Production of proteins in the milk of transgenic livestock:
problems, solutions, and successes\textsuperscript{1,2}

Alan Colman

**ABSTRACT** The milk of livestock can be modified dramatically by introducing foreign DNA into the germline. Exclusive expression of this DNA is ensured by the presence of regulatory sequences from mammary gland–specific genes. In sheep > 50% of the protein in milk can be encoded by a transgene and it appears that the foreign protein is additional to the normal complement of proteins. However, many technical hurdles (DNA configuration, low efficiency of transgenesis, and transgene stability) still prevent the routine use of this technology. In addition, such milk products have not yet received regulatory approval. These difficulties are not insurmountable. Transgenic methods can also be used to study the molecular basis of milk biogenesis. The effect on milk production in mice with all endogenous \( \alpha \)-lactalbumin genes removed and in mice in which the murine genes were then replaced with human homologues is described. \( \alpha \)-Lactalbumin, and consequently lactose, is essential for normal milk formation, and the human gene is expressed more efficiently than the murine gene. *Am J Clin Nutr* 1996;63:639S–45S.

**KEY WORDS** Transgenic livestock, \( \alpha \)-lactalbumin, gene knockout

**INTRODUCTION**

Genetic engineering has made it feasible to clone the protein-encoding genes from any organism. These recombinant genes can be expressed in a variety of systems ranging from simple microbial systems such as bacteria, yeast, and cultured mammalian cells to complex eukaryotic systems such as transgenic plants and animals. When the primary objective is to produce and purify a specific protein or group of proteins, the choice of system is dictated by many factors, including cost of production, amount of protein required, intended use, length of time required for development, and nature of the target proteins. As I examine below, many eukaryotic secretory proteins that are complex or are required in large amounts are best made in the milk of transgenic animals. To date, most transgenic proteins that have been expressed in milk normally originate in tissues other than mammary gland. However, the most natural use of this technology must include modifying the protein content of milk by manipulating the milk-protein genes themselves. I explore ideas for such a program that involve overexpression of \( \alpha \)-lactalbumin.

Finally, it is axiomatic that greater knowledge of a biological system facilitates designing experiments to manipulate the system for useful purposes. \( \alpha \)-Lactalbumin has two functions: in several species, including humans, it is an important nutritional component of milk and in most species it is responsible for the synthesis of lactose, which has long been implicated as the principal osmotic factor in milk formation (1). In the last section of this article I discuss the effect on milk formation in mice of removing the murine \( \alpha \)-lactalbumin gene and replacing it with its human homologue.

**TRANSGENIC TECHNOLOGY: FUNDAMENTAL ISSUES**

**Choice of protein**

Most of the proteins that are targets of the biotechnology and food industries are secretory in nature and many of them assume a highly convoluted but reproducible conformation as they pass through the secretory pathway. In eukaryotic cells, many of these proteins undergo complex posttranslational modifications, leading to alterations that may be essential for functionality. It has long been known that prokaryotic systems like bacteria cannot perform most of these modifications, and that even the folding of a protein (eg, of human serum albumin) can be subtly different when the protein is made in bacteria. Even though yeast and higher plants can make many of the modifications, they are restricted in the scope of the modifications conferred; this restriction can prove unacceptable, particularly when the protein is intended for therapeutic use. A choice must be made between mammalian cell culture, with its proven track record of producing safe and effective therapeutic products, and transgenic animal technology leading to milk production, which is clearly superior in production yields and costs, but has yet to clear regulatory hurdles and may have some disadvantages in the length of time required for development and in consumer acceptance.

**Choice of transgenic species**

The generation times and relevant developmental events for the major domestic livestock as well as for mice and rabbits are shown in Table 1. A significant feature is the time to the birth of the \( G_2 \) and \( G_3 \) animals, because these times correspond to the start of lactation in what could be the production animals. The start of lactation of the \( G_0 \) females is also a salient feature of these generation times because traditionally this is the earliest guide to the potential productivity of the animals and their

\textsuperscript{1} From PPL Therapeutics Ltd, Roslin, Edinburgh, United Kingdom.

