Transgenic technology as it applies to animal agriculture

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1. Introduction

In the past decade, biotechnology fully emerged and was applied to biomedical, diagnostic, and food-related products. In terms of human medicine, recombinant DNAs have been introduced into a variety of expression systems that have yielded important therapeutic proteins. Recombinant proteins such as human growth hormone (hGH), tissue plasminogen activator, hepatitis B vaccine, and interferon have been synthesized, purified, have completed clinical trials and been approved by the FDA. These recombinant products are being sold for the treatment of human disorders. A plethora of other recombinant proteins are in various stages of clinical trials. In addition, genetic engineering strategies have been used in the development of products used for the diagnosis of human and animal diseases.

Examples of diagnostic biotechnology products include test kits that detect *Mycobacterium tuberculosis*, *Salmonella spp.*, and a variety of viral pathogens including human papilloma virus and human immunodeficiency virus. The development of the polymerase chain reaction (PCR) technology as applied to genetically engineered diagnostic test protocols has increased the speed and detection limits of the tests. Using this technology, infectious agents can be detected directly in human specimens. Together, genetically engineered therapeutic and diagnostic products have generated a multi-billion dollar market.

A logical extension of biotechnology is its application in agriculture, particularly in areas that result in the more efficient production of food, in safer

food products and in the use of animals as production systems for recombinant proteins. Biotechnology, as it relates to animal agriculture, includes aspects of animal growth, management, processing and selection, and development of genetic resources to produce desired products. Biotechnologies used to address the above issues include those to produce foods, those evident in the modification of foods, those which ensure the safety of foods, and those that will enhance food production. In this regard, bovine somatotropin (bST) has recently been approved in the United States as an agent to enhance milk production. Also, the ability to alter the genome of animals has led to the use of animals as "production" centers for desired recombinant proteins.

The focus of this discussion will be the use of recombinant DNA technology as an avenue for efficient production of transgenic animals with desirable body composition traits (reduced fat content for example) and in the generation of transgenic animals to be used as production systems for recombinant proteins.

2. Production of transgenic animals

The ability to incorporate exogenous DNA into the germ line of animals and to subsequently develop lines of animals which retain this DNA loosely defines the term "transgenic animal". Recombinant DNA has been inserted into the germ line of many animals including mice, sheep, rabbits, pigs, chickens, and fish (Hammer et al., 1985). Although a variety of technologies have been employed to generate these animals, by far the most commonly used technology has been direct microinjection of the foreign DNA into the pronucleus (usually male) of fertilized mammalian eggs. This work was developed in the late 1970s and early 1980s (Brinster et al., 1981; Wagner et al., 1981; Gordon, Ruddle, 1981) and is used routinely in many laboratories throughout the world. To date, thousands of transgenic mice have been generated that harbor a variety of genes. The overall efficiency of transgenic mice production ranges from 1 to 40% (June Yun, personal communication). The reasons for these differences are not apparent but they may reflect the nature or quality of the DNA, expertise of the injector, quality of the mouse eggs, or a number of undefined variables. In general, the efficiency of transgenic mouse production is good. Other methods employed for the production of transgenic animals include the use of retroviral vectors, embryonic stem cell technology, particle "gun" mediated gene transfer, and electroporation. A variety of transgenic farm animals is listed in Tab. 1.

TABLE 1
TRANSGENIC FARM ANIMALS 1991-1995

Species	Gene	Author & Year
Cattle	1. Cc -ski	Bowen (1994)
Sheep	1. Trf-bGH & mAlb-hGRF	Rexroad (1990)
	2. mIgA	Lo (1991)
	3. Prg	Nancarrow (1991)
	4. Kg	Rogers (1993)
Goat	—	—
Pig	1. mIgA	Lo (1991)
	2. mAb	Weidle (1991)
	3. mTF-bGH & Mt-bGH	Guthrie (1991)
	4. mCC10	Margraf (1993)
	5. RhHA	O'Donell (1993)
	6. FhCl	Fodor (1994)
	7. hDAF	Cozzi (1994)
	8. Mt-RF-hGH	Kazakov (1994)
Rabbit	1. mAb	Weidle (1991)
	2. hapoA-1	Perevozchikov (1993)
	3. CRPV & EJ-ras	Peng (1993)
	4. hHL	Fan (1994)
	5. MT-hGH MT-hGH-RF	Kazakov (1994)

C — chicken; c — cellular; TrF/mTF — mouse transferin enhancer/promoter; bGH — bovine growth hormone; mAlb — mouse albumin enhancer/promoter; hGRF — human growth releasing factor; mIgA — mouse immunoglobulin antibody; PRG — performance regulating genes; Kg — keratin gene; MT — mouse metallothionein promoter; mCC10 — mouse Clara cells 10 kD; RhHA — recombinant human hemoglobin A; FhCl — functional human complement inhibitor; hDAF — human decay accelerating factor; RF — releasing factor; hGH — human growth hormone; h apoA-1 — human apolipoprotein A; CRPC — cottontail rabbit papillomavirus; EJ-ras — ras-oncogene; hHL — human hepatic-lipase.

