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EXPRESSION OF RECOMBINANT PROTEINS IN THE MILK OF TRANSGENIC ANIMALS

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Introduction

Introduction of foreign DNA into the murine genome by microinjection and the generation of transgenic offspring were first reported by Gordon et al. (1980) and Gordon and Ruddle (1981). This technique has been applied to the production of transgenic livestock. Using mammary gland-specific promoters, a wide range of proteins of biopharmaceutical interest have been expressed in rodents, pigs, and dairy animals. An expression vector, comprising a gene encoding the target protein of interest fused to a milk promoter gene, is introduced by microinjection into the pronucleus of a one-cell embryo. Upon germ line integration and expression, the transgene becomes a dominant Mendelian genetic characteristic that is inherited by the progeny of the founder animal. The transgenic offspring may express the target protein in gram per liter quantities, most frequently as a soluble whey protein. Mammalian mammary epithelial cells have the capacity to carry out complex protein synthesis with a variety of posttranslational modifications and proper folding. Coexpression of modifying enzymes in the epithelial cell Golgi apparatus may allow the heterologous protein to be engineered to confer specific or desired pharmacokinetic characteristics. The milk of transgenic livestock presents an excellent starting material from which human diagnostic or pharmaceutically active, therapeutic proteins may be purified using established technologies of the dairy and biopharmaceutical industries.

To date (1997), probably more than 50 proteins have been expressed in the milk of transgenic mice, rats, rabbits, goats, sheep, pigs, and dairy cows. Phase I clinical trials with antithrombin III produced in transgenic goats were completed successfully in 1996, and phase II clinical trials are currently being performed in the United States. α1-Antitrypsin produced in transgenic sheep is currently in phase I clinical trials in the United Kingdom. In addition, human lactoferrin and fibrinogen expressed in the milk of transgenic cows and sheep, respectively, are in the late stages of development or preclinical evaluation.

The field of transgenic research has been reviewed periodically since 1990. The reader will find the following articles, which trace the development of transgenic technology applied to dairy animals,
of interest: Henninghausen (1990), Henninghausen *et al.* (1990), Bialy (1991), Wilmut *et al.* (1991), Wall *et al.* (1992), Jänne *et al.* (1992, 1994), Logan (1993), Ebert and Schindler (1993), Lee and de Boer (1994), Houdebine (1994, 1995), and Echelard (1996).

This chapter discusses the expression of therapeutically useful proteins in mammalian milk with a focus on dairy animals as the production systems of choice and reviews expression constructs, milk-specific transgenes, transgene insertion, transgenic animal production, protein biosynthesis and secretion, lactation, milk, protein purification, and quality and regulatory issues.

### Expression of Heterologous Proteins in Milk

Laboratory mice have served as a model expression system for foreign proteins since the inception of animal transgenesis and are used frequently for feasibility studies concomitant with or prior to the generation of larger, founder transgenic animals. Many recombinant proteins of interest in human therapy are, by their very nature, biologically active in most mammals. The murine model is useful in determining, at an early stage of research, the transgene expression characteristics and potential effects on animal health. Transgenic mice are generally predictive of what will be observed in larger animals. Many transgenic proteins have been expressed in the milk of transgenic livestock. Table 1 summarizes the published results of expression in transgenic farm animals during the period from 1990 to 1996. Several of these proteins have been expressed at high levels, demonstrating the usefulness of the production system. Examination of a great number of transgenic lines has shown that mammary gland-specific expression of a target protein is associated with increased plasma levels of this protein, even in the absence of ectopic expression. The most likely explanation is leakage of the protein from the milk into the circulation through the junc-<ref>tional complexes of the mammary epithelial cells. Therefore, certain proteins and peptides, such as highly active hormones and cytokines, cannot be expressed in the mammary system as their secretion into the blood may have severe detrimental effects on the host.
Table 1
Biologically Active Proteins Expressed in Caprine, Porcine, Ovine, and Bovine Milk

| Expressed protein                        | Animal | Promoter          | Expression level | Reference                     |
|------------------------------------------|--------|-------------------|------------------|-------------------------------|
| Human growth hormone                     | Goat   | Retroviral promoter | 12 ng/ml         | Archer et al. (1994)          |
| Human long-acting tPA                    | Goat   | Murine WAP        | 3 μg/ml          | Ebert et al. (1991)           |
| Human long-acting tPA                    | Goat   | Caprine β-casein  | 3.5–8.0 mg/ml    | Ebert et al. (1994)/GTC       |
| Human protein C                          | Pig    | Murine WAP        | 1 mg/ml          | Velander et al. (1992)        |
| Human α-antitrypsin                      | Sheep  | Ovine β-lactoglobulin | 35 mg/ml      | Wright et al. (1991)          |
| Human α₁-proteinase inhibitor            | Goat   | Caprine β-casein  | 20 mg/ml         | GTC                          |
| Human antithrombin III                   | Goat   | Caprine β-casein  | 20 mg/ml         | GTC                          |
| Human factor VIII                        | Sheep  | Ovine β-lactoglobulin | Not given     | Halter et al. (1993)          |
| Human factor IX                          | Sheep  | Ovine β-lactoglobulin | 5 μg/ml       | Clark et al. (1989)           |
| Human fibrinogen                         | Sheep  | Ovine β-lactoglobulin | 5 mg/ml       | Carver (1996)                 |
| Colon cancer MAb                         | Goat   | Caprine β-casein  | 10 mg/ml         | GTC                          |
| Human lactoferrin                        | Cow    | Bovine αs1 casein | Not given        | Krimpenfort et al. (1991)     |

*Gene insertion via teat canal using retroviral vectors GaLV and MoMLV.

Data from Genzyme Transgenics Corp. (1996).
Milk-Specific Transgenes

Transgenes containing sequences of several milk protein genes, reviewed by Maga and Murray (1995) and Echelard (1996), have been used to direct the expression of exogenous proteins to the lactating mammary gland. These transgenes are usually chimeric, being derived from the fusion of a target protein gene and mammary-specific regulatory sequences. Although both genomic DNAs and cDNAs coding for target proteins have been used for expression, higher levels are normally obtained with genomic DNAs. The incorporation of untranslated exons and introns may contribute to increased expression of the transgene (Whitelaw et al., 1991). Addition of a signal sequence is necessary if the exogenous protein is not normally secreted. This will cause the protein to be secreted out of the mammary tissue into the milk.

Regulatory sequences from several milk-specific genes have been isolated and tested in transgenic animals: ovine β-lactoglobulin; murine, rat, and rabbit whey acidic protein (WAP); bovine α-s1 casein; rat, rabbit, and goat β-casein; and guinea pig, ovine, caprine, and bovine α-lactalbumin. Of these promoters, several have permitted grams per liter expression of target proteins in the milk of transgenic offspring, sometimes in large dairy animals; some of this work is summarized next.

The ovine β-lactoglobulin gene contains seven exons and six introns spanning a 4.2-kb region (Harris et al., 1988). The first reported ovine β-lactoglobulin chimeric transgenes (Archibald et al., 1990) were composed of 4 kb of 5' flanking fused to the α1-antitrypsin genomic sequences. Other configurations using variable amounts of 5'- and 3'-flanking sequences have also been used (Whitelaw et al., 1992) and reviewed by Maga and Murray (1995). With the ovine β-lactoglobulin gene, high-level expression (g/liter) of α1-antitrypsin, fibrinogen, and HSA have been reported (Wright et al., 1991, Shani et al., 1992; Prunkard et al., 1996). However, similar results have not been observed with transgenes containing cDNA sequences (Clark et al., 1989, Shani et al., 1992, Hansson et al., 1994, Yull et al., 1995).

Rodent WAP genes consist of four exons and three introns: the middle two exons encode the two cysteine-rich regions, which probably form separate protein domains (Campbell et al., 1984). WAP is present in the milk of mouse, rat, rabbit, and camel, but is
absent from the milk of cow, sheep, pig, goat, and human. No rec-
ognizable WAP gene homologues have been isolated from these
species. Rat [Wei et al., 1995, Yarus et al., 1997], mouse [Gordon et
al., 1987; Ebert et al., 1991, Reddy et al., 1991; Velander et al.,
1992; Drohan et al., 1994; Hansson et al., 1994; Limonta et al.,
1995], and rabbit [Bischoff et al., 1992; Devinoy et al., 1994; Thépot
et al., 1995] WAP regulatory sequences have been used to direct ex-
pression of exogenous proteins to the mammary gland. High-level
expression of the target protein was observed with mouse and rab-
bit WAP transgenes. Surprisingly, relatively high expression levels
(up to 1 g/liter) were observed in transgenic pigs with a construct
containing 2.6 kb of 5' and 1.3 kb of 3' mouse WAP sequences
linked to a human protein C cDNA [Velander et al., 1992]. This re-
sult seems to indicate that mWAP regulatory sequences can func-
tion efficiently in species that do not have an endogenous WAP
gene.

