The germline manipulation of livestock: progress during the past five years

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ABSTRACT

A number of techniques for directly manipulating the mammalian germline have been established in the mouse. During the past five years these have been applied to domestic livestock but, so far, only pronuclear microinjection has proved successful. The considerable resources required and the low efficiency of the procedure are major limitations and, worldwide, relatively few laboratories have produced transgenic livestock. In applying transgenic technology to farm animals, a major effort has gone into the manipulation of growth. This is a complex trait and progress has been constrained by the inability to control the expression of transgenes in a precise manner, and by the lack of basic knowledge at the interface between molecular biology and physiology. By contrast, the production of biomedical proteins from transgenic animals involves the manipulation of a relatively simple trait, milk composition. So far, only relatively low levels of protein production have been reported for transgenic sheep. However, very high levels of expression of human a1-antitrypsin have been obtained in transgenic mice, demonstrating the feasibility for this application of transgenic animals.

Keywords Germline; transgenic; mice; livestock; growth; biomedical proteins.

INTRODUCTION

In 1974 the feasibility of manipulating the mammalian germline was first demonstrated by Jaenisch and Mintz who injected SV40 DNA into the blastocoel cavity of mouse embryos and showed that it became incorporated into the genome of the adult mice. During the 1980's the techniques for manipulating the germline of mice were established in many laboratories and used to address a variety of fundamental biological questions (for reviews, see Palmiter and Brinster, 1986; Jaenisch, 1988). Subsequently, the techniques developed in the mouse were applied to domestic livestock, and in this paper we discuss the progress that has been made during the past five years both with regard to the technology and its exploitation.

GERMLINE MANIPULATION TECHNOLOGY

Four techniques for the manipulation of the mammalian germline have been described; pro-nuclear injection (Gordon *et al.*, 1980), retroviral-mediated gene insertion (Jaehner *et al.*, 1985), embryonic stem (ES) cell colonisation of the embryo (Robertson *et al.*,1986) and sperm-mediated genetic transformation (Lavitrano *et al.*, 1989). All four of these approaches have been developed in mice and, subsequently, applied to domestic livestock. The details of these techniques have been described elsewhere (for reviews see Clark, 1988; Pinkert *et al.*, 1989; Wilmut *et al.*, 1990).

Pronuclear injection is the most commonly used procedure for the generation of transgenic mice and is, still, the only proven route for gene transfer in domestic livestock (Figure 1). However, there are a number of factors involved in working with large animals that make the use of this technique more difficult than with mice. In general, fewer eggs are available, they are more susceptible to damage during the microinjection procedure, there is greater variation in the stages of the embryo that are recovered, and the granular nature of the egg cytoplasm can complicate visualisation of the pronuclei. Additionally, in sheep and cattle, litter size is limited to one or two and, therefore, large numbers of recipient animals have to be employed. Finally, the

TABLE 1 Summary of gene transfer experiments carried out in pigs

Construct	Embryos Injected and Transferred	Births	Transgenics (%)	Reference
MThGH	2035	192	20 (0.98)	Hammer *et al*, 1985a
MThGH	268	15	1 (0.37)	Brem *et al.*, 1986
MLVrGH	170	15	1 (0.59)	Ebert *et al.*, 1988
PEPCKbGH	1057	94	2 (0.18)	Wieghart *et al.*, 1988
MTpGH	423	17	6 (1.4)	Vize *et al.*, 1988
MThGRF	1041	-	6 (0.58)	Brem *et al.*, 1988
MTMx	1083	-	6 (0.55)	Brem *et al.*, 1988
MTbGH	2330	150	9 (0.39)	Miller *et al.*,1989
MThIGF-I	387	34	4 (1.0)	Pursel *et al.*, 1989
MThGRF	2236	177	7 (0.31)	Pursel *et al.*, 1989
PRLbGH	289	20	5 (1.7)	Polge *et al.*, 1989
Total	**11,319**		**67 (0.59)**	
SNV	222	(21)	17	Petters *et al.*, 1989*

* Retroviral-mediated gene transfer. Cells producing spleen necrosis virus (SNV) injected into the blastocoel of early embryos. Analysis was carried out by PCR on 42-day old foetuses.

logistics of working with large animals, and the relatively long time scales involved, impose considerable demands upon resources.

FIG 1 Microinjection of a fertilised sheep egg.

The egg is held by gentle suction on the holding pipette (left) and readied for injection with the micro-pipette (right). The two pronuclei are clearly visible in the centre of the egg.

