frequently become incorporated into all cells of the individuals including the germ cells, and are thus capable of being transmitted to one-half the offspring of that animal. Alternatively, it is also possible to insert the new genetic material into embryo-derived pluripotent stem cells and then to reinsert these cells into the blastocyst (6). In this case the animal will be a chimera and not all cells of the mature animal will contain the gene. Therefore, only some of the offspring will receive the genes from their parents (34, 36).

An alternative technology is being developed that uses an engineered retrovirus to incorporate the foreign genes into the developing embryos (22, 35, 42, 43). This technique offers considerable promise in that the efficiency of producing transgenic animals can be much higher than in the mechanical injection methods that are currently in widespread use. The advantages are a higher embryo survival rate, integration with a low copy number in the host genome, and integration of the vector without rearrangement of the gene to be inserted (35). However, one of the major limitations is the size of the genetic constructs that can be carried with retroviruses, and this may ultimately limit their utility (43).

Another method of producing modified milk would be by transfection of genes into the mammary secretory epithelium (e.g., through a retroviral infection). The mammary gland has the unique characteristic of undergoing successive developmental cycles where the epithelial cells secrete milk during one lactation and most are sloughed off during the quiescent dry period. They are replaced by a new group of cells during the subsequent lactation. If a method were available to introduce genetic information into the epithelial cells during the prepartum period, this would drastically alter the time it would take to produce new kinds of proteins. The genetic information within the udder could then be altered before the onset of every lactation. This scenario would be a variation on genetic therapy, where cells are removed from an individual, given some new genetic characteristic, and then put back into the individual (19).

#### PRODUCTION OF NOVEL MILK PROTEINS

Examples of genes introduced into the mammary gland secretory cells with the resultant production of different types of proteins are shown in Table 1. To date, studies have only used the mouse and the sheep to test the expression of proteins in the mammary gland (40, 41). Many kinds of proteins, from virus to humans, are represented in this list. There is no reason to think that similar results could not be attained with cows once various technical details have been worked out. Because of the generation interval and the costs of working with large animals, most of these techniques are

TABLE 1. Mammary expression in transgenic animals.

Protein	Gene source	Expressed		References
		in	Promoter	
$\beta$ -Lactoglobulin <sup>1</sup>	Sheep	Mouse	Sheep $\beta$ -lactoglobulin	(41)
Thymidine kinase	Herpes simplex virus	Mouse	Mouse metallothionein-I	(8)
Clotting factor IX <sup>1</sup>	Human	Sheep	Sheep $\beta$ -lactoglobulin	(41)
$\alpha$ -1 antitrypsin <sup>1</sup>	Human	Sheep	Sheep $\beta$ -lactoglobulin	(41)
Tissue plasminogen activator <sup>1</sup>	Human	Mouse	Rat whey acidic protein	(17)
Chloramphenicol acetyl transferase	Bacteria	Mouse	Rat $\beta$ -casein	(28)
Ha-ras	Human	Mouse	Rat whey acidic protein	(2)
Casein <sup>1</sup>	Rat	Mouse	Rat $\beta$ -casein	(29)

<sup>1</sup>Secreted into the milk.

apt to be worked out in laboratory animals and only subsequently will the technology be adapted to cows (38, 39).

As more information becomes available, it is becoming apparent that the elements that confer mammary specificity, i.e., the production of proteins only in the milk of lactating animals, reside in the upstream and downstream flanking regions of the gene. This is not particularly surprising, since this work draws upon a large body of similar information obtained from other molecular genetic research. What is striking, however, is the relative similarity of the genetic control sequences and introns of the genes expressed in the mammary glands of various species of animals as shown in Figure 2 (18, 44). From gene sequencing work on bovine and rat caseins, some common sequences have already been determined. The mutation rate in the flanking regions is lower than that in the coding regions of the genes (5, 44). This implies a stronger functional requirement for these regions than for the structural coding region of casein itself. As an increasing amount of gene sequence data becomes available, more commonality will probably be found. In the  $\beta$ -globulin gene system, it has become apparent that sequences residing hundreds and even thousands of base pairs away from the coding region are critical for the expression of the gene (32). Because there is considerably less known about the genetic loci for the milk proteins, future research is needed in this area. With consideration of the success in mammary specific expression of proteins with our relatively limited knowledge, an increase in information should make the technique even more powerful.