Retroviral-mediated gene transfer involves the use of mammalian or avian retroviruses (RNA, tumor viruses) as delivery systems of foreign DNA into the genome of the animal cell. Usually, the retroviral vector has been altered such that it is replication incompetent or defective. Such vectors have been used to successfully deliver genes to mice and chickens. However, the major drawback of these systems is that the defective virus possesses a formal possibility (albeit low) to recombine with endogenous viral sequences and form replication competent viruses.

Embryonic stem cell technology involves the use of *in vitro* cultured cells derived from an embryo, which when reintroduced into a developing embryo,

can give rise to a population of cells in various tissues of the animal. Animals generated by this approach are mosaic, that is, only a portion of the cells of various tissues of the animal arise from the cultured ESC. Recombinant DNA can be introduced into the cultured ESC and cell lines can be developed *in vitro* that contain and express this foreign gene. When these cells are introduced into the embryo, the resulting mosaic animal possess cells derived from the ESC cells that harbor the recombinant DNA. Because a portion of the germ cells of the animal usually arise from the ESC, the animal can pass the acquired trait to the offspring. These offspring will then differ from their parents in that the transgene will be present in all cells. Thus, lines of animals derived from the original ESC can be developed. To date, ESC technology has been used successfully in mice, however, vigorous research programs are in place to develop ESC technology for pigs, cows, and sheep.

3. Transgene expression and animal growth

Recent efforts to augment the production characteristics of domestic livestock have been by the use of growth hormone (GH) or somatotropin (ST). This has been made possible, in part, by advances in biotechnology. For example, recombinant DNA technology has provided a means for production of large quantities of pure GH for administration to animals at a fraction of the cost of pituitary-derived GH. Furthermore, the physiological effect of foreign GH gene expression on growth and development of transgenic farm animals are currently under investigation.

A variety of genes which impact the endocrine status of the organism have been introduced into the germ line of animals and lines of these animals have been generated. Examples of these genes include growth hormone (GH), insulin, insulin-like growth factor-I (IGF-I), a variety of receptor genes, and others (Chao et al., 1983; Magram et al., 1985; Selden et al., 1986; Palmiter et al., 1987; Tremblay et al., 1988; Mathews et al., 1988; Berg et al., 1988). Usually, the gene of choice is injected into the fertilized egg in the form of a fusion gene; that is, a transcriptional regulatory element (TRE) of a different gene is attached or ligated to the protein-coding region of the gene to be studied. Selection of the TRE depends on the type of study to be performed. For example, one may wish to present that the gene should be expressed only after birth. In this case the phosphoenol pyruvate carboxykinase (PEPCK) TRE has been successfully employed (McGrane et al., 1988). Additionally, one may want to express the gene in a tissue-specific manner, for example in the beta-acinar cells of the pancreas or in the mammary gland. In this case, the murine elastase and whey acidic protein TRE's have been used, respectively (Berg et al., 1988; McKnight et al., 1995). Thus, transgene expression as a function of animal development or tissue specificity can vary depending on the TRE's utilized for a given gene.

Transgenic mice that contained the human or rat GH (rGH) genes were first produced in 1980 (Palmiter et al., 1982). Expression of these genes was under control of the mouse metallothionein-I (mMT) transcriptional regulatory sequence. This transcriptional regulatory sequence directs gene expression in nearly all tissues of the mouse, with high levels of GH expression found in the liver, intestine, and kidney, and is inducible by heavy metals such as zinc and cadium. Ectopic production of GH in these mice escapes the normal physiologic control mechanisms regulating GH production. When the GH gene was expressed and the protein secreted into the serum of the animals, a large mouse phenotype was produced. Mice containing and expressing either hGH, bGH, rGH, or ovine GH (oGH) genes grow to approximately 2 times the size of control littermates. Maximal growth rates of these animals were found to occur when they were between 5 and 11 weeks old. Surprisingly, expression of the acquired GH gene was not related to the number of copies of the transgene inserted into the animal's genome, or to serum GH concentrations (Palmiter et al., 1982). These exciting results have been reproduced in many laboratories including ours (Kopchick et al., 1990; Chen et al., 1990, 1991 a, b, c).