It is, therefore, not too surprising that only a few research groups have described the production of transgenic livestock. Using the available data, details of the transgenic pigs, sheep and cattle that have been produced during the last five year are given in Tables 1-3. Most effort has gone into the production of transgenic pigs and, worldwide, more than 11,000 eggs have been injected and transferred with an average frequency of success (eggs injected and transferred/transgenic animals) of 0.59%. A similar frequency of success (0.74%) has been obtained with the ≈4,500 sheep eggs used in gene transfer experiments. These frequencies compare poorly to the ≈5% that can be obtained with mice (Brinster *et al.*, 1985), further emphasising that these experiments should not be entered into lightly. In cattle (Table III), only a few reports of successful gene transfer have been published. Many of these have involved assessment of the transgenic status of either early embryos or foetuses and so it is not yet possible to make a reliable assessment of the efficiency of producing live transgenic animals. However, it would seem unlikely that success rates substantially higher than those observed with pigs and sheep will be obtained.

TABLE 2 Summary of gene transfer experiments carried out in sheep

Construct	Embryos Injected and Transferred	Births	Transgenics (%)	Reference
MThGH	1032	73	1 (0.1)	Hammer *et al.*, 1985a
MTbGH	711	38	2 (0.28)	Pursel *et al.*, 1987
oMToGH5	1079	81	4 (0.37)	Murray *et al.*, 1989
oMToGH9	409	23	3 (0.73)	Murray *et al.*, 1989
TFbGH	247	33	7 (2.8)	Rexroad *et al.*, 1988
MThGRF	435	63	9 (2.0)	Rexroad and Pursel, 1988
pMK	108	29	1 (0.9)	Simons *et al.*, 1988
BLG-FIX	245	52	4 (1.6)	Simons *et al.*, 1988
BLG-AAT	298	47	3 (1.0)	Clark *et al.*, 1988
Total	**4,564**		**34 (0.74)**	
FeLV	44	(17)	2	Hettle *et al.*, 1989*

* Retroviral-mediated gene transfer. Fertilised eggs injected with wild-type feline leukaemia virus (FeLV). DNA analysis carried out on 50-day old foetuses.

TABLE 3 Summary of gene transfer experiments carried out in cattle.

Construct	Embryos Injected and Transferred	Births	Transgenics (%)	Reference
a-FP	237	111	4 (1.6)[a]	Church *et al.*, 1986
RSVCAT	175	79	4 (2.2)[b]	Biery *et al.*, 1988
pBPV	156	24	5 (3.2)[c]	Roschlau *et al.*, 1989
MMTV-ADH	130	20	9 (0.69)[a]	Roschlau *et al.*, 1989
MTbGH	250	15	2 (0.8)[d]	Roschlau *et al.*, 1989

[a] DNA analysis performed on embryos

[b] DNA analysis carried out on 60-day old foetuses

[c] 1 dead foetus

[d] 1 live animal

The low efficiency of generating transgenic livestock by pronuclear injection has prompted a number of approaches aimed at improving the procedure. One major limitation is the relatively small number of eggs available for injection and transfer. Developments in embryology have shown that viable embryos can be obtained by the *in vitro* fertilsation of ova dissected out from the ovaries of slaughtered animals and subsequently matured *in vitro* (Lu *et al.*, 1987; Fukui *et al.*,1988). This has the potential to provide large numbers of fertilised eggs for injection. The relatively low viability of embryos produced in this manner (Lu *et al.*,1988), even prior to microinjection, limits the utility of this approach at present. However, improvements to *in vitro* embryo culture may be forthcoming, particularly in cattle where a number of research groups are actively engaged, and this may ultimately prove to be a successful approach.

It may also be possible to screen for the transgenic status of embryos developing from injected eggs at the morula or blastocyst stage. Pre-implantation embryos can be cultured *in vitro* or in temporary recipients and a few cells removed to analyse for the presence of the transgene by the polymerase chain reaction (Siaki *et al.*, 1985), prior to transfer to the final recipient. However, the decrease in viability of embryos treated in this manner may limit the usefulness of the approach. Furthermore, the persistence of unintegrated DNA in these early embryos may lead to an unacceptably high frequency of false positives.

Given the limited success that has been obtained with pronuclear injection, it is, perhaps, not surprising that attempts have been made to apply other techniques for germline manipulation to domestic livestock. Retroviral vectors have been used to generate transgenic mice (Jaehner *et al.*, 1985). Because of problems that may arise with the mobilisation of retroviral vectors by, for example, recombination with wild-type viruses, self-inactivating (SIN) vectors capable of incorporating foreign DNA into the host chromosome but unable to further replicate have been developed . However, substantial problems still remain. Firstly, the production of recombinant retroviruses with suitable titres from packaging cell lines is technically difficult. Secondly, during embryonic development the zona-pellucida forms an effective boundary and so must be removed (alternatively, viral concentrates or producer cells can be injected under the zona). Thirdly, the size of foreign DNA that can be carried by a retroviral vector is limited and it would not be possible to introduce segments of DNA longer than about 8 kb. Finally, the technique usually creates a mosaic animal animal in which only a fraction of the animal's cells carry the foreign DNA; as a consequence the frequency of germline transformation is reduced. These problems have limited the usefulness of the approach and, even in mice, it has not been commonly employed. Nevertheless, there are reports of the use of retroviruses in both pigs (Petters *et al.*, 1988) and sheep (Hettle *et al.*, 1989), although it should be noted that neither of these reports provide evidence for integration of the DNA into the germline.