\textsuperscript{2} Address reprint requests to A Colman, PPL Therapeutics Ltd, Edinburgh EH25 9PP, United Kingdom.
TABLE 1
Livestock generation times

<table>
<thead>
<tr>
<th>Species</th>
<th>( G_0 ) birth</th>
<th>( G_0 ) adult</th>
<th>( G_1 ) birth ( \rightarrow ) first milk</th>
<th>( G_1 ) adult</th>
<th>( G_2 ) birth</th>
<th>( G_2 ) herd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep or goat</td>
<td>5</td>
<td>13</td>
<td>18 ([9])</td>
<td>26</td>
<td>31</td>
<td>44</td>
</tr>
<tr>
<td>Cow</td>
<td>9</td>
<td>23</td>
<td>32 ([12])</td>
<td>46</td>
<td>55</td>
<td>76</td>
</tr>
<tr>
<td>Pig</td>
<td>4</td>
<td>11</td>
<td>15</td>
<td>22</td>
<td>26</td>
<td>37</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mouse</td>
<td>0.75</td>
<td>2.5</td>
<td>3.25</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1 Figures in brackets refer to the time of first milk by using premature induction of lactation.

AUTHOR: COLMAN

protein (WAP), ovine \( \beta \)-lactoglobulin, bovine \( \alpha \)-lactalbumin, bovine \( \alpha_1 \)-casein, and caprine \( \beta \)-casein (6). Although WAP-driven genes have proved problematic in pigs (7) because of premature termination of lactation, genes driven by other milk-protein regulatory sequences are approximately equivalent. However, the nature of the target gene configuration does seem important: a variety of experiments in mice have shown that genomic DNA is almost always superior to complementary DNA (cDNA) (Table 3). This trend was clearly shown in sheep for the production of \( \alpha_1 \)-antitrypsin (AAT) (2, 12, 15; Table 4).

Even with the availability of genomic DNA, problems still arise because of the random nature of the chromosomal integration of the foreign DNA. This randomness leads to unpredictable, and often disappointing, levels of expression. The discovery of autonomously acting and tissue-specific locus control regions (LCRs) that confer copy number–dependent expression on the target gene has led to one remedy (22). Unfortunately, no mammary gland–specific LCRs have yet been isolated. Another remedy might be to use very large pieces of DNA (>100 kb) in the form of yeast artificial chromosomes (YACs) because, in principle, these might contain the requisite LCRs even if they have not been identified formally. Transgenic animals have been made with YACs and their expression phenotypes resemble those expected for genes that are accompanied by LCRs (23).

Choice of founder

Founder transgenic animals usually have one or more copies of the transgene inserted into a locus on a single chromosome. These animals are therefore heterozygous for the transgene (strictly speaking, hemizygous, because there is no corresponding allele on the sister chromosome). Because the insertion process is random, every founder has a different genotype. As a result, expression levels in different founder animals that contain the same gene construct can vary widely (12). In addition, not all founders pass on any or all of their complement of transgenes intact (3, 12). The reasons for this are not completely understood, but the frequent integration of several copies of a gene at a single locus might stimulate recombination events at that locus. Furthermore, many founder animals are mosaic (not all cells or tissues are transgenic); a germ tissue that is nontransgenic could account for the lack of transmission. Because stability of inheritance and protein expression are imperative to any long-term production strategy, several founders have to be maintained and evaluated simultaneously. This situation could probably be resolved with the availability of livestock embryonic stem cells, as discussed in another article in this supplement (24).

TABLE 2
Relative merits of species

<table>
<thead>
<tr>
<th>Species</th>
<th>Volume of milk</th>
<th>Time to milk production</th>
<th>Ease of generating founders</th>
<th>Health</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Goat</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Cow</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Pig</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Rabbit</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

The criticism that followed early attempts at attracting commercial interest in the use of transgenic milk was that specific proteins in milk are difficult to purify to pharmaceutical standards at reasonable cost. We showed that these early concerns were unfounded because it proved easy to purify products such as human AAT to well greater than 99% purity. The secret is in removing the lipid, most of which is removed by low-speed centrifugation and the remainder by differential precipitation and chromatography. Purification challenges in separating

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N-Glycosylation, which is responsible for modifying proteins, has been an area of interest in the production of transgenic milk products. The use of transgenic milk for oral use poses concerns, especially in the case of scrapie, a prion disease that can be transmitted through oral ingestion of infected tissue. The development of strategies to remove traces of prions from milk has been a focus of research. The use of transgenic animals has been a controversial approach, with concerns about the transmission of diseases such as Creutzfeldt-Jakob disease. However, the use of bacteriophage T7 promoters to drive expression in transgenic animals has been shown to limit the expression of transgenes, which could help in reducing potential risks.