Transgenic mice that chronically express high concentrations of GH have been examined for a variety of morphologic and physiologic abnormalities (Quaife et al., 1989). The mass of liver, kidney, spleen, and pancreas in these animals was found to be 2 to 3 times that in control animals. Histologic examination of kidneys from transgenic mice revealed that the tissue contained enlarged glomeruli with sclerosis (Quaife et al., 1989). These lesions are similar to those found in mice with diabetic glomerular sclerosis. Lesions within hepatocytes were found to increase with age, resulting in dysplasia and sclerosis. Grossly enlarged hepatocytes were found around the central vein of the liver and became further enlarged with age. Cells of the spleen were morphologically normal but congested with a mild RBC hematopoiesis (Quaife et al., 1989).

Serum chemical profiles of these transgenic mice were also analyzed (Quaife et al., 1989). The sera from mice containing bGH were opalescent and contained albumin, globulin, and total protein concentrations similar to control animals. Serum alkaline phosphatase were two to threefold lower in transgenic animals relative to controls. Mice with high concentrations of GH (>1 μg/ml) were found to be hypercholesterolemic but triglyceride concentrations were normal. Serum insulin concentrations in bGH transgenic mice were three to eightfold higher than those in control mice, and serum glucose concentrations were normal. As expected, serum insulin like growth factor one (IGF-I) concentrations in these mice were increased approximately twofold (500 to 600 ng/ml).

Several groups have produced transgenic pigs (Vize et al., 1988; Wise et al., 1988; Wieghart et al., 1990; Pursel et al., 1989). One major concern in these combined experiments is the overall low efficiency of production. In mice, 10 to 30% of the injected eggs result in pups, of which approximately

25% are transgenic. This equates to an overall efficiency range from 2.5 to 6% (Brinster et al., 1985). In comparison, the overall efficiency of transgenic pig production ranges from 0.5 to 1% (Vize et al., 1988; Wise et al., 1988; Wieghart et al., 1990; Pursel et al., 1989). DNA molecules in which the mMT promoter was fused to either the hGH or bGH gene were obvious choices for insertion into pigs, since these recombinant DNA molecules "worked" well in mice (Pursel et al., 1989). Eight transgenic pigs, that contained between 1 and 500 copies of the mMT-hGH gene, were generated. Serum hGH concentrations varied between foreign gene copy number and serum hGH concentrations. In addition, 8 transgenic animals that contained the mMT-bGH fusion gene were produced. Serum bGH concentrations in these animals varied from 5 to 1,000 ng/ml with no correlation found between foreign gene copy number and serum bGH concentrations. The IGF-I concentrations were found to be high in all but 1 bGH transgenic pigs. Offspring of mMT-bGH transgenic pigs have been generated. Serum bGH concentrations in these progeny were similar to those in the parent animals. Two pig lines have been established in which bGH concentrations were approximately 1,300 and 85 ng/ml (Pursel et al., 1989).

Growth hormone transgenic pigs did not have the enhanced growth phenotype that has been found in mice (Wieghart et al., 1990; Pursel et al., 1989). However, bGH transgenic pigs were found to be approximately 15% more efficient at converting food into body mass (Pursel et al., 1989; Etherton et al., 1987). This observation is similar to that in pigs that have been injected with exogenous pGH (Pursel et al., 1989). The most striking phenotype of bGH transgenic pigs was the 30% decrease in mean backfat thickness (Pursel et al., 1989; Etherton et al., 1987). In addition, growth hormone transgenic pigs injected with pGH (protein) have been shown to have suppressed appetites. Pursel et al., (1989) have suggested that appetite suppression may limit essential nutrients required for animal growth. In this regard, when bGH transgenic pigs were placed on a diet containing high concentrations of crude protein and supplemented with lysine, growth increased approximately 10 to 15% relative to that in control animals (Pursel et al., 1989).

Many negative side effects have been found in transgenic pigs that express hGH (Pursel et al., 1989). These effects include pericarditis, peptic ulcers, and impaired fertility. Similar to GH transgenic mice, the internal organs of GH transgenic pigs have been found to be enlarged. The mMT-bGH pigs had enlarged hearts, kidneys, livers, thyroid glands, and adrenal glands, as well as an incerase in long bone circumference and weight (Pursel et al., 1989). Glucose concentrations in these animals were slightly high and plasma insulin concentrations were approximately 20-fold higher than controls (Pursel et al., 1989). These conditions were also observed in pigs injected with pGH (Etherton et al., 1987; Evock et al., 1988).

