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Review

Transgenic animals and nutrition research

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Transgenic animals are useful tools for the study of biological functions of proteins and secondary gene products synthesized by the action of protein catalysts. Research in nutrition and allied fields is benefiting from their use as models to contrast normal and altered metabolism. Although food, nutritional products, and ingredients from transgenic animals have not yet reached consumers, the technologies for their production are maturing and yielding exciting results in experimental and farm animals. Regulatory governmental bodies are already issuing guidelines and legislation in anticipation of the advent of these products and ingredients. This review summarizes available technology for the production of transgenic animals, discusses their scientific and commercial potential, and examines ancillary issues relevant to the field of nutrition. (J. Nutr. Biochem. 10:682–695, 1999)

Keywords: transgenic animals; nutrition; transgene; targeted mutants

Introduction

Transgenic animals and targeted mutants

Transgenic animals (TA) express, or may express if properly induced, proteins encoded by cDNA or genes usually appended to heterologous transcription regulatory elements (TRE). These fusion genes are commonly referred to as transgenes. It is important to note that the expression of proteins, which are primary gene products, may or may not be the only acquired features of a given TA. If the transgene-encoded protein is an enzyme and if its substrates are present within the cell, then secondary gene products also will be synthesized.[1] TA may accumulate these products in tissues or biological fluids in which they are not normally present. Expression of peptide hormones such as growth hormone (GH) in TA radically changes carcass composition and induces systemic physiologic alterations. For example, mice that express GH are larger than their nontransgenic littermates, have elevated levels of insulin, and die prematurely due to liver and kidney damage.[2,3] In addition, transgenic expression of hydrolytic enzymes may result in the disappearance or modification of molecules normally present in animal tissues or biological fluids. Closely related to TA are animals into which genetic alterations have been introduced, which in turn result in the suppression of endogenous gene expression. These are termed "targeted mutants" (TM) and include the so-called "knockout" or gene-disrupted animals.[4] Both TA and TM are genetically modified animals and are produced and propagated for the purpose of gaining or losing functions with respect to the wild type or standard animal.

Examples

The relevance of TA to the field of nutrition may be better illustrated by a few examples, including pigs and mice that express human proteins in their milk[5,6] or in their urine[7]; pigs with unique fat/muscle ratios due to the expression of peptide hormones[8]; sheep with altered carcass composition due to transgenic expression of hormones[9]; mice that have altered milk oligosaccharides and glycoproteins[1]; the production of lactose-free milk or milk with reduced lactose content due to elimination of α-lactalbumin[10–12]; and tilapia overexpressing GH.[13] The meat or milk from the animals

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listed in these examples contains measurable molecular differences when compared with meat or milk derived from nontransgenic animals. As these products become accessible to consumers, both nutritionists and dietitians will need to assess the overall impact of their inclusion into the food chain or their utilization to achieve desired nutritional results.

Consequences of the use of transgenic technology

Products obtained from transgenic plants are already in the marketplace, and many vegetables and fruits are being targeted for modifications and improvements through the use of transgenic technologies. These are not necessarily focused on improving the nutritional quality of food products, but often on increasing efficiencies in the production of cultivars, improving resistance to insects and pathogens, and changing or enhancing organoleptic characteristics. Because of the fast pace at which TA are evolving, it is conceivable that a significant portion of food products on supermarket shelves will become targets for functional, compositional, or nutritional improvements. Products derived from TA and TM may be modified in predictable or unpredictable ways. For this reason, they represent an interesting subject of study for researchers in the field of nutrition. At present there are no available TA-derived products in the marketplace; therefore, a review on the subject must deal with the current status of relevant technology as well as with its potential, which will necessarily be framed by regulations, issues pertaining to the ownership of animals and technologies, and consumer perceptions.

Transgenic animals, an evolving concept

The production of TA is based on scientific principles established primarily through research in the areas of molecular biology and embryology. The fast pace of discoveries and technical improvements in these areas is reflected by the constant evolution of prevalent definitions. Page et al.[14] define TA as "a result of the incorporation of a foreign gene such that it becomes an integral part of the natural chromosomal makeup of the animal." Knapp and Kopchick[15] and Kopchick et al.[16] propose definitions in which the key elements are the incorporation of exogenous DNA into the germ line of animals and the preservation of such genetic material in subsequent generations. At present, exogenous DNA can also be incorporated transiently into specific tissues, thus producing TA that express heterologous proteins without incorporating foreign DNA into their germ lines.[17] In addition, homologous proteins that are expressed in certain tissues of a given species can be transgenically expressed in tissues in which they are not normally found. An example of this case is a murine enzyme (α-galactosyltransferase), which is normally expressed in liver and transgenically expressed in lactating mammary glands of mice.[18] For purposes of this review, TA are defined as the nonhuman animals that have incorporated a fusion gene and permanently or transiently express at least one primary gene product encoded by the introduced DNA. This necessarily implies that at least one tissue, cell, or biological fluid has been modified either qualitatively or quantitatively as a result of the expression of the genetic constructs or transgenes.

Generation of transgenic animals

Genetic constructs

Several reviews describe the state of the art in TA production through the years. These reviews address relevant technologies from different perspectives, including molecular biology aspects[19]; the production of pharmaceuticals in TA[20,21]; the general use of TA as research tools[4,22]; and the fields of biotechnology, agriculture, and nutrition.[15,23] All of the mentioned reviews include sections on two fundamental aspects of transgenic technology: the generation of recombinant DNA suitable for transgenic expression and the techniques employed to introduce it into animals. Methods for the identification of TA and propagation of transgenic embryos also are discussed in several of these reviews.

Regulatory elements

General descriptions of the structural features of a transgene can be found in Rosen[24] and Bürki and Ledermann.[4] *Figure 1* is a schematic representation of transgene architecture. Transgenes contain TRE, which control the expression of the protein-encoding DNA sequences. These elements regulate the initiation of transcription of adjacent DNA into mRNA, which is subsequently translated into protein. TA have been excellent tools to identify TRE such as promoters, enhancers, and silencers and to determine if their action is equivalent in different species.[25,26] It should be noted that the term "promoter" has been used as a synonym of TRE. There are TRE that are tissue specific; therefore, the expression of the adjacent DNA is targeted to certain tissues or organs. Examples of these are the lactogenic TRE, which enhance DNA transcription during late pregnancy and lactation and target gene expression to the epithelial cells of the lactating mammary gland.[27,28] Other TRE are inducible; that is, they can be "activated" by applying external stimuli. One example is the metallothionein TRE,[29] which is induced to promote transcription by the addition of heavy metals to the animal's diet. Another is the phosphoenolpyruvate carboxykinase TRE, which is not active during fetal development, but is turned on after birth and can be regulated by the amount of dietary carbohydrate.[30] Other TRE direct expression to many tissues simultaneously.[31] Most transgenic experiments result in insertion of the transgene into chromosomal DNA. For this reason, transgene expression may also be affected by the transcriptional state of surrounding genomic DNA. Dominant control sequences known as locus control regions (LCR) are used to "shield" the transgene from such effects[32] (*Figure 1C*). Transgenes can also be protected from position effects by other type of boundary elements known as matrix attachment regions (MAR) or scaffold attachment regions.[33] In addition to the mentioned elements, it is possible to engineer sequences that have differential susceptibilities to methylation in different species.[34] Hypermethylated regions have