In terms of expression of human proteins in transgenic animals, Table 4 provides some data. The expression of human proteins in milk and cell culture is shown, with modifications such as N-Glycosylation and y-carboxylation noted. The expression levels vary, with some proteins showing higher expression in milk than in cell culture, while others show the opposite trend. The use of transgenic animals for producing human proteins in milk has been a topic of debate, with concerns about safety and regulatory issues.

Overall, the use of transgenic milk for oral use requires careful consideration of potential risks and the development of strategies to mitigate these risks. The use of transgenic animals for producing human proteins in milk has also raised questions about safety and the need for appropriate regulatory frameworks.
be an issue and so will be the production of novel immunogenic proteins; the latter is under review in the United States and the United Kingdom regarding transgenic foods in general.

**TRANSGENIC TECHNOLOGY IN ACTION**

**Overview**

All the published expression levels of protein from milk of transgenic livestock are shown in Table 4. Several different proteins of various complexity have now been expressed successfully in concentrations >1 g/L in large animals. This contrasts with the generally much lower concentrations in mammalian cell culture. More complex proteins have now been elaborated in murine mammary glands. For example, fibrinogen is composed of six polypeptides: two \( \alpha \), two \( \beta \), and two \( \gamma \) chains. These are normally assembled in liver cells into a hexamer. The secreted hexamer is cleaved in the blood by thrombin to form fibrin. Human fibrinogen secreted in the milk of transgenic mice in concentrations >2 g/L (Table 4) is thus far indistinguishable from protein derived from human plasma. Again, mammalian cell culture has proved poor by comparison.

**Production of human AAT in sheep milk**

We reported previously the generation of five founder sheep containing the human AAT gene (2). \( \alpha \)-Antitrypsin is a single-chain glycoprotein consisting of 384 amino acids that is secreted mainly from liver. Its main role is thought to be the inhibition of neutrophil-derived elastase. A common congenital deficiency of active AAT is responsible for the development of emphysema; however, because uncontrolled tissue degradation by elastase is also associated with the progression of other lung diseases like cystic fibrosis and adult respiratory distress syndrome, the provision of large quantities of AAT is likely to be medically useful. Unfortunately, before the sheep milk source mentioned above was available, the only source of significant amounts of therapeutically useful AAT was human plasma donations, and these cannot meet expected needs. Human AAT made in bacteria and yeast proved unsuitable, and mammalian cell culture could not meet the demands for large amounts of the protein at reasonable costs.

The founder females produce from 1 to 35 g AAT/L milk, and these amounts have been sustained through each of three lactations. Unfortunately, the transgenic locus in one of these animals, the highest-producing one, was not stable and her progeny all inherited different AAT copy numbers. However, the genetic locus in one of the founder males has been shown to be stable over four generations, and to date female progeny from three of these generations have produced from 13 to 18 g AAT/L consistently (12). We are now developing a production flock from a set of \( G_2 \) half-sisters that were all produced with the semen of the same \( G_1 \) male of the line. It is hoped that the AAT from these animals will enter clinical trials in 1996.

The data gathered in this AAT program provide the most compelling demonstration of this technology. They also address a fundamental aspect of milk composition: we have now analyzed the milk of several different animals that produce from 35 to 47 g AAT/L. This production does not seem to be at the expense of other milk proteins, which continue to be produced in normal amounts (40–50 g/L), a situation that differs from that seen in "overproducing" transgenic mice (26). We may expect a similar situation in cows, although in the elite production animals (animals of the highest pedigree related to milk-producing ability) an upper limit of protein concentration may be imposed by saturation of upstream processes such as food digestion and metabolic interconversion. Nevertheless, it seems that this technology has the potential to radically remodel the protein content of milk.