In similar experiments, a human metallothionein II a transcriptional regulatory sequence was fused to the pGH gene (a cDNA with pGH genomic 3' flanking sequences) (Vize et al., 1988). Although only 1 pig was produced

which exhibited an elevated GH level, this pig was found to be quite different from MT-GH animals. The pGH concentration in this pig was approximately 2 times those in control animals. This pig grew to 90 kg in 17 weeks versus 22 to 25 weeks in control animals. The investigators proposed that enhanced growth rate was attributable to the use of a fusion gene that encodes the authentic pGH gene rather than heterologous GH genes used by others. Also, that animal did not exhibit any negative side effects such as those pigs expressing the hGH or bGH genes.

A third approach to the production of transgenic pigs has been one in which a different transcriptional regulating sequence, that is, phosphoenolpyruvate carboxykinase (PEPCK) fused to the bGH gene, was used (Wieghart et al., 1990). The PEPCK gene normally encodes a liver and kidney enzyme that catalyzes a key gluconeogenesis regulatory step. Expression of this fusion gene in mice can be regulated by dietary manipulation of carbohydrates (McGrane et al., 1988). Transgenic pigs that expressed the PEPCK-bGH fusion gene were found to possess high concentrations of bGH mRNA primarily in the liver and to a lesser extent in the kidney. All transgenic pigs had serum bGH concentrations >100 ng/ml. One of the transgenic male pigs was able to transmit the fusion gene to the offspring. These progeny were found to have decreased backfat (41% that of controls) and increased feed efficiency, similar to mMT-bGH transgenic pigs. Unfortunately, adverse effects were observed in these animals, including joint lesions, stress, and respiratory distress, although these disorders developed later in life, compared with mMT-bGH transgenic pigs. The investigators suggest that the delayed development of these adverse effects could be attributable to the utilization of the PEPCK transcriptional regulatory sequences. This promoter has been found to be activated postnatally rather than prenatally as is the case for the mMT regulatory sequence.

Various fusion genes have been introduced into sheep and the result of these experiments have been reviewed recently (Rexroad et al., 1990). The genes include hGH, bGH, oGH and hGH-releasing factor, attached to either the mMT, oMT, mouse transferrin or mouse albumin transcriptional regulatory sequences (Murray et al., 1989; Rexroad et al., 1990). The efficiencies of transgenic sheep production ranged from 0.1 to 4.45% (Rexroad et al., 1990). Two animals have been produced which express the mMT-bGH fusion gene. The concentrations of bGH in the sera were 36 and 718 ng/ml (Rexroad et al., 1989). Also, two animals have been produced which contain and express the mouse transferrin promoter-bGH gene with serum bGH concentrations of 42 and 289 ng/ml (Rexroad et al., 1990). Transgenic sheep which expressed either oGH or bGH grew at rates equal to or slightly lower than control animals (Rexroad et al., 1990). However, transgenic lambs had 20 to 50% the levels of body fat of control animals (Ward et al., 1989) and no backfat or depot fat at necropsy (Rexroad et al., 1990). GH expression did not affect the feed/gain ratio in transgenic lambs (Rexroad et al., 1990).

Increased GH in sera of transgenic lambs resulted in increased IGF-I levels. Also, before 100 days of age, plasma glucose and insulin levels were

elevated (Rexroad et al., 1990). After 100 days of age, plasma glucose and insulin levels were below those of control animals. Rexroad and co-workers have suggested that excess GH secretion in lambs may produce diabetes which results in death of the animals within one year of birth. Unfortunately, GH-expressing progeny from transgenic sheep have not been produced.

In summary, research on the production of transgenic food animals is still relatively new. Transgenic pigs and sheep that express GH gene have reduced backfat, unlike transgenic GH mice, which grow to approximately twice the mass of control animals. GH transgenic livestock generally have not shown an enhanced growth phenotype. However, when the diet of GH transgenic pigs is supplemented with crude protein and additional amino acids, a substantial improvement in the average daily gain is observed (Pursel et al., 1989). Therefore, the nutritional requirement of GH transgenic animals is an issue that must be addressed in future experiments. Also, the regulatory issues associated with food-producing transgenic animals are currently being discussed. The issue of the gene construct, gene product, and integration of exogenous DNA into the host cell genome is very important and must be considered.

