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Genetically Modified Livestock for the Production of Human Proteins in Milk

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Introduction

The use of naturally occurring proteins as therapeutic agents has a long history, primarily as replacement therapy for genetic or disease initiated deficiency. The advent of recombinant DNA technology allowed the production of such proteins in non-human organisms and rapidly led to the establishment of a new biotechnology industry which fused fermenter technology with the new molecular genetics. In the last 15 years, more than a dozen recombinant protein products have received marketing approval world wide. A recent survey (Anon, 1996) revealed that there were 284 biotechnology medicines and vaccines in development in the US alone, the majority of which were recombinant proteins.

The choice of production system for recombinant proteins has closely mirrored the research molecular biologists' favourite laboratory organism. The earliest, and still one of the most common, host organism was the bacterium *Escherichia coli* (Roberts, 1997). There are a large number of expression systems based on this bacterial technology and many years of production and regulatory experience. The yeast *Saccharomyces cerevisiae* has also been used extensively as a production organism for biotherapeutics. Both of these production systems are efficient, high producers under ideal conditions, however, they falter in the production of facsimiles of complex mammalian proteins due to their inability to reproduce most or all of the post-translational modifications frequently encountered.

For this reason, attention switched to mammalian cells grown in culture. From the late 1970s to date, the systems available for expression in such cells have developed considerably and several licensed proteins, for example erythropoietin, tPA and Factor VIII, are made in this way. However, mammalian culture processes often fail to achieve the high levels of expression attainable with bacterial and yeast methods and also carry the burden of complex and expensive media requirements (Roberts, 1997).

In 1980, Ruddle and co-workers published the first report of the stable addition of genetic material into the germline of a mouse (Gordon *et al.*, 1980). This work was

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soon followed by results which showed that it was possible to direct tissue specific gene expression from the added genes (Wagner *et al.*, 1981, Morello *et al.*, 1986). In the mid-1980s, it was proposed that mammary specific expression of human genes might be exploited for the production of recombinant proteins in the milk of transgenic livestock (Simons and Land, 1987; Clark *et al.*, 1987), by which time, Brinster and his colleagues had extended the transgenic technology to species other than mice (Hammer *et al.*, 1985). Early reports, using mice, demonstrated the feasibility of the mammary specific approach and identified suitable mammary specific promoter sequences (Simons *et al.*, 1987; Gordon *et al.*, 1987; Pittius *et al.*, 1988). This was quickly followed by demonstrations that conventional dairy livestock could be made transgenic and express human proteins in their milk (Simons *et al.*,1988; Clark *et al.*, 1989).

By 1990, the feasibility of genetically engineering farm animals to produce human proteins in milk was established, however, the levels of expression obtained were still below those levels required for commercial exploitation. The commercial viability was finally demonstrated by two papers published in 1991: Wall and co-workers reported transgenic pigs expressing human protein C at 1–2 g/litre (Wall *et al.*, 1991) and Wright and his colleagues described transgenic sheep expressing human alpha-1-antitrypsin (AAT) at the stunning level of 35 g/litre (Wright *et al.*, 1991). In the same year, Klimpenfort and colleagues demonstrated transgenesis in the ultimate dairy producer, the cow (Klimpenfort *et al.*, 1991).

In the years since 1991, products from transgenic milk have made considerable progress towards the market. PPL Therapeutics Plc have recombinant AAT in phase II clinical trials for the treatment of cystic fibrosis and Genzyme Transgenic Corporation have human Antithrombin III in phase II trials. There has been a considerable amount of work done on integrating the transgenic manufacturing process into the manufacturing and regulatory framework of the biologics industry and there have been technological advances which, arguably, make transgenic technology even more attractive as a method of production. We would like to review some of these areas in turn: the construction of transgenics, control of mammary specific expression, development of the purification process and finally addressing the requirements of the regulatory authorities for product safety.

Generation of transgenic livestock

Several methods can be used to introduce exogenous DNA into the developing mammalian embryo in such a way that it may ultimately become stably integrated into a chromosome of the resulting animal (Hogan *et al.*, 1994; Umland *et al.*, 1996).

Direct microinjection introduces DNA into one of the pronuclei of a single-celled, fertilized, egg by physically injecting it through a microscopically small-bore glass needle. It is labour intensive and technically demanding but would appear to have few limitations on the type or size of DNA that can be introduced. Transgenes designed to express human factor IX, containing over 40 Kbp of DNA, have been introduced into mice (Schnieke *et al.*, 1997). Within the last three years there have been several papers which describe the introduction of hundreds of kilobase pairs of contiguous DNA, in the form of yeast artificial chromosomes, into

the mouse germ line (Schedl *et al.*, 1993; Fujiwara *et al.*, 1997; Manson *et al.*, 1997).