**Modification of milk-protein content in cows**

Clark (24) commented on the possible benefits that could accrue if milk could be custom-modified in several ways. So far there is only one example of the remodeling of bovine milk: the generation of animals that contain the gene for human lactoferrin (27). I discuss some future nutritional targets of interest in more detail.

\( \alpha \)-Lactalbumin is a small (123 amino acids), single-chain milk protein that is present in milk whey. Its prominence varies by species: in humans it is the major whey protein and in cows it is a minor whey component. Because of its well-balanced amino acid composition, a case could be made for increasing the amount of \( \alpha \)-lactalbumin in infant formulas at the expense of \( \beta \)-lactoglobulin (not present in human milk), which has a less-suitable amino acid composition for human infants and the presence of which in bovine-derived milk products may contribute to allergy problems in the young. Site-directed mutagenesis is another valuable genetic engineering technique that now makes it possible to custom-design alterations of amino acids of any protein for which a gene and a suitable expression system are available. This technique can be applied to the design of special foods with therapeutic properties, one example being the production of novel proteins for patients with phenylketonuria (PKU).

PKU is a congenital disease that results from the absence of the enzyme that metabolizes phenylalanine. Patients with PKU require a diet low in phenylalanine. This is particularly so for infants and pregnant women. Formulas low in phenylalanine are available but they are unappetizing and compliance is a problem, especially after infancy. A remedy might be available that exploits our ability to modify milk by transgenic means and the knowledge that \( \alpha \)-lactalbumin contains only four phenylalanine residues. The position of each of these residues can be determined in the three-dimensional protein structure; therefore, site-directed mutagenesis with the goal of substituting other amino acids for the phenylalanine residues without disrupting the protein structure can be envisioned. The modified protein could be expressed in milk and then purified to provide the base for an improved diet for patients with PKU. Obviously, such purification would be helped if the endogenous \( \alpha \)-lactalbumin content could be abrogated by either gene deletion or antisense technology.

The generation of transgenic cows is labor-intensive and time-consuming. However, in many ways it is simpler than the current methods for other transgenic livestock. The most convenient method involves the in vitro maturation and fertilization of bovine oocytes that are removed from the ovaries of slaughtered animals. The fertilized embryos are then centrifuged and one of the exposed pronuclei is injected with the chosen DNA. The embryos are cultured for 7 d in vitro and the surviving embryos (~7%) are transferred to hormonally synchronized recipients. The pregnancy rate we obtain is ~20%.
Published data indicate a transgenic integration rate of \( \approx 5\% \) (27), so roughly 5 of every 100 live births can be expected to be transgenic. At best, one transgenic animal for about every 1500 embryos injected can be expected. Fortunately, we are now finding it possible to inject >800 embryos per week. The limiting factor—and also the most expensive—is the number of appropriate recipients required. However, because no invasive procedure is ever used, the recipients can be recycled. This contrasts with the present manufacture of other transgenic livestock animals, in which surgery is required for both the embryo donors and the recipients, thereby limiting the recycling options. Marginal improvements in the process can already be brought about (at great cost) by, for example, the repeated use of live cow embryo donors (such embryos develop better). However, significant improvements in the efficiency of the transgenic process will be made only when injected embryos can be screened for transgene integration before their transfer to recipients, when the integration rate can be improved, or, best of all, when transformable embryonic stem cells become available.

The chronology of a milk-modification program using the present technology is shown in Figure 1. Significant features of the program are that a high-expressing \( G_0 \) male or female founder makes milk collection from a modest production herd possible within 5 y. However, for this to occur in a cost-effective way, nonroutine procedures such as premature induction of lactation of bulls and transvaginal oocyte recovery from cows are required. Large herds of animals can be expected after 6.5 y. Herb development would be expedited by the generation of homozygous animals (ie, animals in which the transgenes are present on both sister chromosomes) because all female offspring of these animals would be transgenic. However, it would be 9.5 y before these herds would be available for milking.

**\( \alpha \)-Lactalbumin and milk formation**

It has been argued above that in certain situations the transgenic mammary gland is superior to the cultured mammalian cell as a protein-production system. However, the expression of added genes in the mammary gland may have unwelcome effects on both mammary gland function and the physiology of the organism as a whole.