4. Transgenic animals as "bioreactors"

As mentioned earlier, recombinant DNA technology has provided a means to produce numerous important therapeutic compounds which are improving our quality of life. However, other therapeutic recombinant proteins produced in standard laboratory bacteria and yeast have proven to be biologically inactive. The reason for this inactivity is often the lack of post-translational processing of the protein by the microorganism or incorrect folding of the nascent protein. Downstream processing of the bacterial product to yield an active compound makes the process of production complicated and costly. Many of these disadvantages may be solved by employment of large scale *in vitro* animal cell culture systems. However, due to problems with long term culture and the necessity of rich media supplementation, the use of this technology also makes it difficult and expensive.

The ability to introduce foreign DNA's into the genome of animals has provided another avenue for the production of these therapeutic proteins. Not only does this system promise to provide proteins which display correct post-translational modifications and folding patterns but may also do so in an extremely cost efficient manner.

The mammary gland represents an excellent potential site for the production of novel recombinant proteins. At the peak of lactation, the mammary glands secrete substantial quantities of protein into the milk, e.g. 0.1 and 1 kg per day in sheep and cattle, respectively. Most of the protein is synthesized by the mammary epithelium, but some blood serum proteins, such as albumin, are present in milk in smaller quantities. The major proteins of cow, sheep, mouse, and human milk are listed in Tab. 2 (modified from Lathe et al., 1986).

TABLE 2
PROTEIN COMPOSITION IN ANIMAL MILK

Protein	Concentration in Milk (g/l)			
	Cow	Sheep	Mouse	Human
Caseins				
αs_1-casein	10	12	*	0.4
αs_2-casein	3.4	3.8	*	*
β-casein	10	16	*	2
κ-casein	3.9	4.6	*	0.2
Major Ehey Proteins				
α-lactalbumin	1	0.8	Trace	1.6
β-lactoglobulin	3	2.8	None	None
Ehey acidic protein	None	None	2	None
Other Whey Proteins				
Serum albumin	0.4	*	*	0.4
Lysozyme	Trace	*	*	0.4
Lactoferrin	0.1	*	*	1
Immunoglobulin	0.7	*	*	1.4

* No data available

The transcriptional regulatory sequences of the genes that encode these major milk proteins were, therefore, considered for fusion to heterologous genes for high-level expression of the foreign proteins in milk. For example, the whey acidic protein (WAP) gene transcriptional regulatory element (TRE), which is found only in rodents and rabbits, was used for expression of foreign proteins in transgenic mice (see below). Also, goat β-casein gene was used for transgenic goats and sheep; β-lactoglobulin, which is found at high levels in whey, was used for transgenic sheep production. For transgenic dairy cattle, bovine αs_1-casein gene was used for the production of heterologous proteins in milk.

The secretion of heterologous proteins into the milk of transgenic animals was first reported in 1987. Production of active human tissue plasminogen activator (Gordon et al., 1987) and sheep β-lactoglobulin (Simons et al., 1987) in mouse milk demonstrated the potential of transgenic technology for the economical production of proteins. Since these initial studies, considerable progress has been made in identifying and characterizing gene regulatory sequences that are useful for targeting tissue-specific expression to the mammary gland. Consequently, many kinds of proteins, from bacteria to humans, have been expressed in the mammary glands of transgenic mice, rabbits, sheep, pigs, and goats. These results are discussed in the following sections with emphasis on the challenges and opportunities of using transgenic mammary glands of animals as bioreactors.

5. Heterologous milk protein expression in mice and rats

The milk proteins which have been shown to be secreted into transgenic mouse milk include bovine, guinea pig, and goat α-lactalbumin (bαL, Vilotte et al., 1989; gpαL, Maschio et al., 1991; gαL, Soulier et al., 1992), goat β-casein (gβCN, Persuy et al., 1992; Roberts et al., 1992) and mouse and rat whey acidic proteins (mWAP, Burdon et al., 1991; or rWAP, Bayna and Rosen, 1990). Bovine α-lactalbumin was also highly expressed (- 2 mg/ml) in some lines of transgenic rats (Hochi et al., 1992). The highest level of expression of a heterologous milk protein in transgenic mouse milk reported to date was 40 mg/ml of goat βCN (Persuy et al., 1992) using a 18 kilobase pair (kb) genomic DNA fragment.