The isolation of ES cells from mice was first described by Evans and Kauffman (1981), Bradley *et al.* (1984) used them to colonise a host embryo and contribute to the germline by and Robertson *et al.* (1986) used them for the introduction of foreign DNA sequences to the germline by this route. In mice, ES cells offer little advantage over pronuclear injection for the introduction of foreign DNA sequences into the germline. However, in farm animals where major limitations include the supply of embryos and the low success rates obtained by pronclear injection, this route has many attractions for the introduction of new DNA sequences. The appropriate transformed lines could be established, characterised and stored prior to blastocoel injection. Furthermore, since blastula stage embryos can be recovered non-surgically from cattle, the use of ES cell would obviate the need for surgery in this species. To date, there are no published data for the successful generation of ES cells in farm animals although attempts have been reported (Handyside *et al.*, 1987). Nevertheless some progress has been made and, for example, ES-like cells have been established from pig and sheep embryos and maintained in culture for several generations (E. Notariani, unpublished observations.) It seems likely that ES cells from farm animls will be available within the next few years.

The development of ES cells will facilitate gene transfer in domestic livestock. However, more importantly, the use of this approach for germline manipulation enables the targeted mutation of specific endogenous genes (for review, see Capecchi, 1989) . The technology is now, more or less, established in the mouse, and at least nine different genes in the germline have been specifically targeted. At present, these approaches have concentrated on the deletion of specific genes, but methods of targeting more subtle changes e.g. altering specific regulatory regions of a gene are also being developed. In mice, at least we are now in a position to precisely modify the germline. Given the availability of suitable ES lines, this technology will be directly applicable to domestic livestock.

Finally, it was reported recently that transgenic mice could be produced efficiently by mixing DNA with spermatozoa which were then used to fertilise eggs *in vitro* (Lavitrano *et al.*, 1989). Despite attempts by ourselves and other experienced transgenic mouse workers this startling result has not been repeated, even when the very same reagents were used in the experiment (Brinster *et al.*, 1989). Surprisingly, or perhaps not surprisingly, these same workers have also reported that this technique has been used successfully to generate transgenic pigs (Gandolfi *et al.*, 1989).

In summary the last five years have seen the

establishment of gene transfer technology in domestic livestock. The only proven route is pronuclear injection. The frequency of success is low (less than 1%) and, although there are some possible improvements to the methodology, it is not yet clear how useful these will prove. ES cells have not yet been described for livestock, although there is hope that they may be available within the next few years; if so, it can be expected that this approach will be widely adopted and supercede pronuclear injection, particularly, since it can be used to target specific changes in the germline.

MANIPULATING GROWTH IN TRANSGENIC LIVESTOCK

Many of the complex biological processes determining production traits such as growth rate, milk production or fertility are regulated by protein hormones. These mediate their effect by binding to specific receptors on the cell membrane. The genes encoding many of these hormones and more recently their receptors have been cloned. During the last five years considerable effort has gone into attempts to manipulate growth and related characteristics, such as feed efficiency, by exploiting gene transfer techniques in domestic livestock. Thus, of the 67 transgenic pigs and 34 transgenic sheep produced by microinjection listed in Tables 1 and 2, 61 and 26, respectively, were carrying transgenes designed to manipulate growth physiology.

The attempts to manipulate growth, stem from earlier work on transgenic mice that carried and expressed either rat growth hormone (rGH) or human growth hormone genes (hGH) (Palmiter *et al.*, 1982; 1983). In these experiments the GH genes were fused to the regulatory DNA sequences (the enhancer/promoter region) derived from the mouse metallothionein (MT) gene. MT is expressed primarily in the liver and this proved to be the major site of synthesis of the foreign growth hormone gene. The mice had very high serum levels of the foreign growth hormone and, consequently, grew to as much as twice the normal size. The growth rate was also considerably enhanced during maximum growth phase and some transgenic mice grew four time faster than the controls (Hammer *et al.*, 1984). A crucial factor in these experiments was the use of the MT enhancer/promoter to regulate the GH genes, since transgenic mice carrying GH genes under the control of their own regulatory elements do not grow any faster (Wagner *et al.*, 1983). This is because synthesis and release of GH is under tight feedback control in the cells of the anterior pituitary where it is normally synthesised and released. By using the MT enhancer/promoter to control expression, the foreign GH gene was released from the tight feedback control, thus allowing high levels of GH to be produced in the serum. Not surprisingly, the synthesis of endogenous GH is dramatically suppressed in these transgenic animals.

