Transgenic Approaches for Modifying the Mammary Gland to Produce Therapeutic Proteins

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Bioengineering of the mammary gland to produce proteins of therapeutic and industrial value is the result of extensive investigation of the physiology of the mammary gland and the ability to generate transgenic animals. Targeting the expression of heterologous proteins to mammary tissue requires a thorough understanding of the biochemical events that coordinate growth and differentiation of the mammary gland and of the hormonal and developmental regulation of expression of milk protein genes. The characterization of mammary-specific promoter regions in milk protein genes and knowledge of the mechanisms that confer integration site-independent expression of transgenes have significantly contributed to modifying the mammary gland to produce heterologous proteins of therapeutic interest. The generation of large transgenic farm animals provides the opportunity for large-scale production of proteins in milk that have a therapeutic value but are naturally present at low concentrations in biological fluids. Transgenic mammary epithelial cells offer a versatile research model in biomedical, environmental health, and neonatal toxicology research. Key words: chronic and hereditary diseases, environmental and health research, gene expression, lactation, mammary gland, therapeutic proteins, transgenics. Environ Health Perspect 102:846–851 (1994)

Significant advances in transcriptional regulation of gene expression, coupled with the development of transgenic animals, have provided whole-animal systems where the mammary gland is of special interest for experimental (1) and therapeutic applications (2). The characterization of mammary-specific promoter sequences in milk protein genes has been of critical importance to investigating induction of oncogene expression (3,4) and stimulation of cell proliferation by growth factors (5) in mammary tissue. As a consequence of this progress, the mammary gland of experimental animals has been genetically engineered to explore the molecular mechanisms involved in the development of mammary neoplasia. Furthermore, transgenic approaches have been developed for modifying the mammary gland to produce heterologous proteins of pharmaceutical and industrial interest (6).

This article presents some of the recent research advances that define the developmental and hormonal control of expression of mammary-specific promoters and the use of these regulatory regions in directing the production of heterologous proteins in milk of transgenic animals. We attempt to complement earlier reviews (7,8) and critically integrate important aspects of the physiology of the mammary gland with recent observations pertaining to regulation of gene expression of milk protein genes. Finally, we discuss the advantages of manipulating the genetic makeup of mammary tissue, as well as the pitfalls and potential areas of concern associated with bioengineering the mammary gland.

Development of the Mammary Gland: Growth and Differentiation

The development of the mammary gland is brought about by proliferative and differentiative events that begin during embryogenesis and progress into the lactating state (9,10). At birth, the mammary gland is a rudimentary branched-duct system that develops isometrically with the rest of the body. With the onset of puberty, ovarian hormones initiate ductal morphogenesis through cycles of mammary cell proliferation that result in branching of the duct system and fat deposition (11). With pregnancy, the secretory cells of the ductal tree enter a phase of sustained proliferation that continues during early lactation, leading to complete lobulo-alveolar development. This process (mammosogenesis) is largely responsible for most of the volumetric development of mammary tissue.

Lactogenesis, the capacity for mammary tissue to synthesize and secrete milk, is made possible because the mammary gland is a specialized organ that secretes milk components into the lumen of ducts. Embedded in a stromal milieu, alveoli are the working units of this branched-glandular machinery. In vitro, alveolarlike structures, generally referred to as mammospheres (12), can be generated by culturing mammary epithelial cells on components of the extra cellular matrix (Fig. 1) (13). Histological cross-sections of fully developed mammospheres show alveolar morphology characteristic of columnar-shaped epithelial cells that delineate a hollow sphere where milk components are secreted by exocytosis (14).

Proliferation and differentiation of the mammary gland are controlled by a large number of factors that include various steroids, peptide hormones, and growth factors. Endocrine control of mammary development is not only species specific, but also differs in prepubertal growth when compared to pregnancy and lactation. Early studies (15–17) which used ablation of endocrine glands and replacement therapy techniques in rats and mice, provided evidence that mammary development is under the control of the ovarian steroids, estrogen and progesterone. Specifically, during the prepubertal stage, replacement therapy with estrogen, in combination with either growth hormone or prolactin, stimulates ductal growth, which is enhanced further by glucocorticoids. Lobuloalveolar growth in ovariectomized, hypophysectomized, and adrenalectomized mice required replacement therapy with both progesterone and estrogen in addition to growth hormone or prolactin.