The bovine αs1-casein gene contains 9 exons and spans 17.5 kb
[Koczan et al., 1991]. Originally [Meade et al., 1990], a transgene
containing 21 kb of 5' and 2 kb of 3' flanking sequence fused to the
genomic sequences of human urokinase was shown to direct high
milk expression levels (1–2 mg/ml) in mice. Promising results were
also obtained in transgenic rabbits with a construct containing the
human IGF-1 cDNA [Brem et al., 1994], in mice with the human
lysozyme cDNA [Maga et al., 1994], and in human lactoferrin and
human granulocyte–macrophage colony-stimulating factor genomic
constructs [Nuijens et al., 1995; Uusi-Oukari et al., 1997]. Con-
versely, a bovine αs1-casein–human lactoferrin cDNA construct
only permitted low-level expression in milk of transgenic mice
[Platenburg et al., 1994], as was the case with a human tPA cDNA
construct fused to 1.6 kb of bovine α-s1 casein 5'-flanking se-
quencies [Riego et al., 1993].

The bovine α-lactalbumin gene contains four exons and three in-
trons. Early reports [Vilotte et al., 1989; Stinnakre et al., 1991] indi-
cated that a construct containing 750 bp of 5' and 336 bp of 3'
flanking region was sufficient to direct intermediate expression
levels in transgenic mouse milk when fused to bovine α-lacta-
bumin or ovine trophoblastin cDNAs. By using a construct contain-
ing the same amount of 5'-flanking sequence linked to hGH ge-
nomic sequences, higher levels of the target protein (up to 4.3
mg/ml) were obtained in the milk of transgenic rats [Ninomiya et
al., 1994]. However, at this point, results obtained with α-lactalbu-
min-driven transgenes in large animals have not been reported.
The caprine β-casein gene (CSN2) has been cloned and sequenced (Roberts et al., 1992; Persuy et al., 1992). The intron/exon organization of the 9-kb goat gene is similar to that of other CSN2 genes and its expression is limited principally to the mammary gland during lactation. High-level expression was observed in goats transgenic for a construct containing 6.2 kb of 5' and 7.1 kb of 3' goat β-casein flanking noncoding sequence fused to a variant of the human tPA cDNA (Ebert et al., 1991). High-level expression with caprine β-casein-containing transgenes has also been observed, in mouse milk, with bovine κ-casein (Persuy et al., 1995; Gutierrez et al., 1996), antithrombin III (cDNA and genomic, mice and goats), HSA (cDNA and genomic), α1-antitrypsin (genomic, mice and goats), and both heavy and light chains of several humanized antibodies (H. Meade et al., unpublished data).

Insertion of the Transgene into the Germ Line

Transgenic animals may be generated by direct microinjection of the foreign gene into the pronuclei of one-cell stage embryos. Microinjection techniques have been reviewed exhaustively by Hogan et al. (1986) and Pinkert (1994), among others. Techniques initially developed for gene insertion into murine pronuclei (Gordon et al., 1980; Gordon and Ruddle, 1981; Brinster et al., 1985; Palmiter and Brinster, 1986) have been adapted to gene transfer into the pronuclei of ruminants and pigs (Hammer et al., 1985; Pinkert, 1994). If the microinjected DNA integrates into the genome of the recipient before the first cell division occurs, a heterozygous founder can be created. Later integration leads to genetic mosaics consisting of normal cells with a normal genome and cells with transgenomes.

Generally, fertilized eggs are flushed from the oviduct of a superovulated female donor, microinjected with a few hundred copies of the transgene, transferred to the oviduct or uterus of a pseudopregnant recipient animal, and developed to term. The first transgenic offspring, or founder animals, are at best hemizygous as the transgene is not integrated into both copies of a pair of homologous chromosomes. In the case of mosaic founders, germ line transmission is not always observed. In addition, multiple transgene integration sites have been detected in 10–20% of transgenic founders.

To optimize the collection and transfer of microinjectable goat
embryos, Selgrath et al. (1990) established regimens for superovulation/synchronization and timing of pronuclear embryo collection. Does were synchronized with Norgestomet ear implants and superovulation was induced with pregnant mare serum gonadotropin (PMSG) or follicle-stimulating hormone (FSH-P). Does were hand mated with the average female being mated six to eight times by two different males over a 24- to 36-hr period. Embryos were recovered surgically and pronuclei could be visualized without centrifugation. After microinjection the embryos were transferred to the reproductive tracts of recipient females using a surgical procedure similar to that used for the embryo collection.

Ewes may be similarly synchronized and superovulated during the breeding season using Progestin (30 mg fluorogestone acetate) pessaries and FSH injection (Rexroad and Wall, 1987). Fertilized sheep oocytes are semiopaque and not readily visible. However, differential interference contrast microscopy allows visualization, and successful microinjection may be determined by the swelling of the pronucleus, which occurs on injection of DNA (Simons et al., 1988). Microinjected embryos are then transferred to recipient ewes.

Bovine oocytes are generally collected from slaughtered heifers or obtained from cows superovulated with PMSG or prostaglandin. To circumvent surgical procedures and in vivo fertilization, Krimpenfort et al. (1991) have used an in vitro fertilization and embryo production procedure. The technique for DNA microinjection is similar to those described earlier: centrifugation, e.g., at 12,000 × g for 10 min, is necessary to visualize pronuclei. Microinjected cow embryos are usually cultured in vitro to the morula-blastocyst age at which time they are transferred nonsurgically to suitable recipients. The techniques and efficiency of gene transfer in cows have been described by Roschlau et al. (1989) and McEvoy and Sreenan (1990).

Synchronization of sows is accomplished with hormonal treatment and superovulation induced with PMSG or human chorionic gonadotropin. Synchronization, however, has been shown to affect the farrowing rate (Pursel et al., 1990). Pig ova are opaque and no nuclear structures can be seen, even using interference contrast microscopy. Centrifugation at 15,000 × g for 5 min leaves pronuclei visible in the equatorial segment of the cytoplasm (Brem et al., 1985; Hammer et al., 1985). Microinjected embryos are then transferred to recipient pigs.

Factors affecting the success of microinjection as a gene insertion
technique have been reviewed by Rexroad et al. [1990]. Experience in microinjection is an important factor as well as DNA concentration and the gene construct. The stage of egg development and the quality of eggs may affect the efficiency of producing transgenic animals. Three factors contribute to problems of microinjection of livestock embryos compared to mice. Cytoplasmic vesicles may obscure the view of pronuclei, fewer embryos are available for microinjection, and there is considerable variability in the stage of embryo development at the time of embryo collection. Despite these challenges, there has been considerable success in developing transgenic goats, sheep, pigs, and cows. Key reproduction parameters and the success rate of transgenesis are summarized in Table 2.

In summary, oocytes may be fertilized in vivo or in vitro. In vivo fertilization may be controlled by artificial insemination of a superovulated animal at the stage where the oocyte has matured in the ovary. An alternative pathway is to isolate follicular oocytes from the ovary of the donor animal and proceed to in vitro maturation and fertilization prior to microinjection and implantation in the recipient female. In goats, sheep, and cows the rate of transgenic births is 5–10%.