\( \alpha \)-Lactalbumin may exert a strong influence on milk carbohydrate and fluid content through its role as a component of the lactose synthase complex. It associates with galactosyltransferase, a Golgi membrane protein, to change the substrate specificity of that enzyme and promote lactose synthesis. In many species, lactose is thought to be the major osmotic influence in determining milk volume. There is a correlation between lactose, fluid, and \( \alpha \)-lactalbumin concentrations in the milks of different species, but no causative relation has yet been established (28). If milk formation is indeed sensitive to \( \alpha \)-lactalbumin synthesis, this could affect some of the programs suggested above. For example, overexpression of native \( \alpha \)-lactalbumin could lead to extra lactose formation and a more dilute milk, and an overexpressed, mutated \( \alpha \)-lactalbumin might be a competitive inhibitor of lactose synthase and reduce lactose synthesis, resulting in a highly concentrated milk.

Clearly, these issues could be resolved empirically by performing overexpression experiments and analyzing the results.

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**FIGURE 1.** Time required to produce a milking herd of transgenic cows, starting from the injection of DNA into a one-cell embryo.
However, a more fundamental understanding of the role of α-lactalbumin in milk formation would undoubtedly be beneficial to the experimental design. We conducted experiments in which we generated mice with no murine α-lactalbumin genes and mice in which the murine α-lactalbumin genes were replaced by human α-lactalbumin genes (29). A description of the phenotypes of mice that contained different combinations of the α-lactalbumin genes from the two species follows; for the original data refer to the study by Stacey et al (30).

The α-lactalbumin concentrations in the milks of the different mouse lines were estimated by direct visualization on polycrylamide gels as well as by quantitative adsorption chromatography with phenyl-Sepharose columns. A normal (wild-type) mouse is designated “αlac+/αlac−”; this designation refers to the presence (αlac+) or absence (αlac−) of the murine (m) α-lactalbumin gene on each sister chromosome. Wild-type mice (αlac+/αlac−) have only a small amount of α-lactalbumin in their milk (0.9 g/L) and this concentration is halved in heterozygous mice with only one copy of the gene (αlac+/αlac−). Nevertheless, the composition of the two milks is similar and even the small reduction in lactose in the heterozygotes is not significant. These data indicate that, although synthesis of α-lactalbumin is dependent on gene dose, α-lactalbumin concentrations in the cell are not limiting for lactose synthesis.

It is of little surprise that mice with no copies of the α-lactalbumin genes (αlac−/αlac−) make no α-lactalbumin and their milk is radically different from that of the mice discussed above. It is viscous and extremely protein- and lipid-rich and contains no lactose. Milk volumes in mice with no copies of the gene are dramatically reduced and these animals are unable to rear litters successfully, but all other developmental functions appear normal. We conclude that the presence of α-lactalbumin is essential to lactose formation and that this in turn drives milk volume.

The content of α-lactalbumin relative to that of other milk proteins is considerably greater in human milk (16–25%) than in mouse milk (~0.1%). This difference is maintained when the gene is substituted for the mouse gene. The human replacement gene is designated “αlac−.” Humanized (αlac+/αlac−) mice secrete ~1.4 mg α-lactalbumin/L milk. We showed by using heterozygous (αlac+/αlac−) mice that this increased secretion is due to a higher rate of transcription that occurs on the human gene. Milk from totally humanized mice is similar to that from wild-type mice except that the volumes are greater in humanized mice. We conclude that human α-lactalbumin can functionally substitute for the mouse protein in the mouse mammary gland. We speculate that the increased volume is due to increased lactose formation that results from either the surplus of α-lactalbumin protein or improved kinetic properties of the hybrid lactose synthase.

CONCLUSIONS

Transgenic technology can be used to both exploit the impressive productivity of the mammary gland and provide information about the molecular mechanisms that underlie that productivity. Large amounts of a desired protein can be obtained from the milk of transgenic animals. The remaining major hurdles involve increasing the efficiency of generating transgenic animals, successfully addressing regulatory issues, and, particularly in the area of modified milk foods, maintaining the low cost of large-scale purification and overcoming public concerns.

REFERENCES


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