The contribution to mammary development by peptide hormones is also well documented. For example, insulinlike growth factor-I (IGF-I), primarily produced in the liver, mediates growth hormone actions on mammary tissue through an endocrine mode of action. In addition, experimental evidence (18,19) suggests that growth factors, such as IGF-I and epidermal growth factor (EGF) may act locally through autocrine/paracrine pathways. Taken together, these observations offer compelling evidence that several biochemical messengers act coordinately to control morphogenesis and biochemical differentiation of mammary cells necessary for the synthesis and secretion of milk components (20).

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Regulation of Expression of Milk Protein Genes and Characterization of Mammary-specific Elements

Approximately 90% of the protein in milk of dairy species is accounted for by four caseins (\(\alpha_1\), \(\alpha_2\), \(\beta\), and \(\kappa\)), and two whey proteins (\(\alpha\)-lactalbumin and \(\beta\)-lactoglobulin). In rodents, the whey acidic protein (WAP) is the major whey protein (6). Expression of milk protein genes is under the control of various factors that include steroids, peptide hormones, and cell–substratum interactions and requires well-defined stage-dependent conditions. Although the development of alveolarlike structures begins in mid-gestation, the expression of milk proteins is minimal prior to birth. The onset of lactation coincides with a marked decrease in serum progesterone, which is inhibitory to the galactopoietic pathways (21), and an elevation of circulating lactogenic hormones, primarily glucocorticoids and prolactin (22).

Full differentiation requires morphological changes that are triggered by the establishment of cell–cell contacts. These interactions are necessary for mammary epithelial cells to assume correct cellular polarity and form a luminal compartment. In fact, the presence of specific extracellular matrix components induces the formation of lobulo-alveolar structures and promotes coordinately with lactogenic hormones the expression of milk protein genes (23).

Induction of expression of milk protein genes is mediated through activation of hormone responsive elements (HRE) localized in 5’ flanking promoter regions. A variety of promoter elements have been characterized that direct the expression of the \(\alpha\)-casein (24), \(\beta\)-casein (25), \(\alpha\)-lactalbumin (26), \(\beta\)-lactoglobulin (27), and WAP (28) genes. Interestingly, the \(\alpha\)- and \(\beta\)-casein genes have most likely evolved from a common ancestor gene and the 5’ flanking region of both genes contains putative HRE sequences that are highly conserved. The evolutionary conservation of the HRE element is of great importance to mammary gland biotechnologists; in fact, the characterization of “universal” transcription regulatory sequences may allow for their general use in different species. For example, various 5’ flanking sequences of the mouse WAP gene have been used successfully to direct the expression of the human protein C in transgenic pigs (29).

Bioengineering the mammary gland requires that transcription modules designed to drive the expression of heterologous proteins should be mammary specific and under the same developmental and hormonal constraints that regulate the expression of endogenous milk protein genes. This is the case of the glucocorticoid-inducible mouse mammary tumor virus-long terminal repeat (MMTV-LTR) promoter element, which is primarily active in the mammary gland. Although it may sustain the expression of heterologous proteins in other tissues (30), the MMTV-LTR contains sequences that are the target for binding not only by glucocorticoid but also sex steroid and mineralocorticoid receptors (31). Consequently, the LTR element has been used extensively to direct transgene expression in intact animals and cell culture of growth factors and proto-oncogenes that may be involved in the development of mammary neoplasia (32). Conversely, lack of mammary specificity could lead to deregulated activation of a transgene in different tissues and impair the normal physiology of the whole animal. Constitutive, tissue nonspecific expression of transgenes would be expected if the transgenes were under the control of strong constitutively active transcriptional elements, such as those found in the simian SV40 or herpes simplex thymidine kinase (TK) promoters (33).

Regulation of Expression of Transgenes

The random integration of chimeric genes into the genome of the host cell may abrogate the correct position-independent expression of heterologous proteins, leading to lack of mammary gland specificity. Many studies have demonstrated that tissue-specific expression of 5’ flanking regions of the WAP gene was copy-number independent and highly affected by the site of integration (6). Moreover, experimental evidence suggests that site-dependent regulation of expression may occur because of the structural organization of the heterochromatin surrounding the integrated transgene (34). This nuclear environment may in turn affect the regulation of transcription of heterologous genes negatively or positively.