Embryonic stem (ES) cells may offer an alternative to pronuclear microinjection for achieving transgenesis. However, pluripotent ES cells able to contribute to the germ line have only been described in mice. There have been descriptions of chimeric animals generated with rat, pig, and cow ES cells [Iannaconne et al., 1994; Wheeler, 1994; Stice et al., 1996], but in the case of rats and pigs no evidence of germ line transmission from these cells has been reported. Cow

Table 2
Comparison of Reproduction Data for Dairy Animals Used to Produce Biopharmaceutical Products by Transgenesis

|                          | Goat | Sheep | Cow  |
|--------------------------|------|-------|------|
| Seasonal breeding        | Yes  | Yes   | No   |
| Number of one-cell embryos | 1-3  | 1-3   | 1    |
| Number of one-cell embryos [superovulated] | 4-8  | 4-10  | 3-6  |
| Gestation time [months]  | 5    | 5     | 9    |
| Rate of transgenic births [%] | 5-10 | 5-10  | 5-10 |
| Litter size              | 1-2  | 1-2   | 1    |
| Time to sexual maturity [months] | 6-8  | 6-8   | 15   |

Data in part from Clark et al. [1987].
fetuses obtained following nuclear transfer of ES cell-derived nuclei in recipient oocytes died in utero, exhibiting major defects in placental development [Stice et al., 1996]. In sheep, the first large animals derived from cultured cells were described in 1996 by Campbell et al. Two healthy phenotypically female lambs were born from embryos generated by transferring nuclei isolated from embryo-derived cells into enucleated oocytes. DNA analysis demonstrated that all the nuclear transfer lambs and fetuses were derived from the cell line. It is not yet clear whether foreign DNA can be introduced in this type of cell line, and there are questions about the health and reproductive fitness of the recovered offspring. Nevertheless, this experiment is certainly a step toward the possibility of replacing pronuclear microinjection as the method of choice for the generation of transgenic large animals.

Another potential alternative to the pronuclear microinjection of the transgene is the use of replication-defective retrovirus vectors. Archer et al. (1994) have described a procedure in which the construct is infused directly into the mammary gland, via the teat canal, during a period of hormone-induced mammogenesis. A gibbon ape leukemia virus was used to deliver the structural gene encoding for human growth hormone resulting in expression of the hormone in goat mammary epithelial cells. The advantage of this method is that expression of the recombinant protein is obtained quickly, without the delay caused by the generation interval required to generate a producing transgenic animal [18 months for goats]. However, reported production levels [Archer et al., 1994] are very low [see Table 1] and at this point in time can only be used for analytical purposes.

Transgenic Animal Production

Transgenic animals for the production of therapeutic and diagnostic proteins are produced by transferring fertilized, transgene-carrying embryos to recipient animals. Following natural gestation and birth, offspring are subject to tissue biopsy and blood sampling: usually ear tissue and blood are screened by polymerase chain reaction and/or Southern analysis for the presence of the transgene. Animals identified as being transgenic are mated with nontransgenic animals: transgenic founder females will produce the protein for
which the DNA codes in their blood or milk depending on the tissue-specific, regulatory promoter sequence of the transgene. Subsequently, transgenic female progeny derived from breeding founder females and males will also express the transgenic protein.

In goats, 16–18 months are required before the first milk is obtained from a natural lactation of a female transgenic animal. However, milk samples can be obtained from founder transgenic females, as well as approximately 30% of male transgenic animals by hormonally induced lactation at about 13 months after microinjection. Induced lactation is useful in checking the expression level and integrity of the heterologous protein.

**Biosynthesis of Milk Proteins**

The original precursors of most of the milk constituents are cellulose, starch, protein, fat, minerals, and vitamins of the plant materials of the ruminant diet. Water is also a prerequisite, and dairy cows require 3–4 liters of water per liter of milk produced. Rumen microorganisms synthesize amino acids, which are adsorbed into the bloodstream. The milk protein precursor amino acids are adsorbed from the bloodstream via the extracellular fluid between the capillaries and the epithelial cells, across the basement membrane of the mammary epithelial cells. Protein biosynthesis is carried out in epithelial cells, and milk proteins are discharged into the lumen of the alveolus by exocytosis and then into the ducts. In ruminants, the ducts empty into a single, primary duct or cistern, which provides extra milk storage capacity. Up to 30% of the milk in the udder is held in the cistern.

As measured by the concentration difference in arteriovenous blood, the mammary gland is particularly efficient at extracting amino acids: 80% of the arterial methionine; 70% of the phenylalanine, leucine, and threonine; 60% of the lysine, arginine, and isoleucine; 55% of the histidine; and 50% of valine are adsorbed. More than 25% of the arterial blood glucose is removed during passage through the mammary gland and is used to power protein synthesis, fat, and lactose production. Intermediates required for protein synthesis are produced in the cytosol and mitochondria. Protein is synthesized at polyribosomes in the rough endoplasmic reticulum where removal of signal peptides occurs. Glycosylation
Section IV. Transgenic Expression

is carried out in the endoplasmic reticulum and the Golgi apparatus where phosphorylation, other posttranslational modifications, and the assembly of casein micelles are carried out. Studies with radiolabeled amino acids indicate that proteins are synthesized in 3–15 min. Radioactivity is seen in the Golgi apparatus after 15–30 min, and the label concentration in the lumen increases after a further 30–60 min. Immunoglobulins and albumin present in milk are not synthesized in the mammary gland but by plasma cells and hepatocytes, respectively, and enter the milk by active transport or filtration. During lactation, B lymphocytes migrate to the mammary gland where they become plasma cells. Plasma cells lodged in the interstitial space may contribute to the high IgG concentration present in the colostrum of early lactation.

In the cow, approximately 500 liters of blood is required to provide the precursors for 1 liter of bovine milk: the blood flow in the udder is ca. 280 ml per second. The goat uses about 400 liters of blood to produce 1 liter of milk: the blood flow in the udder is ca. 1200 liters per day. The mammary gland is able to secrete about 2 g of milk, containing approximately 18 mg of whey protein, per gram of tissue per day. A gram of tissue contains about \(2 \times 10^8\) cells and the milk output is therefore of the order of \(10^{-8}\) g per cell per day. Lymph drains from the udder of the cow at the rate of 1300 ml per hour and in the goat at the rate of 6.5–35 ml per hour. Leukocytes, which account for the major part of the somatic cells found in milk, are derived from lymph.

Morcöl et al. (1994) have calculated the synthesis rate of human recombinant protein C in transgenic swine expressing the protein at 0.1–1 mg/ml milk to be approximately 14 mg per gram of mammary cell per day or about 14 pg/cell/day. In contrast, the rate of normal synthesis in hepatocytes was calculated to be 0.02 mg protein C per gram cells per day.

It has been suggested that mammalian species phylogenetically close to humans may be expected to have more elements of the glycosylation machinery in common (Jenkins et al., 1996). Initial reports indicate that human glycoproteins expressed in the mammary gland of transgenic animals contain glycosylation patterns that differ from those found on human plasma-derived proteins. In general, the glycosylation found on human proteins secreted into transgenic animal milk has been generally similar to plasma protein glycosylation. Sites are mainly biantennary complex oligosaccharides with some variations consistent with the tissue and species of origin. Cole et al. (1994) have shown that human an-
tithrombin III (AT III) and a long-acting form of htpA expressed in goat milk had some GalNAc replacing galactose on complex N-linked oligosaccharides. Both LAtPA and AT III were shown to be more fucosylated than their recombinant or plasma counterparts. Goat plasma AT III contains N-glycolylneuraminic acid and N-acetyleneuraminic acid, as do transgenically expressed AT III and LAtPA. An additional difference observed between plasma and transgenic goat AT III is in the degree of sialylation (Edmunds et al., 1994), with the transgenic protein less sialylated than plasma AT III. Denman et al., (1991) have also noted that significantly lower levels of galactose, N-acetylglucosamine, and sialic acid are present in goat transgenic LAtPA compared to the murine C 127 cell line and Chinese hamster ovary (CHO) cell-derived LAtPA.

The glycosylation of interferon-γ at asparagine 25 and 97 is influenced dramatically by CHO cell culture conditions (Curling et al., 1990); considerable variations in site occupancy are seen. Transgenic mouse-derived interferon-γ has predominantly complex sialated biantennary N-glycans at asparagine 25, and oligosaccharides are α 1-6 core fucosylated similar to the Asn_{25} of CHO cell-derived interferon-γ (James et al., 1995). There is an increased incidence of oligomannose at Asn_{97} compared to the CHO-derived counterpart, suggesting that murine mammary epithelial cells may be deficient in the α 1-2 mannosidase I and GlcNAc transferase I activities in the endoplasmic reticulum.