MThGH and MTbGH transgenic pigs have been produced in a number of laboratories and high levels of exogenous GH demonstrated in the serum of these animals (up to 1 μg/ml). However, the dramatic effects on growth obtained with transgenic mice were not observed, emphasising that caution should be exercised in the extrapolation of results from one species to another. In some cases a growth increase was observed in founder animals, but such results on single animals must be treated cautiously, because wide variations in daily weight gains, as much as 30%, are seen in both controls and transgenics (Pursel *et al.*, 1989).

In a systematic study Pursel and his colleagues (Pursel *et al.*, 1989) have measured average daily weight gain and feed efficiency in two generations of transgenic and control pigs. In two lines of transgenic pigs animals were shown to grow 11% and 14% faster, respectively, than their non-transgenic siblings, but only when the animals were placed on a protein-enriched diet. One of the limitations to increasing the growth rate appears to be that high circulating levels of growth hormone supress the appetite, thus limiting the availabilty of nutrients and, hence, the maximum potential for growth rate. In addition to increases in growth rate, as measured by daily weight gain, a 16-18% increase in feed efficiency was also observed, similar to the increases observed with pigs that have been injected with exogenous growth hormone. Expression of exogenous growth hormone dramatically reduced the carcase fat content, and mean back fat thickness was reduced nearly three times (7.5 mm vs 21 mm) in transgenic pigs as compared to controls.

Although modest improvements in growth performance were observed in transgenic pigs a number of considerable deleterious side effects were also observed, that limited the economic utility of these animals. The most common problems included lethargy, joint abnormalities and exophthalmos (Pursel *et al.*, 1989). In many cases the founder animals did not survive

beyond one year of age. Gilts were found to be anestrus and boars lacked libido. Similar adverse affects were observed in the transgenic mice carrying growth hormone genes although they were not as severely pronounced. However, in particular, female mice expressing rGH and bGH are commonly infertile, due to an an inhibition of the release of prolactin after mating (Bartke *et al.*, 1988).

Experiments with MTGH genes have also been carried out with sheep (Table 2). In general, the results have been similar to those observed for the transgenic pigs. In one set of experiments high levels of growth hormone (0.9 - 2 mg/ml) were present in serum at 20 weeks of age (Nancarrow *et al.*, 1988). No significant enhancement of growth was observed and these animals also suffered from deleterious side effects, such as joint abnormalities. As with the pigs, these transgenic sheep also exhibited a dramatically reduced carcase fat content.

Many of the adverse side effects seen in transgenic animals carrying MTGH constructs seem to stem from the chronic expression of exogenous growth hormone from the MT enhancer/promoter. Although inducible by glucocorticoids and heavy metals, the MT promoter also has a significant basal level of expression that directs the synthesis of GH without the addition of these inducing agents. In experiments carrried out by Kevin Ward and his colleagues a sheep metallothionein promoter linked to the sheep growth hormone (oGH) gene was used. Interestingly, this construct was shown to be highly inducible in transgenic mice, and when zinc was added to the drinking water intense expression was induced in the kidney, intestine and lung (Nancarrow *et al.*, 1988) and the mice were 19-35% heavier at 62 days of age. However, in contrast to the results in mice, this construct was not inducible when introduced into sheep and high constitutive levels of oGH were present from birth. This, again, underlines the dangers of extrapolating results obtained in mice to other species.

In order to circumvent the problems associated with chronic expression of MTGH transgenes, the construction and testing of other inducible GH constructs has been attempted. Phosphoenolpyruvate carboxykinase (PEPCK) is a crucial enzyme in the regulation of gluconeogenesis. PEPCK regulatory sequences have been shown to act as a strong promoter and to be regulated by alteration of dietary carbohydrate or protein, as well as cAMP administration (McGrane *et al.*, 1988). Transgenic mice have been produced carrying the PEPCK promoter fused to bGH. On a low carbohydrate/high protein intake a 30 fold increase in serum bGH was observed and conversely, a high carbohydrate intake resulted in a 90% decrease of serum bGH. However, as for MT regulatory sequences, constitutive expression of GH was also seen. PEPCKGH has also been used in the construction of transgenic pigs. However, the expression pattern of the transgene and, consequently, the phenotype was very similar to that observed with MTGH. Founder pigs expressed the transgene in concentrations ranging from 0.1 μg/ml to in excess of 100 μg/ml and no affect of dietary manipulation was observed on levels of expression (Pinkert *et al.*,1990).