Ideally, each integration event should express the same level of activity in a dominantly-like fashion. Site integration-independent expression may be achieved if a defined transcriptional module could be topologically protected from effects of the surrounding genetic material. Notably, in search of DNA modules that may confer position-independent expression to transgenes, significant progress has been made in defining cis-acting matrix attachment regions (MAR). The MAR elements have been located in border regions of transcriptionally active DNA domains of the human \(\beta\)-globin (34) and chicken lysozyme (35) genes and shown to possess high binding affinity for nuclear matrix molecules (36). Specifically, strong attachment regions were located between 11.1 and 8.8 kb upstream of the transcription start site and between 1.3 and 5.0 kb downstream of the poly(A)” site of the chicken lysozyme (35). The use of one such MAR region, the A element (A =
attachment), in WAP expression vectors confers position-independent, as well as hormonally and developmentally correct, regulation of expression in transgenic mice (37). These studies provided significant evidence of the ability of chicken lysozyme MAR sequences to serve as structural cis-acting elements that confer position-independent and high-level expression of randomly integrated transgenes (38).

It should be pointed out that this strategy presents interesting similarities with that used by the MMTV during its infectious cycle in the mammary gland. The integrated DNA provirus of the MMTV consists of three structural genes (gag, pol, and env), which are flanked on both sides by two LTRs (Fig. 2). Activity of the two LTR elements is position independent and primarily under control of the trans-acting glucocorticoid-hormone receptor complex (38). These are peculiar structural features of the replicating strategy of the MMTV that may offer significant hints to biotechnologists in their effort to manipulate the expression of transgenes in mammary epithelial cells.

The expression of heterologous proteins in milk of transgenic animals has been commonly targeted by cloning cDNAs that encode for the protein of interest in-frame with 5' DNA fragments that contained defined transcription units. However, this strategy has not always been successful in attaining high expression levels due to absence in the expression vector of essential cis-acting regulatory sequences. The inclusion of additional 5' DNA domains and 3' untranslated regions and use of genomic clones or minigenes have conferred enhanced and proper hormonal regulation of expression of chimeric constructs (Table 1). We can speculate that a foreign DNA sequence may be correctly transcribed, although integrated in the extraneous genomic material of the host cell, provided that 1) the transgene is within the context of its regulon that contains a dominant locus control region (LCR) sensitive to differentiative signals (36), and 2) the expression construct carries sufficient sequence information to assure appropriate developmental and hormonal regulation of expression. The use of MAR- and LCR-based expression vectors may prevent loss of expression if a transgene integrates in a silent region and lack of hormonal regulation due to influence of negative or positive neighboring cis-acting control elements.

Trans-acting Regulation of Expression

An additional level of regulation is represented by interactions of regulatory sequences with trans-acting mammary cell-specific nuclear factors. This aspect should be taken into consideration when choosing a particular promoter element to direct the expression of heterologous proteins. It is instructive to consider that a 0.4-kb promoter element of the sheep β-lactoglobulin gene contains binding sites for both the nuclear factor I (NFI) and milk protein binding factor (MPBF). As expected, both factors affected the transcriptional activity of the β-lactoglobulin promoter in transgenic mice (39). Similarly, the hormonal stimulation by lactogenic hormones of the bovine and rodent β-casein genes required removal of transcriptional repression and binding to a mammary gland-specific transcription factor (MGF) (40). The presence of binding sites in the 5' region of the MMTV-LTR for the mammary cell-activating factor (MAF) and transcription factors of the CTF/NFI family indicates that activity of the viral LTR element is also regulated by mammary cell-specific trans-acting regulators. This is not surprising, as the MMTV relies on the cellular machinery of mammary epithelial cells to assemble retroviral particles and trigger mammary tumor formation. Of interest is that homologous binding sites for the MAF and CTF/NFI nuclear factors are present in the 5' flanking region of the WAP gene normally expressed only in rodents and rabbits (41). These observations are in keeping with evolutionarily conserved molecular mechanisms that control expression of milk protein genes in different species. This knowledge can now be exploited to
Table 1. Expression of heterologous proteins in the mammary gland under the control of mammary promoter elements