Although an ultimate goal may be to produce proteins with authentic human glycosylation patterns, a more realistic and possibly more desirable objective is the production of proteins with defined, engineered glycosylation characteristics and therefore predictable pharmacokinetics. Glycoproteins can be remodeled in situ by the transgenic coexpression of human glycosyltransferase. Prieto et al. (1995) have demonstrated that the heterologous, transgenic expression of human α1,2-fucosyltransferase results in expression of both transgene and secondary gene products. Their work also suggests that the mammary gland may be a unique bioreactor for the production of biologically active oligosaccharides and glycoconjugates.

In studies of the expression of rh protein C in transgenic pigs, Subramanian et al. (1996) have shown that there are rate limitations of γ-carboxylation in mice and pigs, partly dependent on the transgene. Their study indicates that a rate limitation of γ-carboxylation in mammary epithelial cells occurs at expression levels of >20 μg/ml in mice and at >500 μg/ml milk in pigs.
Milk Secretion from the Mammary Gland

Mammary glands are skin glands that have no counterpart in non-mammals and are located in the inguinal region of cows, sheep, and goats. In the sow they are located along the thoracic, abdominal, and inguinal walls. Streak canals link the internal milk secretory system with the external environment. The number of teats, teat orientation, and length of the let-down reflex affect the ease and periodicity of milking. Milk is released as a result of a neuroendocrine reflex of the nervous system to tactile, auditory, and visual stimuli. Negative psychological and environmental disturbances have a detrimental effect on milk production. Milk let-down is a response to the release of oxytocin, synthesized in the hypothalamus, by the posterior pituitary gland. Oxytocin is transported to the mammary gland in the arterial blood and binds to myoepithelial cells that contract, causing a release or let-down of the milk. Sheep and goats have let-down periods of 1-2 and 2-4 min, respectively. The let-down reflex in the cow lasts for 5-8 min. In the sow, however, the reflex is extremely short, of the order of 10-20 sec, with a frequency of 1 hr or less.

From the point of view of primary milk production, goats and cows are preferred animals because of the relatively long let-down reflexes, vertical teat orientation, milk volume, and duration of lactation. However, other factors, such as protein concentration, may favor a choice of sheep. Goats, sheep, and cows respond to familiar signals, such as the sound of a milking machine, whereas lactating, nonsuckling sows require injections of oxytocin to elicit the let-down response.

Lactation and Milk Output

At parturition, a series of programmed hormonal changes take place that transform the mammary cells to the fully secretory state. Stage 2 lactogenesis, or the copious production of milk, is brought about by a synchronous drop in progesterone, an increase in estrogen, and the release of prolactin from the anterior pituitary and follows the immediate postpartum production of colostrum.
In the cow, milk production increases in the first 3–6 weeks of lactation and then slowly declines. A similar pattern is seen in goats, sheep, and pigs. Milk secretion continues as long as milk is regularly withdrawn, although production declines during lactation. Dairy animals are capable of undergoing estrus and pregnancy while maintaining their lactation. This enables dairy management to breed the animal such that it will give birth and begin lactation on a yearly basis. In order to maximize production, the animals are allowed to lactate until 2 months before they are due to give birth. They are then "dried off" by cessation of milking. The 2-month rest before the restart of lactation allows the animal to rebuild her energy reserves for the coming birth and lactation. Following birth of the progeny, the animal once again begins the yearly lactation cycle. In all species, yield and energy content are related to body size.

The expression level of exogenous protein tends to follow the normal milk output as can be seen from the plots in Fig. 1 which shows (a) the production of a recombinant monoclonal antibody over a 220-day lactation of a transgenic goat and (b) the levels of antithrombin III in the milk of a transgenic goat during a 300-day lactation.

It has been noted that "considering the yearly milk output of dairy cattle (6000–8000 liters) and the milk content of αs1-casein (10 g/liter), one cow carrying a transgene under the control of αs1-casein promoter would theoretically produce 60–80 kg/year of the transgene-derived protein" (Jäne et al., 1992). At an expression level of 5 g/liter a transgenic goat is capable of producing about 4 kg of target protein per year; at 20 g/liter the output is 16 kg. It is, therefore, quite conceivable to produce 100-kg quantities of target protein in small goat herds or sheep flocks.

**Milk Composition and Purification of the Target Protein**

Milk is a multiphasic fluid composed of a fat emulsion, a micellar casein dispersion, a colloidal suspension of lipoproteins, and a solution of proteins, mineral salts, vitamins, organic acids, and minor components. When collected, milk is not sterile and contains
Figure 1  (a) Milk and recombinant antibody production during the first natural lactation of a transgenic goat during the 1996 season. (b) Milk and Antithrombin III production during the second natural lactation of a transgenic goat during the 1995 season. Work from Genzyme Transgenics Corp. (1996).
bacteria derived from the teat as well as somatic cells derived mostly from the lymphatic ducts of the udder. According to the Pasteurized Milk Ordinance [U.S. Department of Health and Human Services, 1993], the bacterial plate count should be less than 100,000 per milliliter.

Milk composition is species specific; major differences are seen between human and ruminant milk. Within a species, the composition varies with breed, diet, and other factors. Volume and composition also vary during lactation. Table 3 gives the average percentage compositions of livestock milk. Milk is approximately 85–90% water; the pH is 6.5–6.7 and as high as pH 6.8 in ewe’s milk.

It is important from a purification point of view that the fat is present in globular form in the size range of 0.1–10 μm and with a density higher than the other constituents. The fat globule is enclosed in a membrane of polar lipids and proteins. Triglycerides make up 97% of the fat.

The three subgroups of casein, αS-, β-, and κ-casein, display genetic polymorphism and consist of two to eight variants. Casein is present as 10 to 300-nm micelles formed of submicelles held together by phosphate and hydrophobic bonding. Each submicelle has a polar core and a heterogeneous distribution of αS- and β-casein with surface κ-casein, αS- and β-caseins are almost insoluble, whereas the glycoprotein, β-casein is highly soluble in water. Casein may be precipitated somewhat below the isoelectric point range (pH 5.1–5.3) at about pH 4.6–4.7 by acidification or by the addition of chymosin, which attacks the 105 [phenylalanine] and 106 [methionine] peptide bond of the κ-casein. The hydrophilic amino

| Animal | Total protein (%) | Casein (%) | Whey protein (%) | Fat (%) | Lactose (%) |
|--------|------------------|------------|-----------------|--------|-------------|
| Goat   | 3.6              | 2.7        | 0.9             | 4.1    | 4.7         |
| Sheep  | 5.8              | 4.9        | 0.9             | 7.9    | 4.5         |
| Pig    | 5.8              | 4.9        | 0.9             | 8.2    | 4.8         |
| Cowb   | 3.5              | 2.8        | 0.7             | 3.7    | 4.8         |

aData from Bylund (1995).

bHolstein.
acid terminal peptide [106–169] of κ-casein solubilizes in the whey fraction and all other caseins precipitate.

The soluble whey proteins consist primarily of α-lactalbumin and β-lactoglobulin with serum albumin and immunoglobulins being derived from the bloodstream. β-Lactoglobulin is the major protein, accounting for 50% or more of the total whey protein in ruminants and pigs. α-Lactalbumin accounts for approximately 25% of the whey protein fraction and is essential for lactose synthesis and the control of milk secretion: α-lactalbumin binds calcium and zinc. Both α-lactalbumin and β-lactoglobulin have an amino acid composition close to the nutritional optimum and provide amino acids that are essential for the neonate. Whey acid protein is present only in rodent milk.

The temperatures of raw milk at collection is about 37°C. To prevent bacterial growth, oxidation, and proteolysis, milk should be chilled immediately to 4°C and processed within 48 hr unless it is frozen and stored. In the dairy, milk is standardized with regard to the fat content by the centrifugal separation of cream and skim milk: 100 kg of 4% (fat) bovine milk yields 90.35 kg of 0.05% fat skim milk and 9.65 kg of 40% fat cream. The cream fraction is remixed with the skim milk to the required fat content. In processing for a target protein in the whey it is clear that the bulk of the fat can be removed using this standard procedure. However, other standard dairy procedures, particularly microfiltration, may be used to remove fat, casein, and cellular components in a single step. The high lactose and salt content may also be reduced by ultrafiltration. Membrane techniques are generally the initial recovery methods of choice and, when correctly used, may yield a 60% pure protein in a single or a tandem microultrafiltration step, thus providing a clarified whey concentrate for further processing. The use of such techniques also provides a barrier to the entry of adventitious viruses, bacteria, and other microorganisms into the final product.