### POTENTIAL CHANGES IN MILK

Because one of the major products of the mammary gland is protein, it is thus legitimate to consider the production of additional proteins for which there is a demand and in which composition changes are desirable. Several potential changes are listed in Table 2.

One of the most obvious changes is the selective increase of a component that is already present in milk. For example, an increase in one of the casein components in milk might provide a method of increasing the value of milk for the production of cheese. A variation of this is the introduction into milk of a casein with some of its properties modified. The potential importance of such a concept has been pointed out previously (24, 37).

It might be desirable to eliminate selectively certain components from milk. An example of a milk protein that might be targeted for extinction is  $\beta$ -lactoglobulin. It is only present in the milk of ruminant animals and is apparently not required for lactation per se. It is related to retinol-binding proteins and several other proteins (1) but has no known function in the process of milk secretion. Its presence in milk confers some undesirable manufacturing properties (37), and thus, its elimination could provide an opportunity for new types of manufacturing practices. The removal from milk would also mean that the spared amino acids provided to the protein synthesis machinery in the secretory cell could be utilized to produce other (more desirable) proteins.

Other proteins that might be targeted for either reduction or extinction are key enzymes in the synthesis of milk components. An exam-

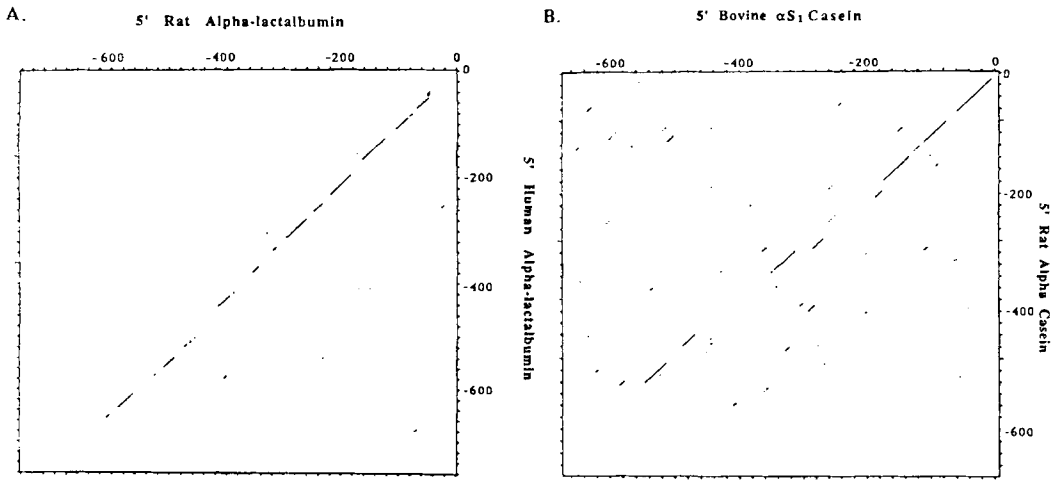


Figure 2. Similarities of 5' flanking regions of milk protein genes from different species. In this type of comparison regions of sequence identity appear as a diagonal (18, 44).

ple might be acetyl CoA carboxylase, which is a large and highly complex enzyme whose genetic sequence has recently been determined (31). This enzyme regulates the rate of de novo fat synthesis from acetate within the mammary gland. A reduction in the amount of this enzyme would lead to a dramatic reduction in the fat content of milk with a concomitant energy-sparing effect on the entire animal producing the milk.