Retroviruses can be genetically modified to act as vectors allowing the infection of embryos with exogenous DNA (Jaenisch *et al.*, 1975; Gilboa *et al.*, 1986). Superficially, this is an attractive option as the process is relatively efficient; however, the size of exogenous DNA that can be incorporated is limited to a few kilobase pairs, a high degree of mosaicism is often obtained necessitating extensive breeding to isolate true lines, multiple sites of integration can occur and, in some cases, the transgene may be inactivated during this process (Gilboa *et al.*, 1986).

Sperm has been claimed as an efficient mediator of exogenous gene transfer into mammalian embryos by one group (Lavitrano *et al.*, 1989). Despite the obvious attractions of such an approach, this work has been dogged by the inability of other workers to repeat its claimed successes (Nancarrow *et al.*, 1993).

The final method of note is DNA recombination, homologous or non-specific, in embryonic stem cells. These are pluripotent or totipotent cells derived from a mammalian embryo that are able to be grown in culture and which can contribute to a developing embryo (Evans and Kaufman, 1981; Robertson, 1987). The resulting animal possesses a number of cells derived from the embryonic stem cells, some of which may populate the germline and result in transmission of genetic material to the offspring (Robertson, 1987). Embryonic Stem cells can also be genetically manipulated whilst in culture. DNA can be introduced into the cells by using a variety of techniques (e.g. electroporation, lipofection, microinjection, retroviral infection and calcium phosphate precipitation). By employing the antibiotic G418, or exploiting the biochemistry of the enzyme thymidine kinase, transformed cells, that have incorporated exogenous DNA in a specific or non-specific way, can be positively and negatively selected (Bradley, 1991; Hooper, 1992). Very subtle alterations of the genome are possible, such as point mutations, as well as the generation of specific deletions (so-called 'knock outs', e.g. Stacey *et al.*, 1994) or replacements (Stacey *et al.*, 1994, 1995). However, to date this method has only been successful in the mouse. Embryonic stem cells have only demonstrably been isolated from murine embryos, the search in other species continues (McWhir *et al.*, 1996).

In February of this year (1997), a major advance was reported which promises to bypass the requirement for livestock embryonic stem cells in order to perform the genomic manipulations described above. Wilmut and co-workers (Wilmut *et al.*, 1997) described the successful cloning of a sheep by nuclear transfer from both foetal and adult cells. The adult cell in question was derived from a culture which had been grown *in vitro* for many passages. This new technology takes an enucleated, unfertilized, egg and places another cell into the space between the zona pellucida and the cytoplasmic membrane. The membranes of the two cells are fused with an electric pulse. Reconstructed embryos are allowed a brief development period in a temporary sheep recipient before final evaluation and introduction into a synchronized ewe.

Thus, it is now conceivable that adult or foetal livestock cells can be manipulated *in vitro*, with much the same technology as is used for the alteration of murine embryonic stem cells, and then used to generate transgenic animals. Indeed, there are now transgenic sheep which have been made by such an approach, whereby a human factor IX gene under the control of BLG has been introduced into cells *in vitro*, prior to nuclear transfer (Schnieke *et al.*, 1997).

Table 1. Reproductive features of different species

Species	Eggs per super-ovulation	Times (months) G0 Birth	G0 Adult	G1 Birth (first milk)[1]	Potential milk volume (l)	Times (months) G1 Adult	G2 Birth	G3 Birth (G2 Herd)
Sheep/Goat	6–10	5	13	18 (9)	>100	26	31	47
Cow	N/A*	9	23	32 (12)	>10,000	46	55	78
Pig	30–50	4	11	15	>100	22	26	37
Rabbit	~30	1	6	7	~1			
Mouse	~30	0.75	2.5	1	~0.001			

* Not applicable, generally use IVM/IVF.
[1] Artificial induction of lactation in parenthesis.