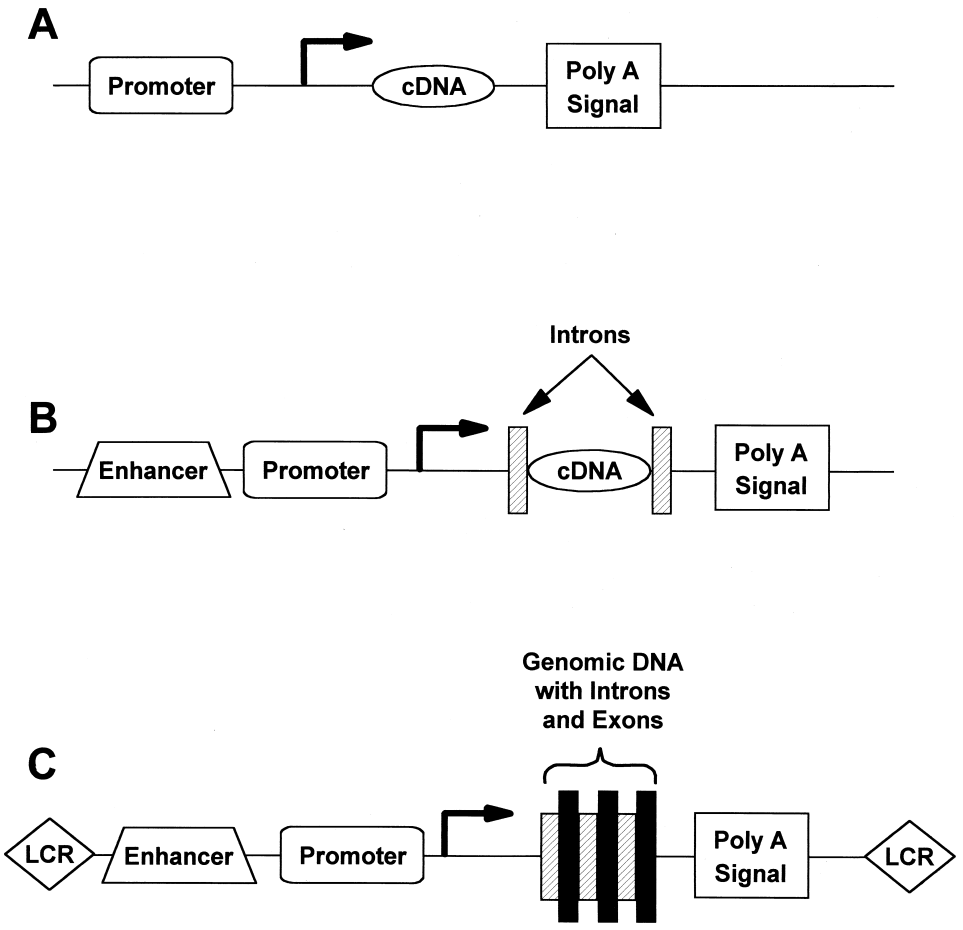

Figure 1 Schematic representations of different fusion genes for transgenic expression. *(A)* Simple construct containing a short transcription regulatory element, a cDNA, and polyadenylation encoding sequence. *(B)* More complex construct showing a larger regulatory element including an enhancer and strategically placed introns. *(C)* A construct with "shielding" elements, in this case, locus control regions (LCR), which contains a full genomic sequence. On occasions the full genomic sequence including transcriptional regulatory elements have been successfully used.

been shown to inhibit gene transcription whereas hypomethylated DNA does not.[35]

Protein-encoding DNA

The core fragment of the transgene is the DNA sequences that encode protein. Sometimes the only available DNA is a cDNA, which is generated from mRNA libraries by reverse transcription (*Figure 1A*). In contrast, most genomic DNAs contain intervening sequences called introns, which are not translated. Introns tend to stabilize and promote protein expression[36,37] and can be engineered into a fusion gene (*Figure 1B*). Alternatively, genomic DNA with all its introns is preferred (*Figure 1C*). On the other hand, there are practical limitations to the size of the transgene. In general as the transgene becomes larger (i.e., $>$20 kb) the rate of production of TA is decreased. This is thought to be due to fragmentation of DNA during the microinjection procedure (Yun, personal communication). In some instances, this has precluded the routine use of genomic DNA.[24] In some cases, "minigenes" that contain a subset of the genomic introns are used in the transgene construct.[38]

Although naturally occurring primary gene products are the most frequent targets for transgenic expression, it is also possible to produce chimeras or fusion proteins. Signal sequences that target the nascent polypeptide to intracellular compartments, secretory signals, or entire reporter genes[39] can be engineered into the protein-encoding DNA fragment.

Incorporation of the transgene

The most commonly employed technique to introduce transgenes into animals is microinjection of the fusion gene into the male pronucleus of embryos as reviewed by Jänne et al.[40] and Velander et al.[21] These embryos are then implanted in pseudopregnant females and the resulting offspring are assessed after birth for the presence of the transgene. It is not possible to regulate the number of copies or the sites in which the transgene will be inserted into the chromosomal DNA of the recipient. Likewise, the influence of the surrounding chromatin cannot be predicted. That is the reason why shielding elements such as the LCR and MAR described above are used in transgene construction. The number of copies of the transgene may or may not affect the final level of transgene-encoded protein produced.[41]

Because of their short generation times, mice have been frequently produced using embryo microinjection. The use of this technique for the preparation of larger transgenic farm animals with significantly longer gestation periods requires long waiting periods before the results of an experiment can be evaluated. For this reason, techniques have been developed to determine if microinjected embryos at advanced stages of development have incorporated the transgene prior to implantation into surrogate mothers.[42–44]

A second technique currently used in freshwater fish and

marine organisms is electroporation. Eggs are incubated in the presence of the transgene and electrical pulses are applied.[45] Alternatively, sperm can be electroporated, thus acquiring the transgene.[46] This sperm is then used to fertilize eggs, which results in transgenic organisms. Electroporation is also used to introduce transgenes into somatic mammalian cells and pluripotent embryonic stem cells.[47] These cells can be cultured and tested for transgene incorporation. Cells that carry the transgene can then be introduced into host morulae, which are then implanted into recipient mothers. The resulting TA are chimeric; that is, the transgene is present in some, but not all, of the cells of the progeny including gametes. Homozygous animals for the transgene can be produced by crossbreeding selected animals. Embryonic stem cells are frequently used to generate targeted gene mutations.