| Element                      | Transgene | Type   | Clone   | Transgenic species | Accessory regions | Expression level/phenotype | Reference |
|------------------------------|-----------|--------|---------|--------------------|-------------------|---------------------------|-----------|
| 7-kb WAP gene                | WAP       | Genomic| Mouse   | 3-kb MAR           | Position independent | (38)                      |          |
| 5’ 2.6-kb WAP                | hTPA      | cDNA   | Mouse   | —                  | Position dependent  | (29)                      |          |
| 5’ 2-kb β-casein gene        | ILK-2     | Genomic| Rabbit  | Poly(A)            | Low                | (57)                      |          |
| 7.2-kb WAP gene              | WAP       | Genomic| Mouse   | —                  | Site dependent     | (58)                      |          |
| 5’ 2.4-kb WAP                | hGH       | Genomic| Mouse   | Poly(A)            | Repressed at birth | (59)                      |          |
| 7.2-kb WAP gene              | hPC       | cDNA   | Pig     | Poly(A)            | High               | (29)                      |          |
| 5’ 0.9-kb WAP                | WAP       | Genomic| Mouse   | SV40-poly(A)       | Deregulated        | (1)                       |          |
| 4-kb β-lactoglobulin gene    | hox-AT    | Genomic| Mouse   | Poly(A)            | High               | (27)                      |          |
| 5’ 17.6-kb WAP               | hox-AT    | Genomic| Mouse   | —                  | High               | (60)                      |          |
| 8.9-kb β-lactoglobulin gene  | hFIX      | cDNA   | Sheep   | —                  | Low                | (61)                      |          |
| 5’ 1.5-kb MMTV               | hTFβ-fx   | cDNA   | Mouse   | β-globin LTR       | Mammary tumors     | (32)                      |          |
| 5’ 1.3-kb MMTV               | oIGF-I    | cDNA   | Bovine  | SV-splice [poly(A) LTR] | High             | (5)                       |          |
| 3.3 α₁-casein gene          | hERY      | Genomic| Mouse   | —                  | High               | (2)                       |          |
| α₁-casein gene               | hLF       | cDNA   | Mouse   | —                  | High               | (62)                      |          |
| 5’ 2.5-kb WAP                | c-myc     | Genomic| Mouse   | —                  | Mammary tumors     | (4)                       |          |
| 5’ 2.5-kb WAP                | Ha-ras    | Genomic| Mouse   | —                  | Mammary tumors     | (3)                       |          |

Abbreviations: WAP, whey acidic protein; MAR, matrix attachment region; hTPA, human tissue plasminogen activator; ILK-2, interleukin-2; hGH, human growth hormone; hPC, human protein C; hox-AT, human α₁-antitrypsin; hFIX, human factor IX; MMTV-LTR, mouse mammary tumor virus-long terminal repeat; oIGF-I, ovine insulin-like growth factor-I; hERY, human erythropoietin; hLF, human lactoferrin.

Bioengineering the Mammary Gland

Bioactivity of many proteins usually requires post-translational modifications, such as glycosylation and carboxylation. These complex modifications may not be carried out in bacterial systems in such a way to yield products of desirable biological equivalence or potency. As a result, chemical manipulation of proteins produced in prokaryotic cells may be needed before recombinant proteins can be used for therapeutic or industrial purposes. Various animal cell culture systems have been developed since mammalian cells are equipped with the cellular machinery to produce fully processed biologically active peptides. An exciting alternative approach is provided by the knowledge of mammary-specific elements and the ability to generate transgenic animals; scientists now have the opportunity to target expression of heterologous proteins to the mammary gland. Although initial studies focused on rodent models, the generation of large transgenic farm animals such as sheep, pigs, and dairy cows offers the opportunity for large-scale production of heterologous proteins in milk.

Emphysema and α₁-Antitrypsin.

Emphysema occurs as a consequence of destruction of lung parenchyma with potential reduction of surface area of the alveolar walls. This pathological state is the result of excessive proteolytic degradation of elastin fibers by the protease elastase released by neutrophils. The condition is linked to α₁-antitrypsin deficiency. Under normal conditions the liver produces sufficient quantities of α₁-antitrypsin, which is present in serum at levels of approximately 200 mg/dl. α₁-Antitrypsin exerts its protective action by complexing with the enzyme elastase, which prevents unbalanced proteolytic breakdown of lung tissue.

Deficiency of α₁-antitrypsin is a hereditary disorder resulting from mutations in the α₁-antitrypsin gene located on chromosome 14. In homozygous individuals with the genotype P2P2 (P = protease inhibitor, Z = type) arising from a single base mutation, serum levels of α₁-antitrypsin drop to about 25 mg/dl. The PiZ mutation affects about 1 in 1000 persons in the United States. Such low levels of α₁-antitrypsin in serum are not sufficient to protect the respiratory tract from enzymatic proteolysis. This leads to chronic obstructive emphysema and decrease in longevity (42). Clinical strategies to counteract lung destruction rely on intravenous treatment with α₁-antitrypsin or respiratory administration of this protein in an aerosol form (43).