Choice of livestock species

Several difficulties arise when the mouse technology is transferred to domestic animals, and in particular farm animals. The first of these is the generation time of the chosen species. Gestation time and the attainment of sexual maturity greatly influence the time at which milk and hence product may be attainable. Indeed, realistic commercial producers will only be available at the birth of G2 animals. *Table 1* summarizes the important features of the major dairy species. Because of its rapid gestation and maturation, the earliest that one can obtain milk is in the mouse. Milk volume in this species is extremely limited although yields are sufficient to justify its use as a test system. The next most rapid is the rabbit, which will produce about 8–10 litres of milk per year over progressive lactations and yield first product after about 7 months (R. Page, personal communication). The pig can produce more than 100 litres of milk from a single lactation; however, this volume is somewhat more difficult to harvest than milk from the more established dairy animals. The sheep and the goat produce similar quantities of milk (200–500 litres per lactation) in a similar time frame to the pig but are much more amenable to the milking process. The ultimate producer to be considered is the cow which may produce around ten thousand litres of milk during a lactation but only after an extensive gestation and maturation phase (Barth and Schlimme, 1989; Jensen, 1995).

Sheep and cows are amenable to hormonal induction of lactation which can shorten the time to first milk but volumes of product obtained are generally much less than that obtained from a natural lactation (PPL Therapeutics, unpublished data). This does not provide a satisfactory source of material for production purposes but may allow early characterization of the product from which the purification processes can be developed. All of these factors, together with the estimated volume of recombinant protein required, must be taken into consideration when choosing the species with which to work as they influence the amount of and the speed with which a given volume of milk is attainable.

Generation of transgenic livestock

The process of making a transgenic farm animal by microinjection is almost exactly the same as that used to produce transgenic mice (Hogan *et al.*, 1994). Fertilized eggs

for microinjection must be generated in large numbers and suitable recipient animals hormonally synchronized to the correct time in the reproductive cycle. All the main farm animals, and rabbits, can be superovulated with appropriate hormonal regimes, thus it is possible to get large numbers of embryos for microinjection (Hammer *et al.*, 1985; for review see Wall *et al.*, 1992). It is also relatively straightforward to synchronize the oestrus cycles of farm animals, thus preparing suitable recipient females. However, the logistics of the experiment are considerably more demanding.

Rabbits are the closest to the mouse yielding similar numbers of embryos. Sheep generally produce around 6–10 eggs per ewe compared to about 30 per female mouse. Cows and goats are similar to sheep. One advantage of pigs is that they are polytocous or litter bearing, thus, the number of recipient animals is reduced. Sheep, goats and cows do not generally bear more than twins and indeed the life of the mother and offspring can be compromised by multiple births. While sheep, goats and pigs are relatively cheap, good dairy cattle are very expensive, for this reason, and to avoid the need for surgical intervention, most groups now utilize slaughterhouse derived ovaries which are manipulated *in vitro* to produce eggs which are then fertilised *in vitro* (so-called IVM/IVF; Gordon and Lu, 1990).

Microinjection of pronuclei in farm animals is technically more demanding than in mice. Fertilized eggs from pigs and goats contain granular inclusions which obscure the target. In sheep, the pronucleus is very close to the cytoplasm in refractive index and thus difficult to see to all but the expert eye. For cattle the problem is that the pronucleus is frequently loosely associated with the cytoskeleton and very sharp needles are needed to penetrate the pronuclear membrane without moving the target. Some of these difficulties can be alleviated by centrifugation of the eggs prior to microinjection to reveal the pronucleus (Wall *et al.*, 1992).

Finally, the frequency of transgenesis, as measured by the percentage of live births that are transgenic, in larger animals is usually reduced compared to mice (e.g. Hammer *et al.*, 1985; Simons *et al.*, 1988; Wright *et al.*, 1991; Krimpenfort *et al.*, 1991; Ebert *et al.*, 1991). It may be that integration of foreign DNA in these eggs happens less frequently or possibly eggs which integrate foreign DNA quickly are at a developmental disadvantage and preferentially die. The end result is that the frequency of transgenesis in livestock is usually much closer to 5% than the 15% or more associated with the mouse.

Targeting gene expression to the mammary gland

There are a relatively small number of proteins secreted into milk. In artiodactyls and man, these consist primarily of two or more caseins and the whey proteins β-lactoglobulin and α-lactalbumin (Barth and Schlimme, 1989; Clark, 1992). The number and levels of distinct casein proteins in milk varies from species to species but always includes a β-like casein and κ-casein. The casein genes are tightly linked in the genome of all species studied so far (Tomlinson *et al.*, 1996) including human (PPL Therapeutics, unpublished data). In rodents, there is no β-lactoglobulin, but an additional major milk protein is present, the so-called Whey Acidic Protein (WAP; Campbell *et al.*, 1984).