An interesting attempt to control exogenous GH expression has been attempted by using regulatory sequences derived from the prolactin gene. A prolactin bGH construct has been introduced into pigs (Polge *et al.*, 1989). Five transgenic founders were produced. No expression of the transgene was observed in the these animals under normal conditions, and consequently there were no ill effects. However bGH expression could be induced either by the external administration of thyroid stimulating hormone or sulpiride. A pulse of exogenous GH was produced as a result of the administration of these agents. Although, as yet, no effects on growth rate have been observed in these animals, the fact that the release of growth hormone was regulated externally opens up the possibility of controlling the pattern of secretion of GH which will be clearly essential for enhancing growth optimally.

The gene encoding growth hormone releasing factor (GRF), the hypothalamic hormone which controls the synthesis and release of GH from the anterior pituitary has also been introduced into transgenic animals under the control of MT regulatory elements (Hammer *et al.*, 1985b). Transgenic mice expressing detectable levels of MThGRF and exhibiting elevated levels of mGH and showed accelerated growth rates (up to 1.5 times the normal rate) and, in contrast to the results with MTGH, the females proved to be fertile. Transgenic pigs carrying MThGRF have also been produced (Table 1), and two out of the seven founders that were produced were shown to have high concentrations of hGRF in their plasma (Pursel *et al.*, 1989). However, despite the high levels of human hGRF in

these animals no elevation of pGH was observed. Since the administration of synthetic hGRF to pigs does, indeed, stimulate growth hormone release in pigs (Kraft *et al.*, 1985), the absence of an effect in expressing transgenic pigs may be due to the inability of pig cells to correctly process the GRF precursor peptide (Pursel *et al.*, 1989).

Another approach for the manipulation of growth in transgenic animals has been to use sequences encoding insulin-like growth factor I (IGF-I) through which, it is believed, many of the somatogenic effects of GH are mediated. One possibility of using IGF-I directly as opposed to GH was that, whereas somatogenesis would still be stimulated, many of the adverse consequences caused by GH (and presumed not to be mediated by IGF-I) would be lost. MThIGF1 transgenic mice have been produced (Matthews *et al.*, 1988). These mice expressed elevated levels of serum IGF-I and grew significantly larger with an apparent reduction in the plasma GH levels. Furthermore, in a comparison to the GH transgenic mice they showed very little of the pathology normally associated with GH over-expression. However, some of the positive effects of GH were also absent, including increased skeletal growth which may possibly be due to a synergism between GH and IGF-I. Transgenic pigs have been produced containing MThIGF-I. Only one of the four founders expressed elevated levels of plasma IGF-I but died before a detail analysis of its growth performance could be carried out (Pursel *et al.*, 1989).

Overall considerable effort has gone into attempts to manipulate the growth physiology of pigs and sheep using gene transfer techniques. Many experiments have involved the introduction of MTGH constructs. However, as a consequence of the high chronic levels of GH expression a number of severe side-effects have been observed in the transgenic animals. Attempts have been made at manipulating growth by using IGFI or GRF. Although apparently successful in mice, these results have not been repeated in farm animals. Finally, workers have tried to develop inducible transgenes so that the expression of GH can be externally regulated. So far, this approach has not met with much success in domestic livestock, at least in terms of producing animals with enhanced growth performance.

TRANSGENIC ANIMALS FOR THE PRODUCTION OF PHARMACEUTICAL PROTEINS.

Attempts to manipulate production traits such as growth rate and feed efficiency by gene transfer will have to compete with the genetic gains that can be accomplished by conventional breeding. To be useful a transgene must confer a large genetic gain (considerably larger than can be obtained by conventional breeding), yet not be offset by deleterious side-effects. As a general point, any potentially useful transgenic stock will require considerable evaluation before release into the gene pool (Smith *et al.*, 1987). Not only will it be necessary to test for possible deleterious effects, but also it will be important to evaluate the performance of a transgene on different genetic backgounds; it is becoming clear that such background effects may have dramatic effects on transgene function (Allen *et al.*, 1990; M.McClenaghan, unpublished observations). Such evaluation programmes are likely to prove extremely costly and will take a considerable amount of time, during which genetic gains will still accrue in conventionally bred livestock.

Gene transfer has opened up the possibilities of producing genetically engineered animals with entirely novel traits. One example of this is the utilisation of farm animals for the production of valuable proteins such as human therapeutics (Lathe *et al.*, 1986; Clark *et al.*, 1987). Using transgenic animals in this manner removes the need to compare performance with conventionally bred animals and they will, by and large, be kept separate from the gene pool.

The DNA sequences for a wide variety of biomedical proteins have been cloned and characterised during the last decade. Expression of these genes in bacteria or yeast is not an option for the production of many eukaryotic proteins since these micro-organisms do not carry out the post-translational modifications required for biological activity or stability. Production in mammalian cell culture is an option into which a considerable amount of research and development effort has been invested. However, commercial scale fermentation of mammalian cells is expensive and requires an input of sophisticated technology. We have argued that if sufficient levels of expression of biologi-

cally active proteins can be attained, and if suitable downstream processing developed, then animal bioreactors will be a cost effective option for the production of some, but not necessarily all, human biomedical proteins (Lathe *et al.*, 1986; Clark *et al.*, 1987).