A number of human proteins have also been evaluated for expression in the milk of transgenic mice. Expression was initially demonstrated by Gordon et al., (1987) utilizing regulatory sequences from murine whey acidic protein (mWAP) to direct the expression of the human tissue plasminogen activator gene. While the level of protein produced from this fusion gene was very low (up to 0.4 μg/ml), the protein was found to be biologically active. Pittius et al., (1988) reported that another transgenic mouse line, that carried the same fusion gene, secreted 50 μg/ml of the active protein into the milk. Another non-milk protein, human CD4, was successfully secreted into the milk of transgenic mice utilizing the mWAP regulatory sequences (Yu et al., 1989). Expression of this fusion gene also resulted in low (0.2 μg/ml) protein concentrations in milk. In 1990, two groups succeeded in secreting high levels of human proteins in transgenic mouse milk. Meade et al., (1990) produced human urokinase at 1 to 2 mg/ml using the bovine αs_1-casein TRE and Archibald et al., (1990) produced human α-antitrypsin at 7 mg/ml using the sheep βLG TRE.

Various transcriptional regulatory elements from milk protein genes have been used for the production of other human gene products in the milk of transgenic mice; goat βCN for cystic fibrosis transmembrane conductance regulator (DiTullio et al., 1992); mWAP for hGH (Günzburg et al., 1991; Reddy et al., 1991); human β-interferon (Schellander, Peli, 1992); human protein C (Velander et al., 1992) and human extracellular superoxide dismutase (Hansson et al., 1994); rabbit WAP for human α-antitrypsin (Bischoff et al., 1992) and sheep βLG for human γ-interferon (Dobrovolsky et al., 1993); and human serum albumin (Hurwitz et al., 1994). The expression levels of these foreign genes varied widely and the highest level of production reported in milk was that of human serum albumin at 10 mg/ml. Human milk proteins, such as lactoferrin (Platenburg et al., 1994; Krimpenfort et al., 1993) have also been produced in the milk of transgenic mice. Also, transgenic mice producing human lysozyme transgene have been reported (Maga et al., 1994).

6. Heterologous proteins expressed in milk of large animals

Since mice and rats can only serve as model animals for transgenic production, larger animals have been tested for production of heterologous proteins in their milk. Transgenic rabbits expressing rabbit β-casein TRE-human interleukin-2 (Bühler et al., 1990) and bovine αs_1-casein TRE-human IFG-I (Brem et al., 1994) fusion genes have been reported. The production level of hIGF-I was reported to be 1 mg/ml in rabbit milk.

Since the initial report of transgenic modification of swine and sheep (Hammer et al., 1985), several transgenic farm animals have been generated. Six transgenic swine lines containing a 7.2 kb fragment of mouse WAP genomic DNA produced milk containing mWAP at concentrations greater than 1 mg/ml. Analysis of mRNA from various tissues indicated that mWAP expression was specific to the mammary gland in all but one line. This exception also exhibited low level of mWAP expression in the salivary gland (Wall et al., 1991). Also, Velander et al., (1992) reported generation of transgenic swine which produce human protein C in their milk. The human protein C cDNA was inserted into the first exon of mouse WAP gene. This construct was expressed at high levels and produced up to 1 mg/ml, 1000-fold higher than mice containing the same transgene construct. The human protein C produced in these animals possessed an anticoagulant activity equivalent to that of human plasma-derived protein C. The above reports indicate that transgenic swine are capable of producing high levels of foreign proteins in their milk in a tissue-specific manner.

Success in gene transfer into sheep (Simons et al., 1988) led to the production of transgenic sheep containing the human antihemophilic factor IX (Clark et al., 1989) and human α-antitrypsin (McClenaghan et al., 1991; Wright et al., 1991) genes fused to sheep βLG regulatory TRE. Human factor IX requires glycosylation and γ-carboxylation for activity and, hence, cannot be produced in bacteria. In both cases, the cDNA's were expressed and proteins accumulated in the milk. Unfortunately, the levels of expression were ~5 orders of magnitude lower than the expression of the endogenous lactoglobulin gene whose regulatory sequences were used for expression. However, transgenic sheep containing the genomic sequence of human α-antitrypsin produced up to 35 mg/ml (50% of the total protein) of the recombinant protein in their milk. This remarkable expression had no profound effect on the animal and did not affect lactation. However, analysis of the transgenic founder animals and their progeny revealed that variable levels of human α-antitrypsin were present in the plasma of all animals, including males (Carver et al., 1992). Generally, the levels were only a fraction of those found in the milk, but the transgenic founder with highest level of α-antitrypsin in milk exhibited higher levels in plasma during lactation, possibly through blood vessel rupture in mammary gland (Carver et al., 1992). In this case, the level of α-antitrypsin was not harmful to the animal. However, even a small leakage of biologically more potent transgene products may adversely effect the animals.