All of these genes have the classic architecture of eukaryotic, protein coding, genes. They are controlled by tissue specific promoter regions which respond to RNA

Table 2. Summary of expression of transgenes in milk

Milk gene promoter	Protein expressed	Expressed DNA	Expression	Animal	Citation		
α-Lac Bovine	α-Lactalbumin	genomic	0.2–2400 μg ml^{-1}	rat	Hochi, S. *et al.*	*Mol. Reprod. & Devel.*	33: 60–164, 1992
α-Lac Bovine	Bovine α-lactalbumin	cDNA	0.45 mg ml^{-1}	mouse	Vilotte, J. *et al.*	*Eur. J. Biochem.*	186: 43–48, 1989
α-Lac Bovine	Bovine α-lactalbumin	genomic	1.5 mg ml^{-1}	mouse	Bleck & Bremel	*J. Dairy Sci.*	77: 1879–1904, 1993
α-Lac Bovine	Ovine troph interferon	cDNA	1 μg ml^{-1}	mouse	Stinnakre, M.G.	*FEBS*	284: 1922, 1991
α-Lac Caprine	Caprine α-lactalbumin	genomic	1.2–3.7 mg ml^{-1}	mouse	Souler, S. *et al.*	*FEBS*	297: 13–18, 1992
α-Lac Guinea pig	Guinea pig α-lactalbumin	genomic	not quoted	mouse	Maschio *et al.*	*Biochem. J.*	275: 459–467, 1991
α_{s1}-casein Bovine	EPO	cDNA	3–205 ng ml^{-1}	mouse	Uusi-Oukari *et al.*	*Transgenic Res.*	6: 75–84, 1997
α_{s1}-casein Bovine	GMCSF	cDNA	0.2–4.6 mg ml^{-1}	mouse	Uusi-Oukari *et al.*	*Transgenic Res.*	6: 75–84, 1997
α_{s1}-casein Bovine	hIGF-1	cDNA	1 mg ml^{-1}	rabbit	Brem *et al.*	*Gene*	149: 351–355, 1994
α_{s1}-casein Bovine	hLactoferrin	cDNA	No results yet	cow	Krimpenfort P. *et al.*	*Bio/Technology*	9: 844–847, 1991
α_{s1}-casein Bovine	hLactoferrin	cDNA	36 μg ml^{-1}	mouse	Platenburg *et al.*	*Transgenic Res.*	3: 99–108, 1994
α_{s1}-casein Bovine	hlysozyme	cDNA	0.71 mg ml^{-1}	mouse	Maga *et al.*	*Transgenic Res.*	3: 36–42, 1994
α_{s1}-casein Bovine	hUrokinase	genomic	1–2 mg ml^{-1}	mouse	Meade *et al.*	*Bio/Technology*	8: 443–446, 1990
β-casein Goat	β-casein	genomic	21–24 mg ml^{-1}	mouse	Persuy *et al.*	*Eur. J. Biochem.*	205: 887–893, 1992
β-casein Goat	CFTR	cDNA	Not stated	mouse	DiTullio *et al.*	*Bio/Technology*	10: 74–77, 1992
β-casein Goat	hLA tPA	cDNA	1–2 mg ml^{-1}	goat (induced)	Ebert *et al.*	*Bio/Technology*	12: 699–702, 1994

Table 2 continued

Milk gene promoter	Protein expressed	Expressed DNA	Expression	Animal	Citation		
β-casein Goat	hLA tPA	cDNA	0.85 mg ml^{-1}	goat (induced)	Ebert *et al.*	*Bio/Technology*	12: 699–702, 1994
β-casein Rabbit	hIL2	genomic	≥450 ng ml^{-1}	rabbit	Buhler *et al.*	*Bio/Technology*	8: 140–143, 1990
β-casein Rat	bovine FSH	cDNA	15 μg ml^{-1}	mouse	Greenberg *et al.*	*PNAS*	88: 8327–8331, 1991
BLG Bovine	BLG-EPO fusion	cDNA	0.3 mg ml^{-1}	mouse	Korhonen *et al.*	*Eur. J. Biochem.*	245: 482–489, 1997
BLG Bovine	BLG-EPO fusion	cDNA	0.5 mg ml^{-1}	rabbit	Korhonen *et al.*	*Eur. J. Biochem.*	245: 482–489, 1997
BLG Sheep	hAAT	genomic	7.3 mg ml^{-1}	mouse	Archibald *et al.*	*PNAS*	87: 5178–5182, 1990
BLG Sheep	hAAT	cDNA	5 μg ml^{-1}	mouse	Whitelaw *et al.*	*Transgenic Res.*	1: 3–13, 1991
BLG Sheep	hAAT	genomic	21.3 mg ml^{-1}	mouse	Carver *et al.*	*Bio/Technology*	11: 1263–1269, 1993
BLG Sheep	hAAT	cDNA	5 μg ml^{-1}	sheep	Simons *et al.*	*Bio/Technology*	6: 179–183, 1989
BLG Sheep	hAAT	minigene	35 mg ml^{-1}	sheep	Wright, G. *et al.*	*Bio/Technology*	9: 830–834, 1991
BLG Sheep	hFIX	cDNA	60 μg ml^{-1}	mouse	Yull, F. *et al. PNAS*	92: 10899–10903, 1995	
BLG Sheep	hFIX	cDNA	25 nm ml^{-1}	sheep	Clark, A.J. *et al.*	*Bio/Technology*	7: 487–492, 1989
BLG Sheep	hIFN-γ	cDNA	20 ng ml^{-1}	mouse	Dobrovolsky *et al.*	*FEBS*	319: 181–184, 1993
BLG Sheep	hSA	minigene	2.5 mg ml^{-1}	mouse	Shani, M. *et al.*	*Transgenic Res.*	1: 195–208, 1992
BLG Sheep	hSA	minigene	35 μg ml^{-1}	mouse	Shani, M. *et al.*	*Transgenic Res.*	1: 195–208, 1992