Many therapeutic proteins are secreted into body fluids and, indeed, it is during the process of secretion from the cell that the important, and often essential, post-translational modifications take place. Directing expression to the liver and secretion into blood is a possible route for the production of proteins from transgenic animals. Choo *et al.*, (1985) have described transgenic mice that carry a MT human factor IX (FIX) gene that secrete significant levels of human FIX into the blood. However, many human biomedical proteins are themselves plasma proteins and it may prove difficult to purify them from the equivalent plasma proteins of the animal. Furthermore, the high circulating levels of a biologically active protein that will be necessary for efficient production may not be compatible with the health of the producer animal. An attractive option is to to target the expression of foreign proteins to the mammary gland. During lactation, this organ synthesises and secretes large amounts of protein into the milk which can be repeatedly harvested. Milk protein genes are single-copy and capable of very high levels of expression. For example, in a single cow during a single lactation, the αS1 casein gene directs the synthesis of about 60 kg of protein. Even in a smaller dairy species such as the sheep the levels of protein production are high, e.g. up to 3kg/sheep/lactation for αS1 casein. The aim has been to utilise the large biosynthetic capacity of milk protein genes in the mammary gland of dairy animals for the production of biomedical proteins. This has involved the isolation of regulatory DNA sequences from milk protein genes that direct high levels of expression in the mammary gland, the construction of fusion genes in which these sequences are used to drive expression of DNA sequences encoding the protein(s) of choice and the generation of the appropriate transgenic animals.

A number of milk protein genes have been cloned and evaluated for their capability of expression in the mammary gland (Table 4), including the rat whey acidic protein (WAP) gene (Rosen *et al.*, 1989), rat ß-casein (Lee *et al.*, 1987), bovine ß-lactalbumin (Vilotte *et al.*, 1990) and ovine ß-lactoglobulin (Simons *et al.*, 1987). Because of the expense and time-scale of working with domestic animals, transgenic mice have often been used as a model system. Even so such experiments are relatively long term, taking a minimum of half a year between egg injection and the analysis of expression in generation 1 (G1) lactating females. Bearing in mind that each experiment should involve an analysis of a number of independently derived lines of transgenic mice to assess transgene function, then each experiment becomes quite a considerable undertaking. However, in the absence of suitable mammary-gland cell lines that can be used to reliably assess the expression of introduced milk protein genes, transgenic mice are still, for the present, the best model system.

In our laboratory in Edinburgh we have been developing the ovine ß-lactoglobulin gene (BLG) for the purpose of targeting expression to the mammary gland. BLG is the major whey protein in ruminant species and is expressed abundantly and specifically in the mammary gland during pregnancy and lactation. There is no equivalent gene in rodent species. The gene comprises seven exons which encompass 4.9 kb of the chromosomal DNA (Ali and Clark, 1988). This gene and associated flanking regions was used to generate transgenic mice. The results were very encouraging and a number of lines of transgenic mice were produced which expressed very high levels of the sheep protein in their milk (Simons *et al.*, 1987). Levels as high as 23 mg/ml of the foreign protein were obtained. Analysis of the distribution of RNA in a variety of tissues showed that the expression of the gene was specific to the mammary gland. Indeed, *in situ* immunostaining showed that the expression BLG was entirely specific to one cell type within the mammary gland, the secretory epithelia.

Two human plasma proteins were chosen as candidate products for expression in the mammary gland. Factor IX (FIX), the zymogen of a serine protease is used routinely for the treatment of haemophila B, a blood clotting disorder which afflicts about 1 in 30,000 males in the population (Brownlee, 1987). α_1-antitrypsin (α_1AT) is a serine protease inhibitor, whose major substrate is neutrophil elastase. It has considerable potential for the treatment of emphysema, for which it has been employed in clinical trials, a common respiratory disease, especially among smokers.

cDNA sequences encoding these two proteins

TABLE 4 Targeting the expression of proteins to the mammary gland in transgenic animals.