TA can also be generated by infection with retroviruses and retroviral vectors.[48] Embryos or pluripotent cells can be transfected using this technique. An interesting application of retroviral transfection described by Archer et al.[17] involved the direct transfection of animals through the teat canal using such vectors. In this case, the exogenous DNA was incorporated only into the genome of mammary gland cells and was not transmitted through the germ line. Transfection takes place during hormone-induced mammary gland differentiation and transgene-encoded proteins can be found in the milk. The transgene is transcribed at least for the duration of lactation.

Finally, once cells containing transgenes or targeted mutations are available, it is possible to obtain derived TA or TM by nuclear transfection. In this technique nuclei are obtained from cell cultures of transfected cell lines such as fibroblasts and are transferred into enucleated oocytes. These oocytes are then implanted into pseudopregnant animals. This demonstrates that somatic cells can be used to generate TA (in the sense that they still contain a human-made fusion gene). This technology can expedite the development of a productive herd that would be comprised of clones from an original TA.[49] The recent generation of transgenic rats and mice by testis-mediated gene transfer may not only provide a more efficient means of TA production but also allow TA production in currently "resistant" species.[50] This method generates transfected sperm by direct injection of DNA-liposome complexes into the male testis. These animals are then mated to normal females to generate the TA.

Transgenic animals and the field of nutrition

TA can be studied from different perspectives depending on their actual and potential contributions to the field of nutrition. Most of the available reports in the scientific literature describe the use of TA as experimental systems or models for scientific studies or as production prototypes. In only a few instances are protein pharmaceuticals being produced by TA in commercial or near-commercial scale.[21,51] From the perspective of nutrition and its allied fields, TA can be classified as: (1) models for the study of nutritional stages and metabolic diseases, (2) sources of modified food products, and (3) bioreactors that produce ingredients for nutritional products. Occasionally, the difference between animals used as bioreactors and animals as sources of modified food products is subtle and may be illustrated by the following example. Milk from a TA that produces high levels of κ-casein could be directly consumed or used to prepare cheese. In this case the product is derived from the TA with no significant handling other than traditional food processing. In contrast, if κ-casein is purified from milk and is used as an ingredient to improve the function or nutritional characteristics of food or nutritional products, then the TA is being used as a bioreactor. The latter is the approach most favored for the production of pharmaceuticals in milk.

Transgenic animals and transgenic mutants as models for the study of nutrition

Several reviews describing TA as disease and biochemical models have been published. Some of these are described in this section. Barrett and Mullins[52] discussed models for cardiovascular disease based on alterations to renin-angiotensin and other systems. Some of these models could be useful to study diet effects on hypertension, cardiac hypertrophy, and thrombosis. Several models for the study of endocrine disorders, including diabetes, were reviewed by Stewart.[53] Breslow[54] reviewed TA and TM models of lipoprotein metabolism and atherosclerosis. This review catalogues mice with altered lipoprotein transport proteins, lipases, and receptors and is perhaps one of the most illustrative documents on the potential of TA as nutritional models. Stewart[53] reviewed transgenic models for endocrine disorders, and Bray and Ryan[55] describe the history and rationale behind animal models focused on the study of obesity, allowing the reader to compare models obtained through traditional genetic selection techniques with those generated through the use of transgenic technology. In addition, animals that express GH transgenes may be models for the progression of type II diabetes. These animals have normal glucose levels but elevated insulin levels.[2] Although they are not diabetic, they are hyperinsulinemic. This situation parallels that seen in humans who are destined to become diabetic. In a similar manner, animals that express GH antagonists have normal levels of glucose but reduced insulin levels.[2] The effect of nutritional status and either elevated or depressed levels of GH may help determine the glucose/insulin levels in patients with type II diabetes. TA that were originally developed to study muscle mass or growth rates have also turned out to be interesting nutritional models. *Table 1* lists examples of TA and TM models.

Models of tissue differentiation and function that are relevant to the field of nutrition also have been generated. A glycosyltransferase, α1-3/4 FT, was expressed in small intestinal epithelia cells and the presence of glycoconjugates synthesized by the enzyme was confined to crypt cells.[56] Double TA simultaneously expressing the simian virus 40 tumor antigen and the transferase exhibited increased synthesis of the secondary gene product, thus providing a marker that responds to proliferative status of the cells. It is believed that glycoconjugates in mucosal cell surfaces function as receptors for pathogenic microorganisms. In this

Table 1 Examples of mouse models produced through transgenic or targeted mutagenesis technologies

Primary gene product	Application	Reference (year)
Brown adipocyte uncoupling protein	Source of transfected brown fat tumors	Ross et al. (1992)[57]
Expression of human triglyceride lipase	Lowering HDL cholesterol levels	Busch et al. (1994)[29]
Mutant thyroid hormone receptor	Resistance to thyroid hormone	Wong et al. (1997)[31]
Apoliprotein E	Diet sensitive atherosclerosis	Plump et al. (1992)[58]
Bovine growth hormone	Diet (carbohydrate) sensitive growth hormone in plasma	McGrane (1988)[30]
A2 Adenosine receptor	Thyroid hyperplasia, hyperthyroidism	Ledent et al.(1992)[59]
Alpha 1B adrenergic receptor	Growth stimulation, malignancy induction, other	Ledent et al. (1997)[60]
Cholesteryl ester transfer protein and apolipoprotein B	Cholesterol feedback regulation, LDL induction	Liu et al. (1997)[61]
Breast cancer oncogenes	Effect of diet in cancer onset	Rao et al. (1997)[62]
Human renin and angiotensinogen	Atherosclerosis	Sugiyama et al. (1997)[63]
α1-3/4 Fucosyltransferase	Proliferative state of epithelial small intestine cells	Bry et al. (1996)[56]
α-Galactosidase	Fabry's disease	Ohshima et al. (1997)[64]

HDL–high density lipoprotein. LDL–low density lipoprotein.

experiment the cell surface glycoconjugates of epithelial cells of the small intestine were remodeled. By altering the putative receptors of intestinal pathogens, researchers can verify if susceptibility to intestinal infections decreases or increases concomitantly. This suggests that models can be exquisitely fine-tuned to target effects to specific tissues or cells. Perhaps one of the most exciting features of some models of nutrition status related diseases is that many of them are diet sensitive. This allows the researcher to quickly evaluate the effects of diet changes and their impact on concomitant variables.