Table 2 continued

Milk gene promoter	Protein expressed	Expressed DNA	Expression	Animal	Citation		
BLG Sheep	hSA	cDNA	None	mouse	Shani, M. *et al.*	*Transgenic Res.*	1: 195–208, 1992
BLG Sheep	hSA	minigene	10 mg ml^{-1}	mouse	Hurwitz *et al.*	*Transgenic Res.*	3: 365–375, 1994
BLG Sheep	Sheep BLG	genomic	23 mg ml^{-1}	mouse	Simons *et al.*	*Nature*	328: 530–532, 1987
WAP Mouse	bovine TAP	cDNA	5 μg ml^{-1}	mouse	Yarus *et al.*	*PNAS*	93: 14118–14121, 1996
WAP Mouse	EC-SOD	cDNA	0.7 mg ml–1	mouse	Hansson, L. *et al.*	*J. Biol. Chem.*	269: 5358–5368, 1994
WAP Mouse	EC-SOD	cDNA	2.9 mg ml^{-1}	rabbit	Strömqvist *et al.*	*Transgenic Res.*	6: 1–8, 1997
WAP Mouse	hCD4	cDNA	level not stated	mouse	Yu, S. *et al.*	*Mol. Biol. Med.*	6: 255–261, 1989
WAP Mouse	hGH	genomic	3.5 mg ml^{-1}	mouse	Reddy, V. *et al.*	*Animal Biotech.*	2: 15–29, 1991
WAP Mouse	hLA tPA	cDNA	3 μg ml^{-1}	goat	Ebert, K. *et al.*	*Bio/Technology*	9: 835–838, 1991
WAP Mouse	hParathyroid hormone	cDNA	415 ng ml^{-1}	mouse	Rokkones, E. *et al.*	*J. Cell. Biochem.*	59: 168–176, 1995
WAP Mouse	hPC	cDNA	3 μg ml^{-1}	mouse	Velander *et al.*	*Ann. NY Acad. Sci.*	665: 391–403, 1992
WAP Mouse	hPC	genomic	0.7 mg ml^{-1}	mouse	Palyeda *et al.*	*Transgenic Res.*	3: 355–364, 1994
WAP Mouse	hPC	cDNA	1 mg ml^{-1}	pig	Velander, W. *et al.*	*PNAS*	89: 12003–12007, 1992
WAP Mouse	hPS2	cDNA	1.5 μg ml^{-1}	mouse	Tomasetto *et al.*	*Mol. Endocrinol.*	3: 1579–1584, 1989

Table 2 continued

Milk gene promoter	Protein expressed	Expressed DNA	Expression	Animal	Citation		
WAP Mouse	htPA	cDNA	114–460 ng ml^{-1}	mouse	Gordon *et al.*	*Bio/Technology*	5: 1183–1187, 1987
WAP Mouse	htPA	cDNA	100 μg ml^{-1}	mouse	Pittius, C. *et al.*	*PNAS*	85: 5874–5878, 1988
WAP Mouse	Mouse WAP	genomic	100–500 μg ml^{-1}	sheep	Wall *et al.*	*Transgenic Res.*	5: 67–72, 1996
WAP Mouse	WAP	genomic	1–2 mg ml^{-1}	pig	Wall, R. *et al.*	*PNAS*	88: 1696–1700, 1991
WAP Rabbit	hAAT variant	genomic	10 mg ml^{-1}	mouse	Bischoff, R. *et al.*	*FEBS*	305: 265–268, 1992
WAP Rabbit	hGH	genomic	22 mg ml^{-1}	mouse	Devinoy, E. *et al.*	*Transgenic Res.*	3: 79–89, 1994
WAP Rat	Rat WAP	genomic	27% of Endogenous	mouse	Bayna, E. & Rosen, J.M.	*Nucl. Acids Res.*	18: 2977–2985, 1990