Animal	Gene Construct	Milk Conc.[a]	Reference
Mouse	oBLG	23 mg/ml	Simons *et al.*, 1987
Mouse	ba-lac	0.45 mg/ml	Vilotte *et al.*,1989
Mouse	rb-casein	-	Lee *et al.*, 1988[b]
Mouse	rWAP	_	Rosen *et al.*, 1989[c]
Mouse	mWAP-htPA	50 ug/ml	Pittius *et al.*,1988
Mouse	mWAP-hPS2	1.5 ug/ml	Tomasetto *et al.*, 1989
Mouse	mWAP-hCD4	0.2 ug/ml	Yu *et al.*, 1989
Mouse	oBLG-ha1AT	7.2 mg/ml	Archibald *et al.*, 1990
Rabbit	bCAS-hIL2	0.43 ug/ml	Buhler *et al.*, 1989
Sheep	BLG-hFIX	0.025 ug/ml	Clark *et al.*, 1989

[a] *maximum published levels*

[b] no protein data available; maximum mRNA 1% of endogenous b-casein

[c] no protein data available; maximum mRNA 88% of endogenous WAP

were fused to the BLG sequences that had functioned so effectively in the transgenic mice. A variety of different construct designs have now been tested in transgenic mice. In general, most of these constructs have not performed particularly well and only relatively low levels of expression have been obtained (Whitelaw *et al.*, in preparation).

In parallel with the experiments on mice, transgenic sheep carrying either BLG-FIX or BLG-AAT constructs were produced. Four founder transgenic sheep carrying a BLG-FIX fusion gene, in which the FIX cDNA was inserted into first exon of the BLG gene were generated (Simons *et al.*, 1987). All four of these animals have now transmitted the transgene to their progeny and lines have been established. Two of the founders were female and after birth of their first lambs were shown to express human FIX mRNA in the mammary gland and secrete the corresponding protein into their milk (Figure 2). However, the levels of the human protein were low (about 1/250th of that found in human plasma). Nevertheless, after monoclonal antibody purification the FIX was shown to have biological activity, indicating that the mammary gland is capable of performing the post-translational modifications required for function of this protein (Clark *et al.*, 1989). Three founder sheep carrying a similar BLG-AAT fusion gene were produced. Two of the three were shown to express human AAT in their milk but, again, at relatively low levels.

Other workers have also been successful in directing the expression of foreign proteins into the milk of transgenic animals. Thus, Gordon *et al.* (1987) and Pittius *et al.* (1989) have described the expression of human tissue plasminogen activator (a potent anti-thrombolytic agent) in the milk of transgenic mice. These workers used regulatory elements derived from the gene encoding the mouse whey acidic protein (WAP) to target expression to the mammary gland. In general the levels of expression observed in the transgenic lines were relatively low. Yu *et al.* (1990) have also used 5' elements from the WAP gene to drive the expression of human CD4 (an HIV receptor molecule with potential for the treatment of AIDS) in transgenic mice. In these experiments the production of functional, soluble CD4 was described, although again the levels of expression were low. The use of transgenic rabbits for the production of human proteins has also been described (Buhler *et al.*, 1990). Rabbits have an advantage over larger animals in that they have a favourable reproductive performance in terms of generation time and litter size. They can produce significant amounts of milk proteins (≈0.5 kg/animal/lactation) although the overall levels of protein production are still considerably below those attainable with dairy cows (≈240 kg/animal/lactation) or dairy sheep (≈12 kg/animal/lactation). Nevertheless, it may prove economical to use rabbits for the production of

some proteins. In the rabbit experiments 2kb of 5' flanking sequences from rabbit ß-casein were fused to the structural gene encoding human interleukin 2 (IL2), a potent cytokine. Only low levels of expression were obtained. In this case, a possible reason for the low levels of expression may be the absence of essential cis sequence elements required for expression in the mammary gland. Thus, Lee *et al.* (1987) have introduced the entire rat ß-casein gene plus 3.5 kb of 5' flanking and 3.0 kb of 3' flanking sequences into mice, yet only observed expression at levels between 0.1-1 % of the endogenous ß-casein gene.

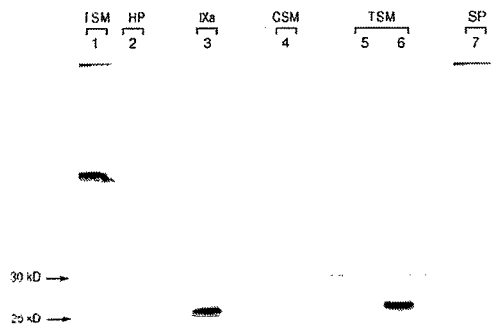

FIG 2 Detection of human FIX in transgenic sheep milk. Monoclonal antibody (MAb) purified human FIX from transgenic sheep milk was analysed by gel-electrophoresis under reducing conditions and Western blotting. In this experiment cleaved (lanes 5 & 6) and uncleaved (lane 1) preparations of sheep milk-derived FIX were co-electrophoresed with human plasma (lane 2), a preparation of activated (cleaved) human FIX (lane 3), a Mab-purified preparation of control sheep milk (lane 4) and sheep plasma (lane 7). The Western blot was reacted with a polyclonal antibody against human FIX. The 25 kD light chain and 30 kD heavy chain of human FIX, evident in the sample of activated human factor IX (lane 3), are also specifically detected in sheep milk-derived samples in lanes 5 and 6. Data from Clark *et al.*, 1989.