The applications described in this section are just a few of the areas related to nutrition in which TA and TM models are being generated. It is precisely in their roles as models that TA and TM have fulfilled the expectations of scientists. In addition, the ectopic expression of a transgene in the developing embryo may prove to be lethal or the TA may fail to thrive postpartum. Even in those cases, TA generate knowledge about the function of gene products and elicit research in new lines of inquiry.

Transgenic animals as sources of functionally modified food products and as bioreactors

The development of transgenic tomatoes[65] constitutes perhaps one of the most exciting recent events in the area of food science. A business intelligence report[66] suggested that products from TA would be on the market at the same time transgenic tomatoes were introduced. As of June 1998 products from TA have not yet reached the marketplace. Several reasons may be responsible for the apparent lag of commercial introduction of products from TA, but a few seem to be particularly important: (1) As opposed to plants, TA were first targeted as bioreactors for pharmaceuticals and these products are subjected to lengthy regulatory processes; (2) expression of certain transgenes in farm animals has resulted in deleterious effects; (3) the regulatory hurdles for a transgenically-produced food or nutritional product are unknown, whereas pharmaceuticals have been produced from a variety of sources including genetically-modified organisms; and (4) it is possible that the current state of the technology and peculiarities of animal systems require longer periods of time for their development.

Carcass and meat improvement

The prospective contributions and problems of TA as improved livestock are summarized in a review by Pursel et al.[67] McCracken[68] compares the potential of TA as an option to obtain lean beef with other approaches. Smith and Lewis[66] and Pursel and Rexroad[69] list traits that could be improved through the use of transgenic technology. Those relevant to nutrition are (1) efficiency of meat production, (2) improved quality of meat, (3) changes in the quantity and quality of lipids, (4) efficiency of milk production, (5) improved quality of milk, (6) resistance to parasites and pathogens, and (7) improved quality of poultry and their eggs. In addition, significant efforts are ongoing to improve fish and aquaculture in general. For many years farmers have selected animals that grow faster and convert feed into carcass weight more efficiently. Administration of anabolic hormones has also been used as a strategy to enhance growth and carcass quality[9]; however, animals may develop health problems when subjected to chronic hormonal treatments.[70]

The advent of TA allowed for the determination of effects resulting from the expression or suppression of single genes and for the methodical analysis of the effects of peptide hormones from different species. For these reasons, the most prevalent theme in the area of improved livestock through transgenesis is the expression of GH and related proteins. In addition to GH, somatostatin, which inhibits the release of GH, insulin-like growth factor (IGF-I), which acts on peripheral tissues, and GH release factor, which stimulates GH synthesis and release, have been expressed in experimental and farm animals.[16,67] Several fusion genes containing different promoters have been expressed in different species. *Table 2* summarizes some of these experiments and their results.

Some interesting secondary effects, which frequently include infirmities of different types, have been reported in TA expressing the mentioned proteins.[67] These and other unexpected deleterious effects in TA will be discussed below. In addition, some of these animals can be studied as models of certain endocrine stages, as is the case of GH transgenic lambs, which were hyperglycemic.[71] Mice are generally used as models to predict transgene viability in

Table 2 Transgenic expression of growth hormone (GH) and related proteins

Primary gene product	Transgenic animal	Promoter	Highlight	Reference (year)
Bovine GH	Mice	hMTA-IIA	Increase in growth rate	Palmiter et al. (1982)[75]
Human GH	Mice	hMTA-IIA	Increase in growth rate	Palmiter et al. (1983)[41]
Porcine GH	Pig	hMT-IIA	Increased weight gain	Vize et al. (1988)[76]
Human IGF-I	Mice	MT-I	Somatic growth gain	Mathews et al. (1988)[77]
Ovine GH	Lamb	oMT-IA	Body fat as low as 1/5 of controls	Ward et al. (1989)[78]
Human GH	Pig	MT-I	Decrease in carcass fat	Pursel et al. (1989)[67]
Bovine GH	Pig	MT-I	Decrease in carcass fat	Pursel et al. (1989)[67]
Human IGF-I	Pig	MT-I	Elevated IGF-I	Pursel et al. (1989)[67]
Bovine GH	Pig	PEPCK	41% reduction in backfat depth	Wieghart et al. (1990)[8]
Tilapia GH	Tilapia	hCMV	F1 82% larger than control	Martinez et al. (1986)[13]
Rainbow trout GH	Carp	RSV	20–40% faster growth	Chen et al. (1993)[79]

hMT-IIA–human metallothionein IIA. oMT-IA–ovine metallothionein IA. MT-I–mouse metallothionein IA. PEPCK–rat phosphoenolpyruvate carboxykinase. hCMV–human cytomegalovirus enhancer-promoter. RSV–rous sarcoma virus.

other species. However, mice do not always accurately emulate transgene expression effects that occur in larger animals. This is exemplified by mice that express bovine and human GH, which show accelerated growth rates and are larger than control mice. That has not been the case when GH is expressed in pigs, which may gain weight faster and be more efficient in converting feed into meat, but do not grow larger than control littermates. However, one report does describe GH transgenic pigs with enhanced growth rates.[72] Perhaps the most remarkable feature in both transgenic pigs and lambs expressing GH is that the carcasses are significantly leaner than those of control animals.[69] Recently, a mouse lacking the GH receptor gene has been generated and exhibits a dwarf phenotype.[73] Nutritional studies on this animal may help in evaluating the role of GH as it relates to weight gain, body composition, and so forth. These examples suggest the potential impact of TA in the field of nutrition. Such an impact is further illustrated by the outstanding review of Pursel and Solomon[74] on carcass composition in transgenic swine. In their review, transgenic and control animals are compared with regard to several biochemical composition parameters. For example, transgenic pigs expressing bovine GH not only contained less fat, they consistently contained lower percentages of saturated fatty acids than control animals. In addition, a larger percentage (36%) of the total fatty acids of transgenic pigs were polyunsaturated fatty acids; in control animals the average was 19%.[74]

There are many conceivable ways to alter meat quality through the expression of transgenes. For example, lipid composition could be altered by the transgenic expression of enzymes that act on fatty acids; the inducible expression of proteases could be used to tenderize meat prior to slaughter; proteins such as gelatin could be overexpressed; and muscle proteins from some species could be expressed in others to yield firmer, softer, or more digestible meat. As long as normal function is not significantly altered, the amino acid profile of certain tissues may be altered by overexpressing proteins or by exchanging endogenous genes for selectively mutated ones. However, as in the case of GH, technical subtleties and concomitant changes cannot be evaluated until TA have been generated, and in some cases, until the transgenes have been passed to subsequent generations. Although a significant amount of experience and data are being accumulated in this area, advances in the basic understanding regarding control of transgene expression are still required.