polymerase II, they have an intron/exon structure producing pre-mRNA which is subsequently spliced and they harbour signals for mRNA termination and polyadenylation in their 3' flanking sequences. All of the proteins are secreted by means of a signal peptide in the pre-protein, this being cleaved off. A number of important control elements have been identified in the promoter regions as befits genes under complex hormonal regulation. For example, glucocorticoid response elements have been identified for WAP and β-casein in rat as well as sites for NF-I, STAT5, YY1 and C/EBP in most of the milk gene promoters (for review see Rosen *et al.*, 1996). STAT5 mediates the prolactin response which is a major activation pathway for milk gene expression (Wakao *et al.*, 1994).

Not surprisingly, almost all of these genes have been harnessed to direct expression of heterologous proteins into the milk and it is fair to say that no one promoter is vastly superior to any other in terms of achieving high levels of expression. *Table 2* summarizes the data on heterologous expression published to date. As can be seen, a large number of disparate proteins have been expressed and from several different milk protein promoters. The list includes complex glycoproteins, gamma carboxylated protein C, homo and hetero multi-subunit proteins (for example Fibrinogen, EC-SOD and FSH), the cystic fibrosis transmembrane receptor (CFTR) and even peptides.

Our own work has used the ovine beta-lactoglobulin gene (BLG) which was cloned and characterized by Ali and Clark (1988). Clark and his colleagues subsequently demonstrated consistent, high level, expression of ovine BLG in the milk of mice transgenic for the entire ovine gene (Simons *et al.*, 1987; Harris *et al.*, 1990). Further experiments demonstrated that the BLG promoter region can direct high levels of expression of a heterologous human protein to the milk of transgenic mice (Archibald *et al.*, 1990). The generation of sheep, expressing human proteins in their milk using BLG regulatory elements, indicated that this technology was applicable to transgenic livestock (Simons *et al.*, 1988; Clark *et al.*, 1989) and, finally, the commercial feasibility of this technology, as a means of producing recombinant therapeutics in livestock milk, was confirmed by PPL, who demonstrated high level expression of human alpha-1-antitrypsin in the milk of transgenic sheep (Wright *et al.*, 1991;Carver *et al.*, 1993).

Despite the efforts of many workers over several years, the development of a reliable cell culture system that would faithfully mimic the properties of the mammary gland has proved elusive (Lin and Bissell, 1993; Medina, 1996). Primary cultures of mammary cells have been the most successful, but by their nature these lack a degree of consistency. If stable cell lines were available, they would allow for a rapid and cost-effective validation of constructs designed to express heterologous proteins in the lactating mammary gland of livestock. In their absence, the mouse is used as a model system for mammary expression studies to enable the testing of transgene constructs for high expression and the ability of the mammary gland to process the product correctly prior to the production of larger transgenic animals.

Most transgene constructs follow relatively simple patterns and can be exemplified with the BLG constructs that we have used. As can be see from *Figure 1*, a vector comprising 4.3 Kbp of the BLG promoter and 2.6 Kbp of 3' flanking region has been designed such that any coding sequence (genomic or cDNA) can be inserted in such a fashion as to place itself under the genetic control of these two regions. Unique