The bacterium E. coli has been successfully engineered to produce α₁-antitrypsin. However, the recombinant molecule is not equivalently glycosylated compared with the natural counterpart, and as a result the half-life in serum of E. coli-derived α₁-antitrypsin is greatly reduced. As normal individuals produce approximately 2 g/day of α₁-antitrypsin, large amounts of plasma are required to purify sufficient quantities of this protein for therapeutic use. Therefore, production of α₁-antitrypsin in milk of transgenic animals may provide an alternative route for large-scale production of this protein. High-level expression of active human α₁-antitrypsin in milk of transgenic sheep was first reported in 1991 (44). The genomic sequence of the α₁-antitrypsin gene was under the control of the β-lactoglobulin promoter. Expression levels ranged from 1 to 60 g/l of milk (Fig. 3A). Moreover, the α₁-antitrypsin purified from milk appeared fully glycosylated and displayed normal biological activity.

Anticoagulants: Protein C. Human protein C (hPC) is a vitamin-K-dependent anticoagulant produced in the liver. Human protein C is an important component of one of the many control systems involved in regulation of hemostasis and coagulation. Upon damage of epithelia, hPC proteolytically cleaves in concert with S-thrombomodulin components of the coagulation cascade (45) that act as anticoagulation agents. Human protein C also blocks the activity of tissue-plasminogen activator (tPA) inhibitor, which allows tPA to generate plasmin that in turn lyases clots. Deficiency of hPC due to hereditary disorders, liver diseases, or intake of vitamin K antagonists may lead to hypercoagulating states. In heterozygous subjects, levels of circulating hPC are lower than normal (4 mg/ml), whereas protein C deficiency in homozygous individuals is associated with thrombosis and death in infants.

Secretion of hPC in milk of transgenic mice and swine has been reported by Velander et al. (29) (Fig. 3B). In this study, an hPC cDNA was under the control of a 2.6-kb 5’ flanking region, and 3.0 kb of coding and 1.6 kb of 3’ flanking sequence of the WAP gene (Table 1). Transgenic pigs produced up to 1 g/l of milk of recombinant hPC, which displayed equivalent anticoagulant activity to protein C purified from human plasma. Given the low concentration of hPC in normal plasma, production of recombinant protein C in milk of transgenic animals may prove a
useful alternative for large-scale supply of this anticoagulant factor.

Lactoferrin. Lactoferrin, a member of the transferrin family, is one of the predominant whey proteins in milk. The iron-binding capacity of this protein accounts for most of the iron present in milk. Lactoferrin is also a ubiquitous protein in mammals: it can be found in high quantities in granules of human polymorphonuclear leukocytes and in mucosal and respiratory tract secretions. Structurally, lactoferrin is glycosylated with two iron-binding sites at homologous domains (46). Three primary functions have been speculated for lactoferrin: first, lactoferrin as a carrier of iron complements the nutritional value of milk. The iron-saturated form, however, accounts for only 5% of the total lactoferrin present in milk. Second, the iron-free lactoferrin, which is the primary form (95%), has distinct bacteriostatic effects on growth of gram-negative bacteria (47). In association with lysozyme, lactoferrin is also bactericidal for strains of V. cholerae, S. typhimurium, and E. coli. In this capacity, lactoferrin acts by depriving microorganisms of iron as well as modifying membrane permeability of bacterial cells. The latter modification causes damage of the outer membrane of bacteria species populating the intestine and therefore increases their sensitivity to subsequent exposure to bactericidal substances. Third, lactoferrin displays anti-inflammatory properties through a number of mechanisms (47). Generation of dairy calves transgenic for the human lactoferrin fusion gene has been previously reported (48). In these animals, expression of a human lactoferrin cDNA was under the control of 15 kb of the 5' flanking region and 6 kb of the 3' untranslated region of the bovine αs1-casein gene (Fig. 3C). In addition, regulatory sequences of the bovine αs1-casein gene allowed expression of human lactoferrin in milk of transgenic mice (49), up to 36 mg/ml. The recombinant lactoferrin appeared to have an identical molecular mass and immunoreactivity when compared with lactoferrin derived from human milk.

Future Developments, Animal Welfare, and Potential Pitfalls

Genetic engineering of the mammary gland is the result of progress in several scientific disciplines, including mammary gland biology, embryo transfer, and molecular biology (50). Bioengineering of the mammary gland to produce pharmaceutical proteins may be regarded as an effective strategy to manipulate the physiology of the mammary gland of farm animals to enhance human welfare. Conceptually, bioengineering of the mammary gland could complement traditional strategies of

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