Protein Production in Transgenic Animals

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The agricultural industry is experiencing a period of dramatic change as new developments in biotechnology provide different methods for making products. How the industry responds to the new challenges will, to a large extent, be governed by the attitudes of the consumer. One of the major effects of biotechnology will be the altering of consumers' attitudes towards farm crops. Future farms will produce food more efficiently, perhaps in environments where originally it was considered too difficult. In addition, farms will produce many different raw materials for industrial processes, a variety of pharmaceutical proteins for human and animal use, and serve as a source of vaccines for many of the developing nations.

One of the technologies that will contribute to this expansion in new products is the generation of transgenic species, both plant and animal. The purpose of this paper is to introduce transgenic animal technology by describing what it is, how it is performed, and why it has such potential. A few examples of possible products will be discussed, and the concept of livestock "stem cells" will be introduced.

WHAT IS TRANSGENIC TECHNOLOGY?

A transgenic animal has been defined as an animal that is altered by the introduction of recombinant DNA through human intervention. The first transgenic mice were produced in the early 1980s, but it is only within the past seven years that transgenic livestock have been produced on a routine basis (Wall, R. J. 1996).

PPL Therapeutics is the world's leading producer of transgenic animals. We are based on three continents (Roslin, Scotland; Blacksburg, USA; Whakaru, New Zealand) and have the ability to produce transgenic mice, rabbits, sheep, pigs, and cows. The different species allow PPL to tailor the production route to the product's market volume and the customer's requirements for speed to market (see Table 1).

TABLE 1: CHARACTERISTICS OF DIFFERENT SPECIES

Milestones	Cow	Sheep/Goat	Pig	Rabbit
G0 birth (1)	9	5	4	1
Induced milk (1)	16	9-10	–	4
Volume of induced milk (2)	~1- >100	~1-50	–	0.01-1e
G0 adult (1)	21	13	11	6
G1 birth (1) (natural milk)	31	18	15	7
Milk Volume (3) per lactation.	>10 000	250-700	>100	~1

(1) all times are in months and relate to the starting point of microinjection
(2) all volumes are in liters and can vary considerably between individual animals
(3) all volumes are in liters
e estimate

Each species has its own quirks and difficulties, but essentially, the process is the same. A DNA construct is designed and built to express the desired protein in the animal. The site of expression is determined by the regulatory sequences used in the construct to control the coding sequence. This construct must then be introduced into a single cell embryo to allow incorporation of the transgene into the animal's genome. There are several methods available for this purpose, including retroviral transmission, stem cell transfection, and microinjection into the pronucleus or cytoplasm. The most popular and most successful method involves microinjecting the DNA solution into the pronucleus of the embryo using a very fine glass needle. The injected zygote is then transferred into a hormonally prepared recipient and brought to term. Finally, positive transgenic animals are matured and the level of expression of the transgene is determined (Palmiter et al., 1982).

DNA CONSTRUCT PREPARATION

The DNA (a gene) that codes for a suitable therapeutic protein is cloned and sequenced. Every complete gene that codes for a protein has a control element (a promoter) attached to it. These direct the expression of the gene to specific sites in the body, at specific levels and times, depending on the function of the protein. PPL attaches a milk gene promoter (ovine b-lactoglobulin) to the therapeutic protein coding region to direct expression of the gene specifically to the mammary gland of the animal. In this way, the transgenic protein is produced in the milk of the animal and can be harvested and purified with no adverse effects on the animal. An added advantage is that the protein can be purified using regular dairy techniques, as well as high technology chromatographic procedures.

TRANSGENIC PRODUCTION

After introduction of the construct DNA into the one cell embryo, the construct DNA is incorporated into the DNA of the cell by an, as yet, unknown mechanism, and only in a few cases are transgenic animals produced. The rate of transgenesis can be 5-25 percent of live births; it is generally believed that the larger the animal the lower the rate, although in recent experiments with cows PPL has achieved high rates of transgenesis.

The embryos are cultured *in vitro* for various lengths of time before being transferred back into a hormonally prepared recipient female. The pregnant animals are brought to term, the offspring are tested for transgenesis, and the positive ones are bred for milk production. This whole process of injection to milking takes 13 weeks in mice, 44 weeks in sheep, and 135 weeks in cows, although with the development of hormonally induced premature lactation, information on the transgenic protein's expression level and quality can be obtained within 70 weeks for the cow. Because the quantity of induced milk produced by a cow can be far greater than a natural lactation from sheep or goats (see Table 1), a lot of the purification and pre-clinical studies may be performed without having to wait for the cow's natural lactation to begin.