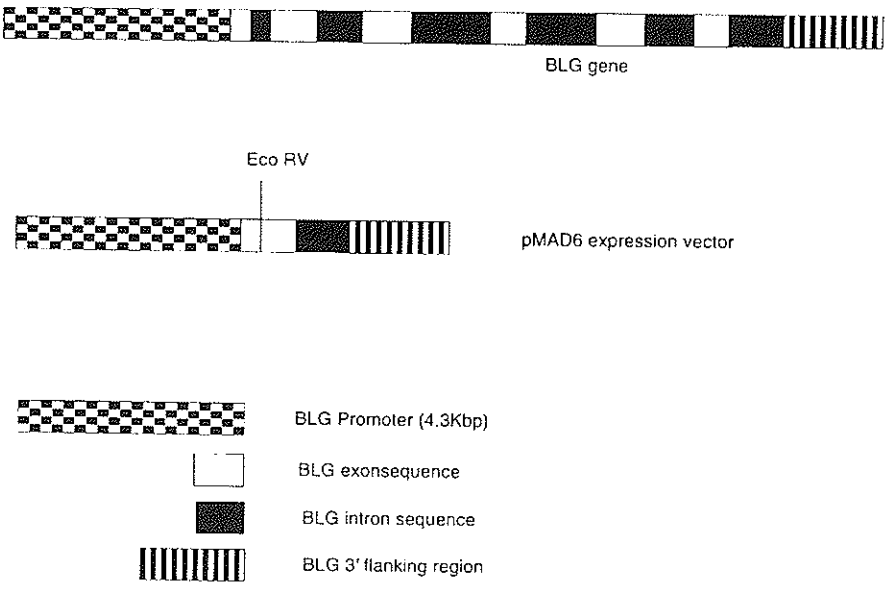

Figure 1. Schematic representation of the BLG gene and expression vector.

restriction sites at the extreme 5' and 3' ends of the hybrid gene are used to excise away the bacterial vector sequences. In our hands, the free transgene is purified from the other plasmid sequences by sucrose density gradient, rate zonal centrifugation. Prior to injection the sucrose is removed and the buffer exchanged to one compatible with transgenesis.

Some limitations in the expression system

The highest levels of expression of heterologous proteins in livestock milk are usually the result of fusing the BLG promoter region to human genomic sequences (Wright *et al.*, 1991). Analogous cDNA based constructs were poorly expressed in transgenic mice (Whitelaw *et al.*, 1991). Despite some notable exceptions in the field as a whole (Ebert *et al.*, 1991; Velander *et al.*, 1992; Brem *et al.*, 1994; Strömqvist *et al.*, 1996) the general inefficient expression of cDNA-based constructs is well documented (Brinster *et al.*, 1988; Palmiter *et al.*, 1991; Whitelaw *et al.*, 1992). Observed problems include the influence of chromosomal position effects and distinct spatial and/or temporal expression in lines transgenic for the same construct. Such constructs can be improved by the addition of some natural or heterologous introns (Palmiter *et al.*, 1988; Choi *et al.*, 1991). However, expression levels from such constructs rarely match levels attained with constructs containing some or all natural introns in the region encoding a heterologous protein. The genetic material encoding many potential target human proteins that may be produced by the transgenic mammary gland is very often, due to availability or the size of the natural gene, limited to cDNAs. As such, a technique giving more consistent expression from cDNA based, intronless constructs is highly desirable.

One solution to the expression of cDNAs is, so-called, 'rescue' technology; an approach developed by Clark and co-workers (Clark *et al.*, 1992) to overcome cDNA-related expression problems. It makes use of the observation that co-injection of the entire ovine BLG gene, together with an intronless construct, results in the expression of the second construct where no expression is achieved when it is injected alone. Clark and colleagues have demonstrated the expression of up to 800 μg/ml of human alpha-1antitrypsin (AAT) in the milk of mice transgenic for both BLG and an intronless human AAT construct. In mice transgenic for the latter construct alone, only one out of eight mice expressed and this at a level of only 3.9 μg/ml. Similarly, using this technology, we have achieved an expression level of 108μg/ml from a human protein C cDNA construct, this represents ~20% the expression level obtained with an equivalent genomic-based construct. The cDNA construct alone gave no expression in 11 lines of transgenic mice (PPL Therapeutics, unpublished data).

The 'rescue' phenomenon has been rationalized as follows. In most cases, co-injected genes integrate at the same chromosomal location. Clark and colleagues propose that this probably results in an open chromatin conformation associated with the actively expressing BLG gene which encompasses adjacent intronless genes. The BLG gene may thus create a permissive domain allowing access to the intronless genes by the transcriptional machinery of the cell. In the absence of adjacent BLG genes, the intronless construct may be inaccessible, probably residing in condensed chromatin. Other possible explanations for this phenomenon include enhancer-like sequences present in the BLG gene but absent from the intronless construct interacting positively with the latter or simply that the BLG gene insulates the intronless gene from the negative effects of adjacent chromatin.

A second limiting phenomenon in attaining high expressing transgenic lines for commercial production is the so-called 'position effect'. Current dogma proposes that transcription in the mammalian genome is coordinated at different levels. Individual promoters can be controlled via specific transcription factors as described above, however, there is superimposed upon this a more global level of control whereby accessability of the gene is determined. Thus, there are permissive and non-permissive states for gene activation (Dillon, 1993). The absolute levels of transgene expression in a transgenic animal will be largely governed by its site of integration into the genome. If integration occurs in a permissive region, expression will be high, if into a nonpermissive region, these strong milk gene promoters will simply not be accessible to the trans-acting factors in the gland (Dillon, 1993).