Ebert et al., (1991) reported the creation of transgenic goats harboring, and expressing in their mammary glands, the human tissue-type plasminogen activator cDNA directed by the mouse WAP promoter sequence. These animals produced — 3 μg/ml of human plasminogen activator which represents only 10% of that produced by a recombinant mouse cell line. However, transgenic goats were generated using a transgene containing a fusion of the cDNA of tissue plasminogen activator to the goat β-casein gene (Ebert et al., 1994). These goats secreted the human tissue plasminogen activator at levels ranging from 1 to 3 mg/ml making the animals economically viable bioreactors. The efficiency of the transgenic production system was exemplified by the calculations that the transgenic goat with the highest level of expression (3 mg/ml) would produce as much human plasminogen activator per day as obtained from a daily harvest from a 1000 liter mouse cell bioreactor (Denman et al., 1991). However, the production 3 mg/ml of active tissue plasminogen activator in one of the animals resulted in the appearance of flocculent material in the milk consisting of the activator and degraded β-casein. Consequently, a rapid decline in milk production was observed in this animal. This indicates that negative side effects may result from extremely high levels of heterologous protein production in goat mammary tissue. Also, 1 to 3 mg/ml of the human tissue plasminogen activator was produced in the milk of 11 to 12 month-old transgenic progeny using a series of estrogen and progesterone injections to induce lactation (Ebert et al., 1994). These experiments indicate that transgenic mammary gland directed expression data can be obtained in a shorter period of time in dairy goats than would be required through normal gestation and lactation schedules.

The production of heterologous proteins in the mammary glands of cattle may represent the largest "living factory" for the production of therapeutic products. One transgenic cow, producing 10,000 liters of milk/year, could serve as an immense source of heterologous protein. To this end, a transgene containing human lactoferrin cDNA fused to a 15 kb fragment bovine αs_1-casein regulatory sequences and 3' flanking sequence (Krimpenfort et al., 1991) was successfully introduced into dairy cattle. Expression data has not yet been reported from the transgenic progeny (the first founder animal was male). It is noteworthy that bovine embryos were produced and fertilized *in vitro* using cattle ovaries from slaughter houses as starting materials and, hence, almost unlimited number of bovine embryos could be produced by this method.

In 1994, there was a report of a calf born after injection of a gene consisting of genomic sequences encoding human erythropoietin combined with bovine αS1-casein regulatory sequences (Hyttinen et al., 1994). There was no report about secretion of human erythropoietin in milk of the transgenic cow because one must wait two years for the first lactation. Future results of transgene expression are more promising since the authors used PCR screening for the presence of gene in the embryo. Also, the sex of the embryo was screened before transfer to the foster mother.

7. Challenges and opportunities of transgenic milk production

The control of transgene expression in mammary glands is far from clear since gene transfer through microinjection inevitably leads to a randon integration of the transgene into the embryonal genome and, as a rule, there is no relationship between the number of integrated gene copies and the rate of expression (Gordon, 1989). This lack of predictability in the level of expression is a significant hurdle in the efficient production of transgenic farm animals since, relative to mice, longer pregnancy times and smaller litter sizes are standard. Therefore, new lines of transgenic animals cannot be rapidly and efficiently generated. The lack of correlation between gene dosage and the rate of expression may be attributable to a chromosomal position effect where the genetic environment rather than the gene copy number determines the efficacy of expression. Various genetic sequences that could potentially improve the transgene expression were reviewed by Janne et al., (1994) but the results are not yet conclusive.