WHY USE TRANSGENIC TECHNOLOGY?

There are various methods of protein production available commercially, including bacterial, insect, fungal, and mammalian cell culture systems. However, they each suffer, to varying degrees, from two major limitations. First, many therapeutic proteins have specific configurations that are necessary for activity. In addition, they frequently require quite complex post-translational modifications (PTM).

Some proteins require glycosylation (the addition of certain sugar residues) for activity or to ensure that they are not cleared from the circulation too quickly (Jenkins et al., 1996). Bacterial expression systems cannot perform

most of these modifications and can subtly alter the folding of the protein. Yeasts and higher plants can make many of the simpler additions, but are limited in their ability to perform complex modifications.

One form of PTM found only in mammalian cells is gamma carboxylation, which is essential for the vitamin K dependent proteins, such as Factor IX, used to treat hemophilia B, and protein C, an anti-coagulant. Although these proteins can be produced in mammalian tissue culture systems, the levels of expression are very low and attempts to increase it have not been successful. Another source is fractionation from human blood plasma, but the quantities available cannot satisfy the market, and possible contamination with human viruses (HIV, or hepatitis B and C) is a significant risk.

Large complex proteins, such as fibrinogen, cannot be produced at commercial levels in any culture system available today. Fibrinogen is an hexameric molecule consisting of two sets of three polypeptides encoded by different genes, and held together by 29 disulphide bridges. For proteins such as this, transgenic animals probably represent the only possible method of production.

The second limitation is that the cost of building and running modern production facilities for cell culture is extremely high. The initial capital investment in a very expensive production facility for a product that has not been through clinical trials, and which may fail, is hard to justify. This gives two compelling reasons for the development of transgenic technology:
Production of complex proteins: The mammary gland is able to perform most PTMs that are needed for therapeutic proteins. Even a protein as large and as complex as fibrinogen can be produced in the mammary gland in gram/liter quantities.
Cost of manufacture: The initial capital investment to produce transgenic animals is one to two orders of magnitude lower than for large production facilities. Operational costs are also considerably lower.

Alpha-1-Antitrypsin

One of the most advanced transgenic products is alpha-1-antitrypsin (AAT), a serine protease inhibitor. The protein is being developed by PPL for the treatment of lung disorders, including cystic fibrosis.

The transgenic sheep line that was chosen for this product has been studied in great detail. Expression levels and transgene copy numbers have been followed over five generations of sheep, over four complete lactations in individual sheep, and between more than seven siblings, all without showing any significant change. All of the production animals are New Zealand sheep, especially flown to Scotland to avoid any possible problems with scrapie, a spongiform encephalopathy of sheep that is not found in New Zealand.

There are now nearly 150 milking ewes in the production flock, producing AAT in the new pilot production facility, the first of its kind in the world. This facility has been designed and built by PPL to combine a high-tech dairy, operating within Good Agricultural Practice (GAP), with a state-of-the-art protein purification plant operating in accordance with Good Manufacturing Practice (GMP) requirements.

Regulatory Issues

A very important part of any new technology is developing procedures and practices to ensure that regulatory concerns are addressed. PPL has been in contact with the regulatory authorities in Europe and the United States for several years to help to establish the highest levels of safety possible. The Food and Drug Administration's (FDA) Points to Consider (Aug. 22, 1995) and the European Community's CPMP Draft Guidelines (July 4,1995) on production of therapeutics in transgenic animals were generally well received by the industry. Using those documents and our own expertise, PPL has developed a regulatory strategy for transgenic animals in the production plant. That strategy begins with checking the sequence of the transgene to ensure there are no known oncogenic sequences present, and continues after the birth of transgenic animals with:

Check Insert: An analysis of copy number, transgene integrity, number of insertional sites, and stability of the insert is carried out.

Seed stock: Semen of low generation male from the same line. This could be compared to the working cell bank of a tissue culture system.

Production flock: As the seed stock comes from low generation males, the production flock will consist of several groups of half sisters with the same integration site.

Highest possible quality animals: PPL's sheep are imported from New Zealand specifically because the health status of these animals is the best in the world.

Quarantine animals: The sheep on our farms are kept in strict quarantine from other animals to minimize the risk of infection.

Control of feed stock: There is strict control over the feed stock and only approved suppliers are used.

Regular health screening: The animals are regularly checked by qualified veterinary surgeons. Animals showing any signs of disease are immediately removed from the milking flock.