Another component whose reduction might be desirable is lactose. This might be accomplished either through removal of  $\alpha$ -lactalbumin or by introduction of an enzyme such as  $\beta$ -galactosidase into milk. Either of these strategies might work. However, unlike  $\beta$ -lactoglobulin, which has no known role in the secretion

of milk, and acetyl CoA carboxylase, which is involved in fat synthesis but not secretion,  $\alpha$ -lactalbumin is one subunit of lactose synthetase that catalyses the synthesis of lactose. It is fundamental to the secretory process because lactose is critical to the movement of water and thus most probably other milk constituents through the mammary cells. This is because lactose is a disaccharide, and once secreted, it cannot permeate the luminal membrane of the mammary alveolus and thereby establishes an osmotic gradient across the secretory cells (26).

Introduction of an enzyme into milk to break down lactose may allow glucose and galactose to re-enter the mammary cell. This might be more successfully implemented than extinction of  $\alpha$ -lactalbumin. However, it is not without its

TABLE 2. Potential changes in milk through genetic engineering.

Gene	Change
Casein	Increase protein
Engineered casein	Manufacturing properties
Anti-sense $\beta$ -lactoglobulin	Reduce/remove
Anti-sense acetyl CoA carboxylase	Reduce/remove fat
$\beta$ -Galactosidase, lactase	Increase solids content
Antibodies of pathogens	Safer food, mastitis prevention

own potential problems. Hydrolysis of lactose into its substituent monosaccharides will increase the osmotic pressure within the alveolar lumen, thus leading to a further dilution of the other milk components, which is opposite of the desired effect—the reduction of lactose and the concomitant reduction in milk volume. For this goal to be accomplished, the monosaccharides would have to diffuse back into the secretory cells at a sufficiently fast rate.

There are several ways that these components might be removed. A premature stop codon might be inserted into the gene leading to abortive synthesis of the gene product. This method requires site-specific mutagenesis within the eukaryotic genome, which, while feasible, has not to our knowledge been done. A second way that a protein might be eliminated is by the introduction of an anti-sense gene to the gene of interest (33). The product of an anti-sense gene is a mRNA whose complementation with the gene of interest is thought to give rise to an RNA/RNA hybrid, which in turn, blocks translation of the original mRNA. Thus, the original protein is no longer produced. Nishikura and Murray (33) demonstrated an example of an antisense gene leading to the production of a "phenotypic null mutant".

Areas that need further consideration are human and animal health that might be addressed through the modification of milk. The presence of pathogenic organisms in milk continues to be a problem, and there are opportunities to use the described techniques in this area as well. It has recently been shown that a gene coding for a monoclonal antibody against a certain antigen can be expressed in other cells under the control of the appropriate promoter (10). It might be possible to produce monoclonal antibodies in the mammary gland by introduction of a gene coding for an antibody under the control of a mammary-specific promoter. Thus, one can envision antibodies against salmonella, listeria, or other pathogens produced with the mammary secretions so as to produce safer milk products. In a like manner, the production of antibodies directed against mammary pathogens would provide defenses against mastitis equivalent to those being attempted through vaccination procedures.

#### PROSPECTS FOR IMPLEMENTATION OF THE TECHNOLOGY

The foregoing discussion has been intended to point out the diverse potential of this technology. However, a number of challenges remain if the composition of milk is to be effectively altered.

More basic information about the actions of promoters and enhancers in eukaryotic cells is needed. This information base is rapidly expanding as sequence information is accumulated (32). This will permit the construction of well understood, highly efficient, mammary-specific promoters and enhancers which are better than those currently available. An example of how an increase in sequence information has been useful in understanding genetic control elements is the steroid hormone, thyroid hormone, vitamin D superfamily of control elements (13). In this case, control regions are predicted on the basis of DNA sequences for which ligands have not yet been discovered.