An alternative pathway is to apply expanded bed technology. After initial processing to remove fat, skim milk may be passed through an adsorbent for the target, allowing the casein, lactose, and the bulk of the whey proteins to pass through the bed. Partially purified target protein may be recovered by desorption from the matrix in a fixed bed mode. This type of separation or direct feed capture may be used in a totally fixed bed mode applied to the whey fraction after tangential flow filtration. Subsequent processing by various chromatographic techniques, including affinity,
hydrophobic interaction, ion exchange, and metal chelate chro-
mamatography, is applicable to achieve a protein of required purity.
The number of steps should be kept to a minimum as even small
step losses can lead to a low yield over an extensive process.

A procedure for the purification of human recombinant protein C
expressed in porcine milk has been described [Degener et al., 1996].
Milk fat was removed by centrifugation, and casein was precipitat-
ed in the presence of zinc. Direct feed capture was performed using
an expanded bed, and the protein was purified by hydrophobic
interaction and anion-exchange chromatography.

Quality Issues in Transgenic Production

Farm activities associated with transgenic production include
founder development, progeny testing, and dairying. To minimize
production risks and achieve validatable procedures, “good agricul-
tural practices” [GAPs], which are in the spirit of good manufactur-
ing practices and good laboratory practice, should be used. GAPs
are based on high standards of animal husbandry, adherence to
standard operating procedures, on-site veterinary care, rigorous ani-
mal health monitoring programs, and state-of-the-art milking prac-
tices to maximize product quality. Standard operating procedures
should cover areas such as generating founder animals, herd main-
tenance, herd health, breeding, milk production, and other special
procedures. Master and working transgenic banks should be kept
under strictly controlled conditions.

Animal feeds should be specially blended free of animal fat and
protein. Hay for transgenic production animals should be screened
for residual chemicals that may have been used in the growing
process. Both written and computerized records should be kept to
track animal lineages, health, and performance records.

Careful attention should be paid to milk facilities and personnel
sanitation. Production animals should be observed on a daily basis,
and animal side testing of milk can be used to detect early indica-
tions of mastitis and other animal illnesses. Milk collection and
processing areas should be physically separated, and state-of-the-art
pharmaceutical grade milking equipment and practices should be
used in dedicated milking parlors.
Regulatory Considerations

"Points to Consider in the Manufacture and Testing of Therapeutic Products for Human Use Derived from Transgenic Animals" [Food and Drug Administration (FDA), 1995] is the result of an iterative process between the FDA and the biopharmaceutical industry, and the FDA is supportive of transgenic production. The European Union's Committee on Proprietary Medicinal Products (CPMP) is similarly positive to the use of transgenic animals, stating in its guidance document: "Transgenic animals may produce higher quantities of material in more concentrated form than existing culture methods, and therefore have considerable advantages in both the cost of producing the starting material and in its downstream processing. In some instances where very large amounts of material are required for therapy the use of transgenic animals may be one of the few viable production strategies" [CPMP, 1995].

The FDA has addressed topics such as the structure of the transgene, creation, characterization, and maintenance of the transgenic herd, fidelity of transgenic inheritance, consistency of transgene expression, analysis of product identity and purity, and the avoidance of contamination by drugs, chemicals, and adventitious agents. Many issues of purity, consistency, safety, and potency of transgenically produced therapeutic proteins overlap with various CBER points to consider for monoclonal antibodies and other biologicals produced by recombinant DNA technology. To quote the FDA: "The considerations that apply are therefore a blend of those relevant to recombinant DNA derived materials and materials from less defined sources" [FDA, 1995].

Despite the fact that goats and other farm animals are susceptible to species-specific viral and prion infections, viruses may be more of a concern for animal health than they are a human risk. A CBER official has pointed out that prions, such as those responsible for bovine spongiform encephalitis and scrapie, have "less than a theoretical" risk of transmission via milk because they do not occur in the mammary gland and, if introduced, they do not persist [Rudolph, 1995]. No transmission of scrapie has ever been reported in humans. Milk and semen are noninfectious for scrapie according to the World Health Organization [1996]. Although scrapie has been detected in significant numbers of sheep in the United States, only five goat scrapie cases have occurred in goats, all comingleing with scrapie-infected sheep. The use of ani-
mals from closed, scrapie-free herds or flocks has been adopted by the leading transgenic production companies to obviate any such concerns. Goat viruses relevant in North America are shown in Table 4.

The risk of viral and bacterial contamination of human biopharmaceutical products expressed in the milk of transgenic goats can be minimized by a multistage or combinatorial approach at three levels: the goat, the milk, and the final product (Ziomek, 1996). Minimization of the risk of contamination of the goat production herd can be accomplished by strict selection, animal husbandry, and adherence to GAPs. Bacterial contamination can be minimized by careful, state-of-the-art milking procedures and rapid GMP processing. Purification processes from raw milk are designed in a manner similar to the manufacturing schemes for recombinant therapeutics from fermentation and cell culture with the inclusion of steps to remove and/or inactivate adventitious agents, microbial contaminants, and pyrogens.

For advantages and disadvantages of transgenic expression in the milk of dairy animals, see Table 5.

Table 4
Goat Viruses Relevant to Production of Proteins in Transgenic Goat Milk

| Family               | Nucleic Acid | Shape      | Size (nm) |
|----------------------|--------------|------------|-----------|
| Enveloped            |              |            |           |
| Rabies (z)           | Rhaboviridae | RNA (ss)   | Bullet    |
| Coronavirus          | Coronoviridae| RNA (ss)   | Unsymmetric |
| Caprine arthritis    | Retroviridae | RNA (ss)   | Spherical |
| encephalitis         |              |            |           |
| Caprine herpes virus | Herpesviridae| DNA (ds)   | Spherical |
| Pseudorabies         | Herpesviridae| DNA (ds)   | Icosahedral |
| Nonenveloped         |              |            |           |
| Bluetongue           | Reoviridae   | RNA (ds)   | Spherical |
| Rotavirus            | Reoviridae   | RNA (ds)   | Spherical |
| Adenovirus           | Adenoviridae | DNA (ds)   | Icosahedral |
| Complex              |              |            |           |
| Capripox (z)         | Poxviridae   | DNA (ds)   | Complex   |
| Contagous ectorhyma (z) | Poxviridae | DNA (ds)   | Complex   |

*aFrom Ziomek (1996).

*z, zoonotic: ss, single stranded; ds, double stranded.
Table 5
Advantages and Disadvantages of Transgenic Expression in the Milk of Dairy Animals

| Advantages                                                                                                                     |
|-------------------------------------------------------------------------------------------------------------------------------|
| High level expression at multigram/liter level                                                                             |
| Expression directed to and located in mammary gland                                                                       |
| Expression can be tested in rodents                                                                                         |
| Genetic and lactation-to-lactation stability                                                                               |
| Animal-to-animal consistency                                                                                                 |
| Mammary epithelial cells carry out post-translational modification                                                          |
| Product recovered at high concentration in milk                                                                             |
| Aseptic milk processing technology well proven                                                                               |
| Bulk impurities removed easily                                                                                                |
| Low cost of pre-purification product                                                                                        |
| Low investment costs                                                                                                        |
| Capability of combining transgenics with nuclear transfer (cloning)                                                         |

| Disadvantages                                                                                                                |
|-------------------------------------------------------------------------------------------------------------------------------|
| Possible adventitious virus and prion issues in dairy animals                                                                  |
| Control of animal environment and feed                                                                                       |
| Microinjection required as a technology for species where cloning is not yet available                                         |
| Time to clinical product slower than cell culture (without process development)                                                |

Current Status and Future Directions

Expression of therapeutically beneficial proteins in the milk of transgenic dairy animals present an unparalleled opportunity for the large-scale production of monoclonal antibodies, plasma, and other proteins.

Mammary gland epithelia have a cell density that is 100- to 1000-fold greater than the cell densities used, for example, in mammalian cell culture using CHO cells. The cells are some of the most productive protein synthesis sources designed by nature to produce large amounts of correctly processed protein and which are switched on and off by hormonal changes. Thirty-five transgenic goats expressing a monoclonal antibody at 8 g/liter in their milk are equivalent to a 8500-liter batch CHO cell culture running 200 days/year with a 1-g/liter final expression level (Young et al., 1997).