Dual site production: In the future there will be several sites of production. This is a precaution in the unlikely event of a flock or herd infection at one site.

POTENTIAL PRODUCTS

Protein C

Protein C is an anti-coagulant that plays an important role in the blood clotting cascade. To be active, the polypeptide must be cleaved into light and heavy chains, N-glycosylated, correctly folded and linked with disulphide bridges, and have nine gamma-carboxyl groups attached. Clearly this is a complex protein. PPL has produced fully active transgenic protein C at levels of 0.3g/l in sheep.

In addition, in blood plasma it can be seen that not all of the polypeptide is cleaved into two chains. Some single chain material remains that cannot be activated. In collaboration with Zymogenetics of Seattle, Wash., we have altered the cleavage site to increase the efficiency of cleavage to 100 percent (Foster et al., 1990). Therefore, all of the transgenic material can potentially be processed to make active protein C.

Fibrinogen

Fibrinogen is a complex plasma glycoprotein required for the final phase of blood coagulation. It is produced in liver parenchymal cells where the six chains are assembled and linked by 29 inter-chain and intra-chain disulphide bonds. Complete molecules are then secreted into the blood stream. Fibrinogen is going to be useful as a therapeutic protein and is one of the main components of surgical tissue sealants. These are being developed as a treatment for wounds as well as surgical procedures. PPL has demonstrated the production of g/l quantities of mature hexameric fibrinogen, which was functional in clotting reactions in the milk of transgenic mice (Prunkard et al., 1996), and also in induced milk from prepubertal sheep.

Neutraceuticals

In the past year, PPL established the technology to produce transgenic cattle, with nine transgenic animals born so far. Another 130 pregnant cows carrying potential transgenics have been generated. Those embryos were injected with a gene to produce a therapeutic food.

THE FUTURE OF TRANSGENICS

The present method of producing transgenic livestock animals, random integration of injected DNA into the genome, has two major disadvantages. First, it is inefficient and relatively more expensive than it could be. Second, currently it is only possible to add genes to an animal with no control over the site of integration. Livestock “stem cells” would address both these problems.

Embryonic stem (ES) cells are cells derived from the inner cell mass of blastocysts, which have been adapted to grow in *in vitro* conditions in the laboratory. The cells maintain their totipotency and when they are reintroduced into another blastocyst they contribute to the animal, and in some cases, to the germ

line. Therefore, any changes introduced into the ES cells in culture can become part of a new line of animals. Because the cells can be propagated *in vitro,* it allows various techniques to be employed to alter the genome of the animals into which those cells will ultimately develop. For example, homologous recombination and targeting are very powerful techniques used in genetic manipulation, which have allowed researchers to mutate, delete, and/or add genes in mice (Fässler et al., 1995).

At present, totipotent ES cells can only be isolated using standard approaches from specific mice strains. It has previously been suggested by others that it may never be possible to use this type of cell for other species. However, alternate techniques are currently being investigated and we have recently achieved the derivation of totipotent ES cells from strains of mice previously shown to be intransigent to classical techniques (McWhir et al., 1996). The application of this technique to different species may allow the future derivation of totipotent ES cells from livestock. That and other approaches are directed towards allowing the transfer of genetic manipulations performed on cell lines into live animals. In some cases, these scientific approaches involve the generation of cells that could not be considered in the classic sense as totipotent ES cells. However, they may allow genetic manipulation of the genome *in vitro* and the subsequent transfer of modifications to the genome of a live animal. Therefore, when people talk of livestock "stem cells," they are usually referring to a method of production, rather than the actual cells used in the process.

Recently, Campbell et al. (1996) demonstrated that a sheep could be produced by nuclear transfer from a cell grown in tissue culture. This is the first time a route from *in vitro* cultured cells to live animals has been demonstrated in a livestock species, and offers the same opportunities for analysis and modification of gene function as are available in the mouse using ES cells.

In practical terms, it will enable transgenics to move on to the next phase in its development; giving precise control over many more useful genetic alterations in livestock animals and increasing the efficiency of production. Clearly, this new approach to transgenic production will raise ethical issues that will need to be addressed in the next few years. One of the most controversial will probably be the use of this technology for the production of transgenic animals for xenografts (Jones 1996). This is the proposed replacement of diseased human organs with those from a transgenic animal, preferably a pig. Several companies are working towards this end already, however, the application of homologous recombination and targeting would undoubtedly improve their ability to realize their goals.

It is important that this debate is conducted in public, not just in academic circles, in a rational and sensible manner, so that the ultimate consumers can have properly balanced information upon which to base their decisions.

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