It will be essential to produce gene constructs analogous to some of the currently available cloning vectors with appropriate cloning sites to permit the ready construction of various genetic systems for introduction into the mammary gland. The promoters that are currently used for the production of transgenic animals and cellular gene-transfection experiments are well characterized and are effectively used as reagents in a variety of systems. Rather limited information is available on mammary-specific promoters. The success that has been reported with sheep  $\beta$ -lactoglobulin expression in mouse milk (40), using regulatory elements  $\beta$ -lactoglobulin, is encouraging, since it points to the development of mammary-specific targeting through the use of promoters for milk proteins. Other work, using the 5' upstream control sequences of rat  $\beta$ -casein to drive mammary-specific expression, demonstrates that this technique may have general applicability (29).

There is a need to dramatically improve the efficiency of gene transfer for the process to be economically practical in agriculture. Current efficiency of transgenic animal production is in the range of .5 to 2% of injected ova (9, 11, 36). Although it is feasible to inject genetic material and transfer several hundreds of mouse embryos to produce a few transgenic lines established, this is unacceptable in any kind of animal husbandry system.

There is a need for improved specificity of gene insertion. The expression of genes in transgenic animals is not at present proportional to copy number inserted (25). For commercial application the improvement in the efficiency of transgenic production must occur concurrently with an increased specificity of gene insertion. The location of insertion into the genome and level of expression are critical to any scenario for introduction of genes into a population of animals. In cases that have been studied, transgenes appear to segregate as Mendelian dominants. An agriculturally useful system of genetic constructs would have to be linked with other useful characteristics of animals such as milk production.

#### **IMPACTS AND CHALLENGES TO THE DAIRY INDUSTRY**

Food chemists have not in the past had the opportunity to design the proteins that went into a food product. This is no longer the case. It will be possible to define the characteristics of food materials for production in either plants or animals. Thus, there is a need for food chemists to identify new and desirable characteristics of milk. Through genetic engineering it is possible to design new proteins very easily. Computer modelling of protein structures is advancing rapidly (12) and is widely used in pharmaceutical development. Its application to food protein design is certainly within reason. What kinds of changes make sense? Traditionally, there has been relatively little coordination of the efforts of food chemists and animal physiologists. Research needs to be carried out on the fundamental chemical properties of milk proteins and how changing these properties might potentially modify the qualities of milk. Progress of the dairy industry will demand that new alliances and research teams be formed with coordination of efforts.

Any system introducing new genes into the population will have potential ramifications on animal breeding. The high productivity of dairy cattle is testament to the breeding strategies that have been developed and implemented over the past several decades. A number of scenarios for the introduction of new proteins into milk have been outlined above. If the method of introduction of these genes is via transgenic animals,

then it will be necessary to consider how these genes will be propagated in the population. Land and Wilmut (27) pointed out that considerable resources will be required to evaluate and introduce desirable traits into the population. Scenarios such as those they describe depend very heavily on the technological capabilities considered to be available when the scenario is developed. It will be possible (if not essential) to evaluate some of the changes in milk in laboratory animals so that there is a higher probability of a desirable outcome when introduced into lactating animals. The production of rare biological with sufficiently high economic values will most likely drive the development of the technological capabilities described in this article. Following this it will become feasible to develop small, specialized populations of dairy cattle, sheep, and goats for the production of specialty milks.

Supplying adequate protein in the diet of high producing dairy cattle is a continuing challenge to dairy nutritionists. The protein content of the milk of laboratory animals is in excess of 10%. If the protein content of the milk of dairy animals could be increased to similar concentrations through genetic engineering strategies, then drastic changes would be required in dairy cattle diets. It should be pointed out that this situation is not limited to dairy cattle. Laboratory animal diets may have to be evaluated for protein sufficiency if additional proteins are to be expressed in their milk.

Dairy animals were domesticated between 3000 to 5000 BC. One can argue that introduction of new genes is a natural progression of the domestication process. It will be necessary, however, to be careful to ensure that the objectives of any work along these lines is clearly defined and is not misinterpreted.

#### **CONCLUSIONS**

We have outlined some potential changes the dairy industry might see in the future. There are still many challenges to be overcome before the changes we have outlined can be implemented. Even though these changes may be a decade away, the possible impact on the dairy industry will be great.

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