With the exception of the last experiment, all the attempts described above for targeting mammary expression used constructs comprising cDNA sequences encoding the protein of interest. There is a practical reason for using cDNA segments for the construction of fusion genes, as opposed to their genomic counterparts. This is because the presence of introns can make the *in vitro* manipulation of genomic clones very difficult. Thus, the segment of cDNA used in the construction of BLG-FIX fusion genes was 1.55 kb in length, whereas the gene that encodes human FIX is 34 kb in length. A segment of DNA of this length presents considerable difficulties in the practicalities of gene construction using conventional DNA manipulation techniques. The situation is even worse for other potential products; for example, the gene encoding human factor VIII (another essential blood-clotting factor) is about 180 kb in length!

Nevertheless, evidence is beginning to accumulate that the inclusion of at least some of the introns of a gene may be necessary for attaining efficient expression in transgenic animals (Brinster *et al.*, 1988; Whitelaw *et al.*; in preparation). A fusion gene comprising the 5' sequences of BLG fused to an α_1AT minigene (a genomic α_1AT DNA segment, minus the first intron) was introduced into transgenic mice. These mice expressed the gene construct relatively efficiently in the mammary gland (Archibald *et al.*, 1990). Indeed, a line of mice was produced that secreted 7mg/ml of human α_1AT into milk, representing a level of expression several hundredfold greater than had been previously reported for expression of this protein in mammalian cell culture. Investigation of the trypsin inhibitory properties of this milk-derived protein showed it to be as biologically active as α_1AT derived from human plasma. This fusion gene has now been injected into sheep eggs. If the transgenic sheep that are generated express the transgene at the same high levels, they will produce substantial amounts of human α_1AT (≈3 kg/sheep/lactation).

Given that high-expressing constructs for other proteins will soon be available, efficient methods for the purification of transgenic products from milk will have to be developed. Proteins destined for therapeutic administration to humans will have to be essentially free from any animal proteins and, as such, rigorously evaluated. It should be noted that, although special protocols may have to be developed for extraction from milk, the same requirements of purity will be encountered for the production of any recombinant-DNA derived proteins, if they are to be used therapeutically in humans. A number of milk protein genes have now been expressed efficiently in the mammary gland of transgenic mice. Fusion constructs designed to target the expression of biomedically important proteins have been constructed and shown to function to produce

biologically active proteins in transgenic mice, rabbits and sheep. In general, the levels of expression have been quite low except for one recent example in which human α_1AT was produced in mg/ml quantities in the milk of transgenic mice demonstrating that transgenic animals are a viable route for the production of recombinant proteins.

CONCLUSIONS

During the past five years gene transfer has been established in domestic livestock. Pronuclear injection is tedious, expensive and highly inefficient, yet still remains the only proven route for manipulating the germline of large animals. If ES cells can be developed in domestic livestock they will have a dramatic impact. Not only will the 'conventional' introduction of new genes be facilitated, but also it will be possible to target specific changes to the germline, such as gene-deletion or gene-replacement.

Two approaches to exploit gene transfer in domestic livestock, developed during the latter half of the eighties, have been described in this paper. As yet, attempts to manipulate growth physiology have not met with great success. A major problem has been that the chronic unregulated expression of transgenes, particularly those encoding GH, leads to deleterious side-effects. Certainly, there is a need to develop far more precise ways to regulate the expression of transgenes. As the understanding of the mechanisms that control gene expression improves, so will our ability to design transgenes with more appropriate patterns of expression. A particularly exciting prospect will be the ability to manipulate the expression patterns of endogenous genes using gene targeting techniques, should ES cells become available. However, there is clearly a need for a more fundamental understanding of the processes underlying complex production traits, such as growth, so that the most effective types of germline manipulation can be identified.

By contrast, generating transgenic animals to produce foreign proteins in their milk involves the manipulation of a relatively simple trait, milk composition. In this case the primary goal is to obtain high levels of expression in the milk and, as such, complex physiological side-effects are not anticipated. This has already been accomplished for α_1AT in mice and, although caution should be exercised in extrapolating to livestock, in this situation it seems reasonable to predict that high-yielding producer animals will soon be available. Nevertheless, substantial research will still be required to purify and assess the utility of the proteins produced by this route.

Advances have been made in the germline manipulation of livestock, although the rate of progress has been relatively slow when compared to the spectacular advances attained in the mouse. Furthermore, the mouse remains an important model system in which to develop approaches for exploiting this technology in domestic livestock. The technical and scientific advances and their exploitation are ultimately dependent upon basic scientific research and this will require sustained funding in areas such as embryo manipulation, the control of gene expression and physiology.

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