In production terms, 170,000 liters of culture is equivalent to 21,000 liters of milk; with the expression levels noted earlier and
process yields of 60%, both of these systems would produce 100 kg of purified monoclonal antibody.

It has also been discussed that the mammary bioreactor is capable of most posttranslational modifications and protein folding and can, therefore, be used to produce complex proteins.

A current limitation of the technology is the low rate (5–10%) of transgenesis. Nuclear transfer techniques, in which embryonic or adult cells are engineered and cultured to produce a master cell bank from which nuclei are transferred to recipient oocytes, are under development; these methods hold great promise for improved rates of transgenesis.

Because of concerns of transmissible spongiform encephalopathies, animal sourced proteins and amino acids are currently under scrutiny. However, it should be kept in mind that the expression system described in this chapter is the mammary gland. Given the safety of milk and milk products in combination with the quality and regulatory precautions described, there is every reason to consider that proteins derived from transgenic animals should be safe with respect to possible virus and prion transmission. Many of the proteins that are expression targets today are currently available as human plasma derivatives, which have a measurable degree of risk for the transmission of HIV, hepatitis, parvo-, and other viruses, and Creutzfeld-Jakob disease.

As indicated, several proteins are in phase I and II clinical trials. This number can be expected to increase dramatically in the near future. Transgenic dairy animals provide a bulk protein production system that is capable of making proteins available that are either not available today or are only recoverable from other sources at very low yields. Transgenic production may thus enable a move from "on-demand" patient treatment to prophylaxis and far wider indications and use of many proteins. In addition, the production of proteins in the milk of transgenic dairy animals is highly cost effective (Young et al., 1997), opening up real possibilities for nutraceutical product development.

References

Archer, J. S., Kennan, W. S., Gould, M. N., and Bremel, R. D. (1994). Human growth hormone (hGH) secretion in milk of goats after direct transfer of the hGH gene into the mammary gland by using replication-defective retrovirus vectors. Proc. Natl. Acad. Sci. U.S.A. 91, 6840–6844.
Archibald, A. L., McClenaghan, M., Hornsey, V., Simons, J. P., and Clark, A. J. [1990]. High level expression of biologically active human α1-antitrypsin in the milk of transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 87, 5178–5182.

Bially, H. [1991]. Barnyard biotechnology: “Transgenic pharming comes of age.” *Bio/Technology* 9, 786–788.

Bischoff, R., Dergryscy, E., Perraud, F., Dalemens, W., Ali-Hadjija, D., Thépot, D., Devinoy, E., Houdebine, L. M., and Pavirani, A. [1992]. A 17.2 kbp region located upstream of the rabbit WAP gene directs high level expression of a functional human protein variant in transgenic mouse milk. *FEBS Lett.* 305, 265–268.

Brem, G., Brenig, B., Goodman, H. M., Selden, R. C., Graf, F., Kruß, B., Springman, K., Hondele, J., Meyer, J., Winnacker, E.-L., and Krässlich, H. [1985]. Production of transgenic mice, rabbits and pigs by microinjection into pronuclei. *Zuechthygiene* 20, 251–252.

Brem, G., Hartl, P., Besenfelder, U., Wolf, E., Zinovieva, N., and Pfaller, R. [1994]. Expression of synthetic cDNA sequences encoding human insulin-like growth factor-1 (IGF-1) in the mammary gland of transgenic rabbits. *Gene* 149, 351–355.

Brinster, R. L., Chen, H. Y., Trumbauer, M. E. et al. [1985]. Factors affecting the efficiency of introducing foreign DNA into mice by microinjection eggs. *Proc. Natl. Acad. Sci. U.S.A.* 82, 4438–4442.

Bylund, G. [1995]. “Dairy Processing Handbook.” Tetra Pak Processing Systems AB, Lund.

Campbell, K. H. S., McWhir, J., Ritchie, W. A., and Wilmut, I. [1996]. Sheep cloned by nuclear transfer from a cultured cell line. *Nature (London)* 380, 64–66.

Campbell, S. M., Rosen, J. M., Hennighausen, L. G. et al. [1984]. Comparison of the whey acidic protein genes of rat and mouse. *Nucleic Acids Res.* 12, 8685–8697.

Carver, A. [1996]. Transgenics on trial. *Scrip Mag.* November, pp. 51–53.

Clark, A. J., Simons, P., Wilmut, I., and Lathe, R. [1987]. Pharmaceuticals from transgenic livestock. *Trends Biotechnol.* 3, 20–24.

Clark, A. J., Bessos, H., Bishop, J. O., Brown, P., Harris, S., Lathe, R., McClenaghan, M., Prowse, C., Simons, J. P., Whitelaw, C. B. A., and Wilmut, I. [1989]. Expression of anti-haemophilic factor IX in the milk of transgenic sheep. *Bio/Technology* 7, 487–492.

Cole, E. S., Higgins, E., Bernasconi, R., Garone, L., and Edmunds, T. [1994]. Glycosylation patterns of human proteins expressed in transgenic goat milk. *J. Cell. Biochem. Suppl.* 18D, 265.

Committee on Proprietary Medicinal Products [CPMP] [1995]. “Use of Transgenic Animals in the Manufacture of Biological Medicinal Products for Human Use.” Ad hoc Working Party on Biotechnology/Pharmacy, Directorate-General III/3612/93 Final.

Curling, E. M., Hayter, P. M., and Baines, A. J. [1990]. Recombinant interferon-gamma. Differences in the glycosylation and proteolytic processing lead to heterogeneity in batch culture. *Biochem. J.* 272, 333–337.

Degener, A., Belew, M., and Velander, W. H. [1996]. High selectivity purification of recombinant proteins from milk using expanded bed chromatography. *Abstr. Recovery Biol. Prod.*, Tucson, AZ, 8th, 1996.

Denman, J., Hayes, M., O’Day, C., Edmunds, T., Bartlett, C., Hirani, S., Ebert, K. M., Gordon, K., and McPherson, J. M. [1991]. Transgenic expression of a variant of human tissue-type plasminogen activator in goat milk: Purification and characterization of the recombinant enzyme. *Bio/Technology* 9, 839–843.
Devinoy, E., Thépot, D., Stinnakre, M.-G., Fontaine, M.-L., Grabowski, H., Puissant, C., Pavirani, A., and Houdebine, L.-M. (1994). High level production of human growth hormone in the milk of transgenic mice: The upstream region of the whey acidic protein (WAP) gene targets transgene expression to the mammary gland. *Transgenic Res.* 3, 79–86.

Drohan, W. N., Zhang, D., Paleyanda, R. K., Chang, R., Wrobie, M., Velander, W. H., and Lubon, H. (1994). Inefficient processing of human protein C in the mouse mammary gland. *Transgenic Res.* 3, 355–364.

Ebert, K. M., and Schindler, J. E. S. (1993). Transgenic farm animals: Progress report. *Theriogenology* 39, 121–135.

Ebert, K. M., Selgrath, J. P., Di Tullio, P., Denman, J., Smith, T. E., Memon, M. A., Schindler, J. E., Monastersky, G. M., Vitale, J. A., and Gordon, K. (1991). Transgenic production a variant of human tissue-type plasminogen activator in goat milk: Generation of transgenic goats and analysis of expression. *Bio/Technology* 9, 835–838.

Ebert, K., Di Tullio, P., Barry, C. A., Schindler, J. E., Ayres, S. L., Smith, T. E., Pellicer, L. J., Meade, H. M., Denman, J., and Roberts, B. (1994). Induction of human tissue plasminogen activator in the mammary gland of transgenic goats. *Bio/Technology* 12, 699–702.

Echelard, Y. (1996). Recombinant protein production in transgenic animals. *Curr. Opin. Biotechnol.* 7, 536–540.

Edmunds, T., Higgins, E., Bernasconi, R., Garone, L., and Cole, E. S. (1994). Tissue specific and species differences in the glycosylation pattern of Antithrombin III. *J. Cell. Biochem., Suppl.* 18D, 265.

Food and Drug Administration (FDA) (1995). “Points to Consider in the Manufacture and Testing of Therapeutic Products for Human Use Derived from Transgenic Animals.” FDA, CBER.