Modified milk and milk ingredients

Milk is a food product that has been extensively modified via expression of specific transgenes and, in many cases, without deleterious consequences for the TA. Several pharmaceutical proteins are already being produced in TA milk and are at different stages of development, from concept testing to preclinical and clinical evaluation. Examples of these are human α-antitrypsin in sheep,[5] human plasminogen activator in goats,[80] human protein C in pigs,[51] and human IGF-I in rabbits.[81] Milk can be modified or improved from different perspectives: (1) as an important dietary component for mass consumption, (2) as a raw material for dairy products, or (3) as a source of ingredients for nutritional products including infant formula. The fundamentals of transgene expression in lactating mammary glands and the fine points of the control of gene expression are discussed in a recent account by Clark.[6] The subject has also been reviewed by, among others, Colman,[82,83] Karatzas and Turner,[10] and Kopchick et al.[16] The architecture and function of milk protein genes was reviewed by Mercier and Vilotte.[28] Wall et al.[84] published a comprehensive review on milk improvement through the use of transgenic technology. This review includes a historical background of the field and contains tables summarizing the primary transgene products and regulatory elements used to express proteins in the milk of TA. Human milk is considered by many to be the gold standard of infant nutrition. For this reason, it is not surprising that several groups are attempting the transgenic expression of human milk proteins and other human milk constituents. Human milk proteins, which are candidates for transgenic expression in milk, were catalogued by Lönnerdal.[85] Blanc[86] published a seminal comparative review of human and bovine milk, which was later updated by Jensen et al.[87] Nonprotein constituents of human milk, such as oligosaccharides, also have been targeted for expression in milk of TA. One of the most comprehensive reviews on

Table 3 Transgenic expression experiments of β and κ caseins (CN) in milk of transgenic mice

Casein-origin	Promoter	Reference (year)
κ-CN bovine	Own*	Rijnkles et al. (1995)[25]
κ-CN bovine	Goat-β-CN	Gutierrez et al. (1996)[26,94]
κ-CN rabbit	Own	Baranyi et al. (1996)[95]
β-CN goat	Own	Persuy et al. (1992)[96]
β-CN bovine	Own	Rijnkles et al. (1995)[25]
β-CN bovine	Sheep-lactoglobulin	Hitchin et al. (1996)[97]
β-CN bovine	Bovine α-lactalbumin	Jeng et al. (1997)[98]

*Was not expressed in mammary gland. "Own" denotes that regulatory elements were casein promoters of the same species.

these carbohydrate structures and their putative functions was published by Kunz and Rudloff.[88]

The mammary gland is a privileged tissue in terms of its ability to synthesize milk proteins and lactose during late pregnancy and lactation. This unique character reflects a degree of biochemical insulation that is tightly controlled by hormones. Caseins, lactoglobulins, and whey proteins are among the primary gene products that are exclusively synthesized during lactation. Their expression is regulated by lactogenesis-responsive TRE. The most abundant protein in the milk of experimental animals, such as mice, rats, and rabbits, is the whey acidic protein (WAP).[89] WAP is a soluble protein whereas other proteins such as κ-casein occur both in solution or as components of micelles. TRE of WAP genes from several species have been identified and used to generate TA that contain transgene-encoded products in their milk. The WAP TRE, pioneered by researchers at the National Institutes of Health,[90,91] has been used to express proteins in lactating mammary glands of animals. Other TRE that are frequently used to generate TA are from the α-lactalbumin,[92] β-lactoglobulin,[93] and different casein genes.[84] Similar to the case of GH transgenic expression as a central theme of studies on carcass improvement, caseins and WAP are perhaps the dominant topics of transgenic expression targeted to the mammary gland. Because of the abundance of WAP and caseins in milk, significant efforts have been undertaken to determine the nature and location of the regulatory elements that direct their synthesis. Discovery and characterization of powerful regulatory elements have the potential to impact the efficiency of expression of other proteins in addition to caseins or WAP. In addiiton, caseins are targets as functional ingredients that may facilitate cheese manufacturing[26,94] and possess important biological activities that are relevant to infant nutrition.[85] *Table 3* summarizes experiments in which caseins have been expressed in the milk of TA. Unfortunately, most of these experiments have been carried out in mice and no transgenic farm animal producing CN has yet been reported. For this reason, it is difficult to assess the impact of TA in the field of nutrition.

As mentioned above, a rather ambitious and significant target for the transgenic modification of animal milk is the emulation of human milk. The production of proteins and secondary gene products characteristic of human milk has been pursued by several groups, including our own. The current state of the technology is illustrated by *Table 4*. From this table it is apparent that only a few human milk proteins have been expressed in TA. Furthermore, only one, lactoferrin, has been expressed in a large animal. The expression of lysozyme could be viewed from at least two different perspectives: (1) the decreased microbial load, reduction of handling costs and processing intensity, or the increase of shelf life and (2) the synthesis of a human milk component that is desirable to have as an ingredient of dairy products including infant formulas. This further exemplifies the difference between a TA producing an improved milk versus one producing an ingredient. The expression of fucosyltransferases is aimed at demonstrating the feasibility of synthesizing secondary gene products and remodeling of milk glycoconjugates and not at producing large quantities of the enzyme. Only catalytic amounts are needed to alter the oligosaccharide and glycoconjugate profile of milk. In each of these cases, available reports describe the technical foundations of a specific aspect of milk modification. Even using modern techniques for embryo propagation, commercial introduction of TA-derived milk products for mass consumption may not be available for more than 3 years. While pursuing emulation of human milk, two main types of tasks must be accomplished: (1) synthesis of characteristic human milk components and (2) elimination of undesirable animal milk components. TM, therefore, may play as important a role as transgenic expression of milk proteins. Even though the present review is not focused on TM, it is important to note that technology is evolving at a fast pace in this area. For example, advances in ribozyme technologies have provided another method of reducing or eliminating specific proteins. Ribozymes are RNA molecules capable of hydrolyzing RNA, thus interfering with the translation of mRNA transcripts.[102,103] Huillier et al.[104] reported the reduction of bovine α-lactalbumin content in the milk of transgenic mice through the expression of a