As described earlier, the cDNA's directed by genomic regulatory sequences of milk protein genes were poorly expressed in transgenic sheep and goats. However, transgene expression has been found to increase when introns are present in the injected gene (Brinster et al., 1988). Also, expression levels were found to improve when a genomic fragment was used as a transgene (α-antitrypsin) or the cDNA was fused within a genomic minigene of a milk protein (tissue plasminogen activator). In mice, α-antitrypsin expression also improved if a genomic minigene was used instead of the cDNA (Whitelaw et al., 1991). The presence of human serum albumin gene introns in the transgene also dramatically improved expression of human serum albumin in milk (Hurwitz et al., 1994). Clark et al., (1992) have shown that under the control of the sheep βLA promoter silent cDNA transgenes can be activated after co-injecting them with a genomic sheep βLG transgene in mice. However, expression from mouse WAP cDNA was marginally increased after co-injection with a mouse WAP genomic transgene (McKnight et al., 1995). This suggests that a permissive integration site capable of supporting transcription can be established by co-integration of genomic and cDNA transgenes but further events are required for full activation of the transgene. At this time, rather limited information is available on interaction among mammary-specific promoters, enhancers, transacting elements, and intragenic sequences. Also, control of the transgene integration event promises to become more refined when embryonic stem cell techniques are in use with embryos of large farm animals and targeted gene transfer can be achieved with the aid of homologous recombination. Probably the greatest hindrance to the immediate commercial application of the transgenic technology is the relatively low efficiency of production of transgenic offspring. The percentage of transgenic offspring from injected eggs is 0.5 to 1 for rabbits (Brem et al., 1994), 0.3 to 4 for pigs, 0.1 to 4.4 for sheep (Pursel, Rexread, 1993), 0.5 to 1 for goats (Ebert et al., 1991), and 0.1 to 0.5 for dairy cattle (Janne et al., 1994). The improvement of effi-

ciency of gene transfer in large transgenic animals is required for the process to be economically practical. There are ongoing efforts to improve the isolation, *in vitro* maturation and fertilization of slaughter house derived bovine oocytes, *in vitro* cultivation of microinjected embryos, embryo sexing by PCR and screening for the transgene integration prior to non-surgical embryo transfer (Janne et al., 1994). With the evolution of methods for introduction of DNA into somatic cells, it might also be possible to produce transgenic animals carrying genes in their germ cells. Recently, replication-defective retrovirus vectors were used for direct transfer of hGH genes into the mammary glands of goats during a period of hormone induced mammogenesis (Archer et al., 1994). This resulted in the secretion of hGH into the milk when lactation commenced. Although the expression levels were low, the time of production of a foreign protein in milk was reduced from years to weeks.

In developing transgenic animals to provide milk for human consumption, it is important to recognize that changes in the amount of a given milk component may have profound effects on the complex interaction between the other milk constituents and may negatively effect their production.

Despite all the challenges discussed above, the opportunities offered by transgenic animal milk are practically unlimited. Theoretically, expression of a bovine αs_1-casein (10 g/l in cow milk) promoter-driven fusion gene could produce 60 to 80 kg/yr per cow of the transgene-derived protein. Such volumes certainly make any compromises regarding low expression rates and modest purifications yields acceptable. Finally, transgenic animals can feed and reproduce in natural settings and have unlimited potential for expansion of the transgene as a dominant Mendelian characteristic.

TABLE 3
MAMMARY GLAND TISSUE SPECIFIC TRANSGENE EXPRESSION

Species	Gene	Protein	Author & Year
1	2	3	4
Cattle	1. bovine αS1-casein human lactoferrin		Krimpenfort (1991)
	2. bovine αS1-casein-human erythropoietin		Hyttinen (1994)
Sheep	1. ovine β-lactoglobulin-human α1-antitrypsin (cDNA)	human α1-antitrypsin	Simons (1988)
	2. ovine β-lactoglobulin-human factor IX	human factor IX	Clark (1989)
	3. ovine β-lactoglobulin-human α1-antitrypsin (genomic construct)	human α1-antitrypsin	Wright (1991)

1	2	3	4
Sheep	4. mg-sp cDNA of human coagulation factor VIII	human coagulation factor VIII	Halter (1993)
Goat	1. mWAP-human tissue-type plasminogen	human tissue-type plasminogen	Ebert (1991)
	2. mammary specific promoters-human growth hormone	human growth hormone	Archer (1994)
Pig	1. mWAP-mouse whey acidic protein	mouse whey acidic protein	Wall (1991)
	2. mWAP-human protein C	human protein C	Velander (1992)
Rabbit	1. αβS1 casein — synthetic human insulin like growth factor 1	human insulin like growth factor 1	Brem (1994)

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Transgenic technology as it applies to animal agriculture

Summary

A variety of recombinant DNA molecules have been introduced into the germ line of animals. The resulting transgenic animals have a diverse range of phenotypes, some of which were expected and some not anticipated. To date, the majority of transgenic animals are mice. However, recombinant DNA has been introduced into agriculturally important animals such as sheep, rabbits, pigs, chickens, cattle and fish. In this report, we will survey the consequences of transgene expression in agriculturally important mammals in terms of their effects on growth. Also, we will review the data concerning the use of transgenic mammals as "bioreactors" for the production of recombinant proteins.

Key words:
transgenic mammals, growth, bioreactors, recombinant proteins.

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