Gordon, J. W., and Ruddle, F. H. (1981). Integration and stable germ-line transmission of genes injected into mouse pronuclei. *Science* 214, 1244–1246.

Gordon, J. W., Scangos, G. A., Plotkin, D. J., Barbosa, J. A., and Ruddle, F. H. (1980). Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci. U.S.A.* 77, 7380–7384.

Gordon, K., Lee, E., Vitale, J. A., Smith, A. E., Westphal, H., and Hennighausen, L. (1987). Production of human tissue plasminogen activator in transgenic mouse milk. *Bio/Technology* 5, 1183–1187.

Gutierrez, A., Meade, H. M., Ditullio, P., Pollock, D., Harvey, M., Jimenez-Flores, R., Anderson, G. B., Murray, J. D., and Medrano, J. F. (1996). Expression of a bovine k-CN cDNA in the mammary gland of transgenic mice utilizing a genomic milk protein gene as an expression cassette. *Transgenic Res.* 5, 271–279.

Halter, R., Carnwath, J., Espanion, G., Herrmann, D., Lemme, E., Niemann, H., and Paul, D. (1993). Strategies to express Factor VIII gene constructs in the ovine mammary gland. *Theriogenology* 39, 137–149.

Hammer, R. E., Pursel, V. G., Rexroad, C. E., Jr., Wall, R. J., Bolt, D. J., Ebert, K. M., Palmiter, R. D., and Brinster, R. L. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. *Nature (London)* 315, 680–683.

Hansson, L., Edlund, M., Edlund, A., Johansson, T., Marklund, S. L., Fromm, S., Stromqvist, M., and Tornell, J. (1994). Expression and characterization of biologically active human extracellular superoxide dismutase in milk of transgenic mice. *J. Biol. Chem.* 269, 5358–5363.

Harris, S., Ali, S., Anderson, S., Archibald, A. L., and Clark, A. J. (1988). Complete
nucleotide sequence of the ovine \( b \)-lactoglobulin gene. *Nucleic Acids Res.* **19**, 10379–10380.

Henninghausen, L. [1990]. The mammary gland as a bioreactor: Production of foreign proteins in milk. *Protein Express. Purif.* **1**, 1–6.

Henninghausen, L., Ruiz, L., and Wall, R. [1990]. Transgenic animals—production of foreign proteins in milk. *Curr. Opin. Biotechnol.* **1**, 74–78.

Hogan, B., Costantini, F., and Lacy, E. [1986]. "Manipulating the Mouse Embryo." Cold Spring Harbor Press, Cold Spring Harbor, NY.

Houdebine, L.-M. [1994]. Production of pharmaceutical proteins from transgenic animals. *J. Biotechnol.* **34**, 269–287.

Houdebine, L.-M. [1995]. The production of pharmaceutical proteins from the milk of transgenic animals. *Reprod. Nutr. Dev.* **35**, 609–617.

Iannaccone, P. M., Taborn, G. U., Garton, R. L., Caplice, M. D., and Brenin, D. R. [1994]. Pluripotent embryonic stem cells from the rat are capable of producing chimeras. *Dev. Biol.* **163**, 288–292.

James, D. C., Freedman, R. B., Hoare, M., Ogonah, O. W., Rooney, B. R., Larionov, O. A., Dobrovolsky, V. N., Lagutin, O. V., and Jenkins, N. [1995]. N-glycosylation of recombinant human interferon-g produced in different animal expression systems. *Bio/Technology* **13**, 592–596.

Jänne, J., Hyttinen, J.-M., Peura, T., Tolvanen, M., Ahlonen, L., and Halmekytö, M. [1992]. Transgenic animals as bioproducers of therapeutic proteins. *Ann. Med.* **24**, 273–280.

Jänne, J., Hyttinen, J.-M., Peura, T., Tolvanen, M., Ahlonen, L., Sincervita, R., and Halmekytö, M. [1994]. Transgenic bioreactors. *Int. J. Biochem.* **26**, 859–870.

Jenkins, N., Parekh, R. B., James, D. C. [1996]. Getting the glycosylation right: implications for the biotechnology industry. *Nat. Biotechnol.* **14**, 975–981.

Koczan, D., Hobom, G., and Seyfert, H. M. [1991]. Genomic organization of the bovine \( a_S \)1-casein gene. *Nucleic Acids Res.* **19**, 5591–5596.

Krimpenfort, P., Rademakers, A., Eyestone, W., van der Schans, A., van den Broek, S., Kooiman, P., Kootwijk, E., Platenburg, G., Pieper, F., Strijker, R., and de Boer, H. [1991]. Generation of transgenic dairy cattle using "in vitro" embryo production. *Bio/Technology* **9**, 844–847.

Lee, S. H., and de Boer, H. A. [1994]. Production of biomedical proteins in the milk of transgenic dairy cows: State of the art. *J. Controlled Release* **29**, 213–221.

Limonta, J. M., Castro, F. O., Martinez, R., Puentes, P., Ramos, B., Aguilar, A., Leonart, R. L., and de la Fuente, J. [1995]. Transgenic rabbits as bioreactors for the production of human growth hormone. *J. Biotechnol.* **40**, 49–58.

Logan, J. S. [1993]. Transgenic animals: beyond ‘funny milk.’ *Curr. Opin. Biotechnol.* **4**, 591–595.

Lonnerdal, B., and Iyer, S. [1995]. Lactoferrin molecular structure and biological function. *Annu. Rev. Nutr.* **15**, 93–110.

Maga, E. A., and Murray, J. D. [1995]. Mammary gland expression of transgenes and the potential for altering the properties of milk. *Bio/Technology* **13**, 1452–1457.

Maga, E. A., Anderson, G. B., Huang, M. C., and Murray, J. D. [1994]. Expression of human lysozyme mRNA in the mammary gland of transgenic mice. *Transgenic Res.* **3**, 36–42.

McEvoy, T. G., and Sreenan, J. M. [1990]. The efficiency of production, centrifugation, microinjection and transfer of one- and two-cell bovine ova in a gene transfer program. *Theriogenology* **33**, 819–828.

Meade, H., Gates, L., Lacy, E. *et al.* [1990]. Bovine \( a_S \)1-casein gene sequences direct
high level expression of active urokinase in mouse milk. *Bio/Technology* **8**, 443–446.

Morcöl, T., Akers, R. M., Johnson, J. L., Williams, B. L., Gwazdauskas, F. C., Knight, J. W., Lubon, H., Paleyanda, R. K., Drohan, W. N., and Velander, W. H. (1994). The porcine mammary gland as a bioreactor for complex proteins. *Ann. N.Y. Acad. Sci.* **721**, 218–233.

Ninomiya, T., Hirabayashi, M., Sagara, J., and Yuki, A. (1994). Functions of milk protein gene 5' flanking region on human growth hormone gene. *Mol. Reprod. Dev.* **37**, 276–283.

Nuijens, J. H., Geerts, M. E. J., van Berkel, P. H. C., Harteveld, P. P., de Boer, H. A., van Veen, H. A., and Pieper, F. R. (1995). Characterization of recombinant human lactoferrin expressed in the milk of transgenic mice. *Proc. Int. Conf. Struct. Funct. Lactoferrin, 2nd.*

Palmiter, R. D., and Brinster, R. L. (1986). Germ-line transformation of mice. *Annu. Rev. Genet.* **20**, 465–499.

Persuy, M. A., Stinnakre, M. G., Printz, C., Mahe, M. F., and Mercier, J. C. (1992). High expression of the caprine b-casein gene in transgenic mice. *Eur. J. Biochem.* **205**, 887–893.

Persuy, M. A., Legrain, S., Printz, C., Stinnakre, M. G., Lepourry, L., Brignon, G., and Mercier, J. C. (1995). High-level, stage- and mammary-tissue specific expression of a caprine k-casein-encoding minigene driven by a b-casein promoter in transgenic mice. *Gene* **165**, 291–296.

Pinkert, C. A. (1994). “Transgenic Animal Technology: A Laboratory Handbook.” Academic Press, San Diego, CA.

Platenburg, G. J., Kootwijk, E. P. A., Kooiman, P. M., Woloshuk, S. L., Nuijens, J. H., Krimpenfort, P. J. A., Pieper, F. R., deBoer, H. A., and Strijker, R. (1994). Expression of human lactoferrin in milk of transgenic mice. *Transgenic Res.* **3**, 99–108.