Table 4 Examples of human milk proteins expressed in transgenic animals

Human milk protein	Animal	Reference (year)
Lactoferrin	Cow	Krimpenfort (1993)[99]
Lactoferrin	Mouse	Krimpenfort (1993)[99]
Bile salt stimulated lipase	Mouse	Strömqvist et al. (1996)[100]
Lysozyme	Mouse	Maga et al. (1994)[101]
Fucosyltransferase α1-3/4 FT	Mouse	Prieto et al. unpublished data
Fucosyltransferase α1-2FT I*	Mouse, rabbit	Prieto et al. (1995)[1]

*α1-2FT I Is not found in human milk. The isozyme α1-2FT II is expressed in human milk

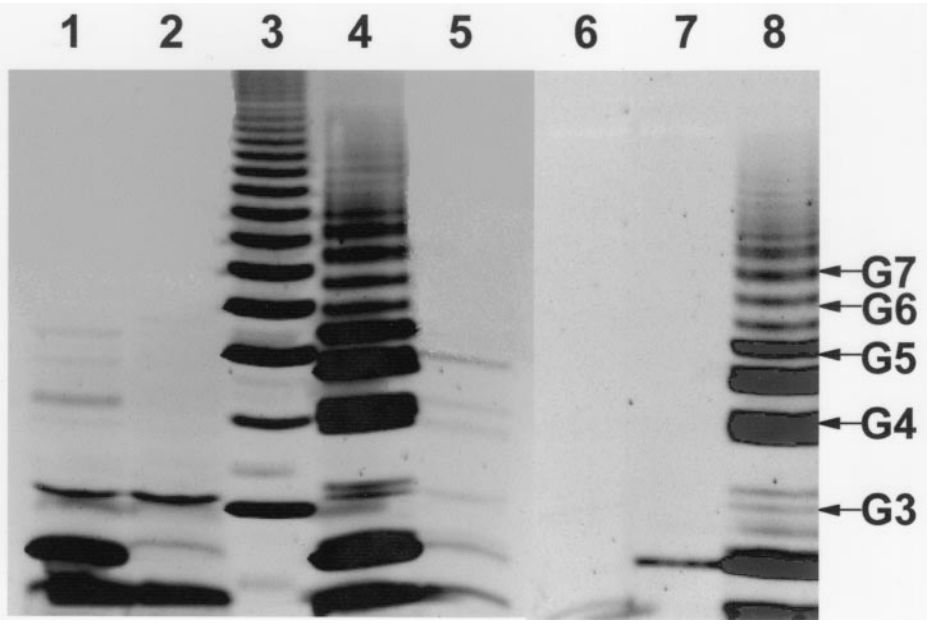

Figure 2 Fluorophore assisted carbohydrate electrophoresis (FACE) image of labeled free oligosaccharides from different milk samples. Oligosaccharide extracts were prepared as previously described.[1] The equivalent to 5 μL of milk were labeled, electrophoresed, and imaged utilizing a Glyko (Novato, CA USA) system developed for the analysis of O-linked oligosaccharides and according to manufacturer's directions. Lactose, the most abundant saccharide in the mixtures, was allowed to run off the gel. Lane numbers indicate the source of the milk samples with the exception of lane 3, which is a molecular weight standard consisting of linear glucose polymers. Lane 7 is a standard of the human milk saccharide 2′fucosyllactose. Lane 1, sow; 2, sheep; 4, human; 5, rabbit; 6, cow; and 8, human. Positions of glucose polymers are indicated at the right side of the image: G3 = three glucose units; G4 = four glucose units; etc.

second transgene encoding an α-lactalbumin specific ribozyme. With the use of these technologies, nonhuman animal milk proteins that are deemed undesirable may be reduced or eliminated. Other components are deemed undesirable for certain nutritional applications. Lactose (Galβ1-4Glc), which is the most abundant milk saccharide, is one of such components.[10]

Remodeling of glycoconjugates in transgenic animals

Lactose is a secondary gene product; its presence in milk reflects the activity of the lactose synthetase complex, which is comprised of α-lactalbumin and Galβ1-4 galactosyltransferase. In a similar fashion, the synthesis of free saccharides in solution and carbohydrate moieties of glycolipids and glycoproteins requires the expression of active glycosyltransferases in the appropriate cell compartments. One of the criticisms that has been raised to the production of glycoproteins in the lactating mammary glands of TA is that co- and post-translational protein modifications vary from animal to animal. A good example was provided by Strömqvist et al.[100] This group transgenically expressed human bile salt stimulated lipase (BSSL) in mouse milk and compared its glycosylation with those of authentic human BSSL and BSSL expressed in C127 mouse cells, CHO cells, and *Escherichia coli*. Although transgenically-expressed BSSL was active, it was demonstrated that O-glycosylation was either reduced or absent. Because the proteins expressed in C127 cells were significantly glycosylated, it was concluded that the differences were not due to the species but to the characteristic glycosylation machinery in the lactating mouse mammary gland. Differences in the glycoconjugates present in milk of different species are dramatically illustrated in *Figure 2*. Free oligosaccharides in solution are synthesized by the sequential elongation of lactose. It is apparent from this figure that human milk contains a uniquely rich and complex oligosaccharide profile when compared with those from other species. The structures of these molecules and their potential functions have been reviewed by Kunz and Rudloff.[88] Glycosyltransferases are the primary gene products responsible for the synthesis of oligosaccharides and other glycoconjugates. We hypothesized that by expressing human glycosyltransferases in lactating mammary glands of mice, oligosaccharides that are not present in mouse milk would be synthesized and would accumulate in the milk of TA. This hypothesis was proven when transgenic mice expressing the human fucosyltransferase α1-2 FT-I, under the control of WAP regulatory elements, synthesized 2′fucosyllactose (Fucα1-2Galβ1-4Glc), which subsequently accumulated in their milk.[1] In addition, endogenous glycoproteins were remodeled as they acquired fucose residues. *Figure 3* summarizes the results of these experiments. *Figures 3A and 3B* are chromatographic profiles of oligosaccharide extracts from milk of a control and a TA, respectively. The presence of a major additional component (2′fucosyllactose) in the milk of TA is clearly demonstrated. *Figure 3C* is a Western blot analysis using a lectin specific for α1-2 fucosylated residues and demonstrates the presence of a fucosylated glycoprotein only in TA milk. Since then, we have expressed other glycosyltransferases in animal mammary glands. In one of these experiments a murine α1-3 galactosyltransferase, which is normally expressed in liver,[105] was transgenically expressed in the lactating mammary glands of mice.[18] The primary product of the transgene was a homologous protein and it synthesized a previously undescribed free oligosaccharide with the structure Galα1-3Galβ1-4Glc. These TA pass the transgene through the germline and it has been possible to obtain double TA through crossbreeding.