Unfortunately, there is no control over the site of integration of a microinjected gene. Therefore, the generation of a high expressing line is basically a numbers game. In mice, stem cell technology allows the precise positioning of new genetic material in the genome (Bradley, 1991; Hooper, 1992), however, such techniques are not available for livestock. The ability to generate live animals by nuclear transfer from cells grown in culture, in principle, opens the door to such manipulations in the sheep and cow.

The existence of locus control elements (LCRs) which may mediate such global control, has been proposed, most convincingly in the β-globin gene cluster (Grosveld *et al.*, 1987). We, and others, are searching for mammary specific LCRs in and around milk protein genes. The identification of small, portable, pieces of DNA with LCR activity would be a great help in reducing the expense of generating transgenic livestock.

Purification of human proteins from milk

There are two principal components of milk which distinguish it from conventional fermenter media, fat and casein micelles. Different species have different levels of fat in their milk. Sheep usually have around 50–70 g/l whilst cows have about 40 g/l (Barth and Schlimme, 1989; Jensen, 1995). Most of the fat in milk is present as a colloidal dispersion of lipid globules which will float as cream. The purification of a recombinant protein from milk is therefore initiated by using a skimming centrifuge (Wright and Colman, 1996). This also has the advantage of removing cell debris and bacteria as a solid pellet and improved the storage properties of the milk if frozen. Skimming removes 95–98% of the milk lipid.

The natural tendency for casein micelles to aggregate, could cause considerable problems for chromatography steps employed later in the process, therefore, it is prudent to remove the caseins early on (Wright and Colman, 1996). Various options are available; however, the addition of precipitating agents such as polyethylene glycol, salts or alcohol are generally cheap and effective. The resulting fraction is equivalent to whey in the dairy industry.

Once a solution clear of lipid and casein is produced, subsequent purification is almost entirely dependent on the properties of the protein of interest.

Regulatory issues in producing biologics from milk

PPL has developed a strategy designed to address safety issues arising from the production of therapeutic agents in transgenic milk (Wright *et al.*, 1996).

The origin and health status of the production animals is rigorously determined. Our sheep are currently sourced from New Zealand which is recognized to be 'scrapie free'. These sheep are kept in strict quarantine on facilities with no adjacent stock farms and secure natural boundaries. Animals are continuously monitored for unusual events by highly trained and experienced staff. The sheep are given feeds free from mammalian derived products and, where possible, are allowed access to natural grazing. Nutritional practices are in line with those used in high-quality dairy flocks. Milking is performed on clinically healthy animals, by healthy operators, using automatic milking equipment similar to that employed in the bovine dairy industry. Various checks are put in place to prevent animals on medication entering the milking parlour. Natural variation in the volumes of milk and expression levels obtained from individual animals is minimized by blending milk from multiple sheep.

Two approaches have been taken to validate the safety of milk derived products and to address the potential for transmission of infectious agents from sheep to man. First, a study was performed whereby sheep were intentionally infected with different virus types and the levels of virus in the milk investigated (Wright *et al.*, 1996). In general, the levels of virus found in the milk were exceptionally low. Secondly, conventional process validation is performed. Infectious agents are purposely spiked into scaled-down versions of the purification process and the processes capacity to eliminate the agents is investigated. Depending on the particular combination of purification steps, many logs of clearance can be demonstrated. PPL has performed such a validation exercise for AAT, in particular to demonstrate the capability of the purification processes to remove spiked scrapie agent.

Conclusions

The commercial reality of producing recombinant protein therapeutics in transgenic livestock is going through its final stages of feasibility testing. Transgenic animals can be produced reasonably effectively by microinjection, much is now understood about achieving good levels of mammary specific expression and the major players have invested heavily in the infra-structure required to harvest and purify the proteins. Large numbers of sheep, goats and cows have been created which express economically viable levels of several proteins; some of these products are now in the clinic undergoing final safety and efficacy testing. With the first marketing approval of a protein derived from transgenic milk, this technology will take its place alongside bacteria, yeast and mammalian cell culture as a production alternative. In the meantime, major improvements in transgenic generation, such as by nuclear transfer, coupled with continuous improvements in expression control, make the technology a highly competitive challenger to fermenter-based methods.

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