Prieto, P. A., Mukerji, P., Kelder, B., Erney, R., Gonzalez, D., Yun, J. S., Smith, D. F., Moremen, K. W., Nardelli, C., Pierce, M., Li, Y., Chen, X., Wagner, T. E., Cummings, R. D., and Kopchick, J. J. (1995). Remodeling of mouse milk glycoconjugates by transgenic expression of a human glycosyl transferase. *J. Biol. Chem.* **270**, 29515–29519.

Prunkard, D., Cottingham, I., Garner, L., Bruce, S., Balrymple, M., Lasser, G., Bishop, P., and Foster, D. C. (1996). High-level expression of recombinant human fibrinogen in the milk of transgenic animals. *Nat. Biotechnol.* **14**, 867–871.

Pursel, V., Hammer, R. E., Bolt, D., Palmiter, R. D., and Brinster, R. L. (1990). Integration, expression and germ-line transmission of growth-related genes in pigs. *J. Reprod. Fertil., Suppl.* **41**, 77–87.

Reddy, V. B., Vitale, J. A., Montoya-Zavala, M., and Robl, J. M. (1991). Expression of human growth hormone in the milk of transgenic mice. *Anim. Biotechnol.* **2**, 15–29.

Rexroad, C. R., Jr., and Wall, R. J. (1987). Development of one-cell fertilized sheep ova following microinjection into pronuclei. *Theriogenology* **27**, 611–619.

Rexroad, C. R., Jr., Powell, A. M., Behringer, R. R. *et al.* (1990). Insertion, expression and physiology of growth regulating genes in ruminants. *J. Reprod. Fertil., Suppl.* **41**, 119–124.

Riego, E., Limonta, J., Aguilar, A., Perez, A., de Armas, R., Solano, R., Ramos, B., Castro, F. O., and de la Fuente, J. (1993). Production of transgenic mice and rabbits that carry and express the human tissue plasminogen activator cDNA un-
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der the control of a bovine alpha S1 casein promoter. *Theriogenology* **39**, 1173–1185.

Roberts, B., Di Tullio, P., Vitale, J. *et al.* (1992). Cloning of the goat beta-casein gene and expression in transgenic mice. *Gene* **121**, 255–262.

Roschlau, K., Rommel, P., Andreewa, L., Zackel, M., Roschlau, D., Zackel, B., Schwerin, M., Hühn, R., and Gazarjan, K. G. (1989). Gene transfer experiments in cattle. *J. Reprod. Fertil., Suppl.* **38**, 153–160.

Rudolph, N. S. (1995). Regulatory issues relating to protein production in transgenic animal milk. *Genet. Eng. News* **15**, 16–18.

Selgrath, J. P., Memon, M. A., Smith, T. E., and Ebert, K. M. (1990). Collection and transfer of microinjectable embryos from dairy goats. *Theriogenology* **34**, 1195–1205.

Shani, M., Barash, I., Nathan, M., Ricca, G., Searfoss, G. H., Dekel, I., Faerman, A., Givol, D., and Hurwitz, D. R. (1992). Expression of human serum albumin in the milk of transgenic mice. *Transgenic Res.* **1**, 195–208.

Simons, J. P., Wilmut, I., Clark, A. J., Bishop, J. O., and Lathe, R. (1988). Gene transfer into sheep. *Bio/Technology* **6**, 179–183.

Stice, S. L., Strelchenko, N. S., Keefer, C. L., and Matthews, L. (1996). Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. *Biol. Reprod.* **54**, 100–110.

Stinnakre, M. G., Vilotte, J. L., Soulier, S., l’Haridon, R., Charlier, M., Gaye, P., and Mercier, J. C. (1991). The bovine a-lactalbumin promoter directs expression of ovine trophoblast interferon in the mammary gland of transgenic mice. *FEBS Lett.* **284**, 19–22.

Subramanian, A., Paleyanda, R. K., Lubon, H., Williams, B. L., Gwazdauskas, F. C., Knight, J. W., Drohan, W. N., and Velander, W. H. (1996). Rate limitations in posttranslational processing by the mammary gland of transgenic animals. *Ann. N.Y. Acad. Sci.* **782**, 87–96.

Thépot, D., Devinoy, E., Fontaine, M. L., Stinnakre, M.-G., Massoud, M., Kann, G., and Houdebine, L.-M. (1995). Rabbit whey acidic protein gene upstream region controls high level expression of bovine growth hormone in the mammary gland of transgenic mice. *Mol. Reprod. Dev.* **42**, 261–267.

U.S. Department of Health and Human Services Public Health Service. (1993). “Pasteurized Milk Ordinance.” FDA, Washington, D.C.

Uusi-Oukari, M., Hyttinen, J.-M., Korhonen, V-P., Västl, A., Alhonen, L., Jänne, O. A., and Jänne, J. (1997). Bovine a-s1 casein gene sequences direct high level expression of human granulocyte-macrophage colony-stimulating factor in the milk of transgenic mice. *Transgenic Res.* **6**, 74–84.

Velander, W. H., Johnson, J. L., Page, R. L., Russell, C. G., Subramanian, A., Wilkins, T. D., Gwazdauskas, F. C., Pittius, C., and Drohan, W. (1992). High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human Protein C. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 12003–12007.

Vilotte, J. L., Soulier, S., Stinnakre, M. G., Massoud, M., and Mercier, J. C. (1989). Efficient tissue-specific expression of bovine a-lactalbumin in transgenic mice. *Eur. J. Biochem.* **186**, 43–48.

Wall, R. J., Hawk, H. W., and Nel, N. (1992). Making transgenic livestock: Genetic engineering on a large scale. *J. Cell. Biochem.* **49**, 113–120.

Wei, Y., Yarus, S., Greenberg, N. M., Whitsett, J., and Rosen, J. M. (1995). Produc-
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- Wheeler, M. B. (1994). Development and validation of swine embryonic stem cells: A review. *Reprod. Fertil. Dev.* 6, 563–568.
- Whitelaw, C. B. A., Archibald, A. L., Harris, S. *et al.* (1991). Targeting expression to the mammary gland: Intronic sequences can enhance the efficiency of gene expression in transgenic mice. *Transgenic Res.* 1, 3–13.
- Whitelaw, C. B. A., Harris, S., McClenaghan, M. *et al.* (1992). Position-independent expression of the ovine β-lactoglobulin gene in transgenic mice. *Biochem. J.* 286, 31–39.
- Wilmut, I., Archibald, A. L., McClenaghan, M., Simons, J. P., Whitelaw, C. B. A., and Clark, A. J. (1991). Production of pharmaceutical proteins in milk. *Experientia* 47, 905–912.
- World Health Organization (1996). "Emerging and Other Communicable Diseases." Report of a WHO Consultation on Public Health Issues Related to Human and Animal Transmissible Spongiform Encephalopathies. WHO, Geneva. http://www.who.ch
- Wright, G., Carver, A., Cottom, D., Reeves, D., Scott, A., Simons, P., Wilmut, I., Garner, I., and Coleman, A. (1991). High level expression of active human α1-antitrypsin in the milk of transgenic sheep. *Bio/Technology* 9, 830–834.
- Yarus, S., Greenberg, N. M., Wei, Y., Whitsett, J. A., Weaver, T. E., and Rosen, J. M. (1997). Secretion of unprocessed surfactant protein B in milk of transgenic mice. *Transgenic Res.* 6, 51–57.
- Young, M. W., Okita, W. B., Brown, M., and Curling, J. M. (1997). Production of bio-pharmaceutical proteins in the milk of transgenic dairy animals. *BioPharmacology* (in press).
- Yull, F., Harold, G., Wallace, R., Cowper, A., Percy, J., Cottingham, I., and Clark, A. J. (1995). Fixing human factor IX (fIX): Correction of a cryptic RNA splice enables the production of biologically active fIX in the mammary gland of transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 92, 10899–10903.
- Ziomek, C. A. (1996). Minimization of viral contamination in human pharmaceuticals produced in the milk of transgenic goats. *Dev. Biol. Stand.* 88, 263–266.

- Contribution of human surfactant protein C in milk of transgenic mice. *Transgenic Res.* 4, 232–241.