The removal of lactose from milk also has been pursued, and different strategies have been proposed.[10] By decreasing or deleting the levels of α-lactalbumin, Stacey et al.[12] were able to decrease the lactose content of milk in TM.

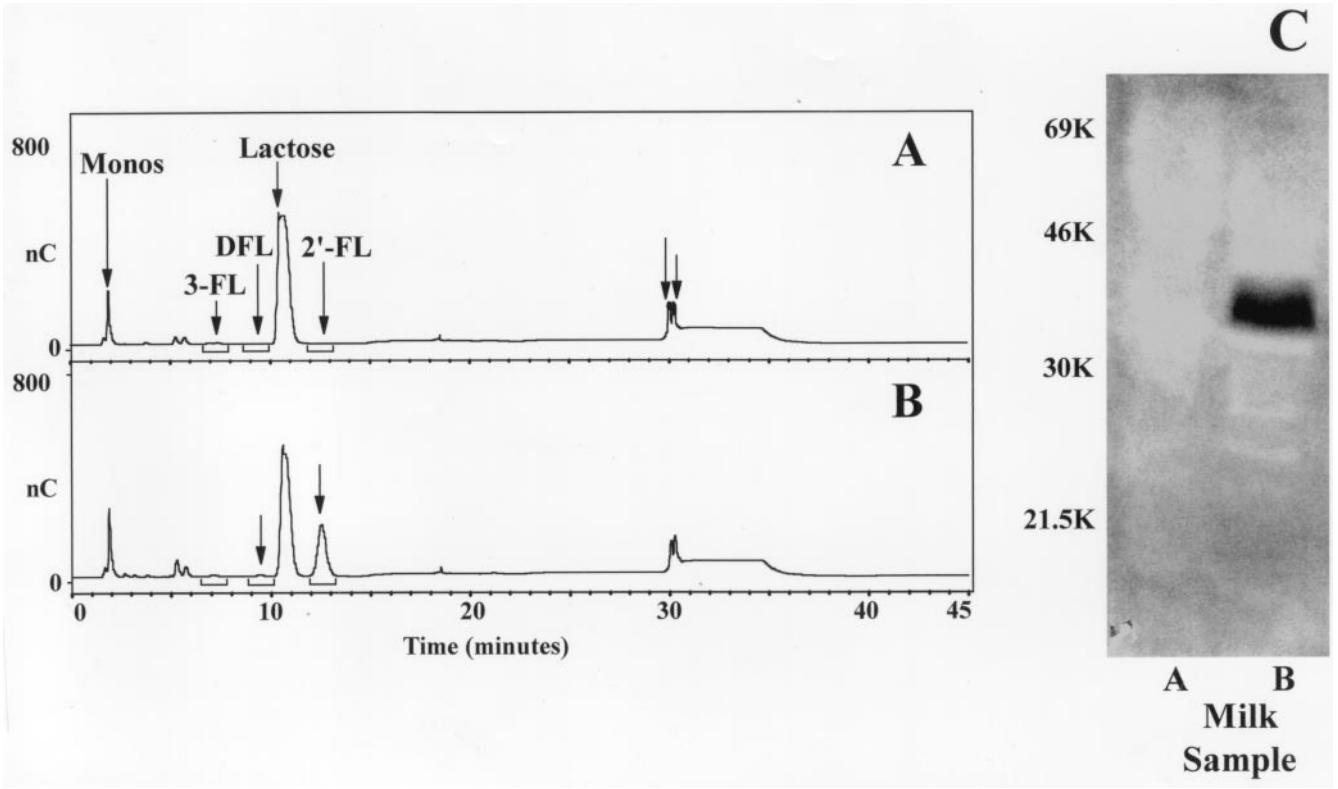

Figure 3 Analysis of control and transgenic mouse milk samples. Mouse milk samples from control *(A)* and transgenic *(B)* mice were prepared as previously described[1] and were assayed for free oligosaccharide content using high performance anion exchange chromatography. Protein pellets from the same samples were subjected to SDS-PAGE using a 10% polyacrylamide gel. After transferring to a polyvinylidene difluoride membrane, the glycoproteins were visualized using *Ulex europaeus* agglutinin I, a lectin specific for fucose α1-2 linkages *(C)*. The first lane corresponds to the control milk sample analyzed in *(A)*, and the second column is the transgenic sample from *(B)*.

However, the total milk output substantially decreased. This may be caused by the absence of lactose in the "milk compartment" of the mammary gland. It is believed that lactose is the major osmolite in milk that is responsible for the movement of water from the plasma into the milk.[106]

The reports and results summarized in this section suggest that it is worthwhile to pursue further experimentation with larger animals. It may be possible to produce glycoproteins with "humanized" or modified glycosylation patterns in the milk or other tissues of TA and TM.

Unexpected effects in transgenic animals

Table 5 summarizes some deleterious effects observed in TA after birth. Some of these unexpected findings have led to further research in the area of gene control and physiologic changes due to chronic expression of certain proteins. It is also not uncommon to find that embryos fail to develop when certain genes are expressed using systemic regulatory elements. A case in point is the expression of glycosyltransferases and enzymes that affect glycosylation in general. The expression of N-acetylglucosaminyltransferase I was lethal to embryos[107] whereas the expression of a sialic acid specific esterase under the metallothionein TRE also resulted in early arrested development.[108] The overexpression of β1-4-galactosyltransferase, also under the metallothionein TRE, resulted in impaired mammary gland development.[109] In addition, experiments in which the human α1-3/4-FT glycosyltransferase was expressed in different

Table 5 Examples of unexpected effects in transgenic animals

Transgene/TA	Effect	Reference (year)
MTh-bovine GH/pig	Gastric ulcer, cardiomegaly, arthritis	Pursel et al. (1989)[67]
mWAP/pig	Failure to lactate (agalactia; characteristic mammary gland phenotype)	Shamay et al. (1992)[112]
baLac-bbcasein/mice	Short lactation	Bleck et al. (1995)[110]
rWAP-EPO/rabbit	Infertility, agalactia, premature death	Massoud et al. (1996)[111]
MTh-β-Gal-transferase/mice	Impaired mammary gland development	Hathaway and Shur (1996)[109]
mWAP-α1-2FUT I/rabbit	Lactose free milk (changes in milk protein quality and content)	Prieto, unpublished results

MTh–mouse metallothionein promoter. mWAP–mouse whey acidic protein promoter. rWAP–rabbit whey acidic protein promoter. EPO–human erythropoietin. baLac-bbcasein–bovine αlactalbumin promoter-bovine β-casein. α1-2FUT–human α1-2 fucosyltransferase "H."

mouse tissues yielded different results with no apparent deleterious effects for the animals. Expression of the enzyme was directed by either the rat intestinal fatty acid binding protein TRE[56] or the WAP promoter (Prieto et al., unpublished observations). The secondary gene products and remodeled glycoconjugates synthesized by the enzyme remained localized to their targeted tissues. However, the affects of the transgene expression may be species-specific. As previously described, we were able to express the α1-2 FT-I in mice under the control of the WAP TRE.[1] When the same fusion gene was used to generate transgenic rabbits, the transgene affected milk production and resulted in lactose-free milk. Several generations of these animals are now under study to understand the effect of the enzyme on rabbit lactation. These examples strongly indicate that many factors influence the final results and the viability of different TA species, even when the same construct or primary gene product is expressed. Other effects may simply be due to the limited capacity of tissues to express proteins, specifically, the lactating mammary gland.[110] Ectopic expression, such as in the case of WAP controlled expression of erythropoietin[111] or extemporaneous expression of WAP in pigs,[112] are interesting from the point of view of development, morphogenesis, and protein function. As indicated above, some of the effects are species-specific. This is dramatically illustrated by the fact that transgenic sheep express mouse WAP in several tissues other than the lactating mammary gland.[113] The examples in *Table 5* and those discussed in this section suggest that in-depth research on gene control in different targeted species is still required.

Alternatives to transgenic technology

Transgenic organisms have captured the imagination of scientists and industrialists alike. However, biotechnology has evolved in other areas. Taking into account the relatively long development times for transgenic production of food products and ingredients, other options may be considered to solve specific problems. An example is the production of human β-casein. This human milk protein is present in several forms or variants containing from zero to five phosphate groups attached to serine and threonine residues. Bovine phosphorylated β-casein that contains all the variants has been produced in the milk of TA.[97] Preparations of human β-casein containing all the variants have also been obtained from *E. coli* by coexpressing the casein and human casein-kinase genes.[114] In addition, β-casein has been produced in transgenic potatoes[115] but the protein was not phosphorylated. These examples indicate that TA may not be the obvious choice to produce large quantities of human milk proteins. From the standpoint of nutrition, milk composition can also be altered by traditional methods such as changes in animal diet.[116] When the goal is to produce specific modifications in animal food products for commercial purposes, all options should be considered and analyzed from at least four perspectives: (1) timeline for commercial scale development, (2) regulatory issues and concerns, (3) economic feasibility, and (4) adaptability of current production methods. In some instances TA and TM may not represent the best available options.

Regulatory and legal issues

The utilization of TA to produce food products and ingredients on a commercial scale does not seem to be achievable in the near term. It is difficult to predict how many years will be required for this to occur. In the meantime, the technology has been divulged and discussed beyond the specialized press. It is plausible that drugs produced from TA bioreactors will pioneer their way to market before food products. Regulatory agencies and consumer groups are already taking steps to prepare themselves for such an event. In the United States, the Food and Drug Administration (FDA) has issued a notice entitled "Points to Consider in the Manufacture and Testing of Therapeutic Products for Human Use Derived From Transgenic Animals."[117] This notice is an important first step to initiate discussions on regulatory issues pertaining to TA and illustrates the approach of the FDA to this particular tool of biotechnology. However, regulatory actions are not given in vacuum. Consumer attitudes toward biotechnology and political pressures result in governmental actions that may influence the development of applications of TA. The reader is referred to a study conducted by Hamstra and Smink[118] on the attitudes of consumers toward biotechnology in The Netherlands. Furthermore, Enserink[119] reports that the Dutch government limited cloning experiments designed to propagate TA. It is clear that a current limitation of TA is the time required to generate a productive herd. This limitation can be circumvented by cloning already available TA. The impact of the mentioned governmental decision may be significant for companies based in The Netherlands. Even definitions in regulations may create controversy. A way to circumvent the long waiting period for natural or induced lactation of a transgenic cow is by expressing transgenes in the lactating gland using viral vectors. This technique can, at a minimum, be employed to determine if it is worthwhile to initiate germline incorporation experiments for a given transgene.

TA have elicited controversy and discussion in areas such as politics, ethics, and law. The reader is referred to two articles that discuss issues regarding intellectual property and patent law as it applies to TA. Irvine[120] asks fundamental questions regarding ownership of TA and their products and discusses the conflict between inventors and corporations and society at large. Marshall[121] describes one example of the on-going struggle between industry and academic institutions for rights to use and transfer TA.

Particular opportunities in the field of nutrition

The advent of TA allows experts in the field of nutrition to think about ways to improve milk, meat, and eggs for nutritional purposes. Present generations are witnessing an unprecedented environment in which foods can be substantially modified without the need of lengthy genetic selection and crossbreeding cycles guided by Mendelian genetics. It is now possible to conceive or conceptualize the ideal milks to be used in different applications, such as production of cheese, infant formula manufacture, or products for undernourished children, and the ideal meat for large scale production of ground beef. These functional characteristics

may be some of the first modifications of animal tissues and fluids that reach the market because they have undisputed commercial value. These possibilities force researchers in academia and industry to reexamine old concepts and standards based on the inherent limitations of currently available production methods.

Limitations and conclusions of the present review

One of the main objectives of the present review was to provide a panoramic view of the technology involved in the generation of TA. It was also an objective to illustrate the technology's current situation and potential by invoking examples and referring the reader to more specialized accounts. Scientific literature was used as the primary source of information. Three reports regarding regulations and business issues are cited because it was deemed that such issues will inescapably impact the development and expansion of transgenic technology. Several interesting experiments and models can be found in patent applications and they may represent some of the most advanced aspects of the technology. It was decided not to include them in this review because in most cases reproducibility and scope of the examples are not addressed. It is the bias of the reviewers that TA are already valued models for the study of metabolic pathways, diseases, and infirmities and that traditional approaches such as diet management can now benefit from the existence of these models. In their review on transgenic dairy cattle, Wall et al.[84] point out a paradox by indicating "Unfortunately, this area of investigation has received less attention in laboratories than it has in review articles." Although this may be true, it is important to consider that the fine line between science and technology is crossed back and forth in areas such as the subject of the present review. Companies and institutes do not disclose all the advances in the area because scientific divulgence becomes secondary to the submission of patent applications. In addition, scientific publications become a marketing tool for nascent technologies and efforts have to be made to separate facts from proposals or from overoptimistic portrays of the future. The following conclusions may also permeate the review as preexisting biases: (1) some aspects of transgenic technology, such as gene expression control, are still under basic scientific investigation; (2) most TA described up to this point are prototypes; (3) each case requires its own evaluation, because results from laboratory animals do not necessarily predict those that would be obtained from farm animals; and (4) TA and TM are powerful tools to study the effect of nutrition in health and disease.

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