THE DEVELOPMENT OF SHEEP EXPRESSING GROWTH PROMOTING TRANSGENES

JAMES D. MURRAY

Animal Science
University of California,
Davis, CA 95616

CAIRD E. REXROAD JR.

USDA ARS Bare East
Building200, Room 13
10300 Baltimore Avenue
Beltsville, MD 20705

The first successful attempts to transfer foreign DNA into mice (Gordone, *et al,* 1980) were rapidly followed by a number of reports demonstrating that foreign DNA, or transgenes, can be permanently incorporated into an animal's genome (Costantini and Lacy, 1981; E. Wagner, *et al,* 1981; T. Wagner, *et al.,* 1981). Once incorporated, transgenes are stable and can be transmitted to an animal's progeny in the course of normal breeding.

The potential for the application of transgenic technology to alter animals used in agriculture was further indicated by Palmiter and his colleagues (1982,1983). These workers produced mice transgenic with a rat growth hormone (rGH) gene that was under the control of the mouse metallothionein I (MT) gene promoter, thus directing the production of GH in the liver rather than the anterior pituitary. The resulting transgenic mice that expressed this transgene grew to approximately 1.5 to 2 times as large as non-transgenic littermates. Thus, they established that the alteration of the pattern of expression of a gene, or the expression of a foreign gene, could result in a marked change in a quantitative trait like body growth. The control of growth, feed efficiency, reproduction, fat deposition and disease resistance are all economically important traits in animal agriculture.

At this point in 1982, laboratories at the USDA/ARS facility at Beltsville, Maryland (C.E.R. Jr.) and the CSIRO, Division of Animal Production in Sydney, Australia (J.D.M.) began experiments designed to transfer growth hormone transgenes into sheep. The goals of both groups were to determine 1—if growth hormone transgenic sheep were more feed efficient, i.e., produced more muscle per unit of food consumed, 2-contained less fat and 3-grew more rapidly than non-transgenic controls, as such modifications would be advantageous to producers. In this paper we will discuss the experimental work concerned with producing growth hormone trans-

genic sheep leading to the present, as well as our thoughts on the ethical and social considerations of this work.

ETHICAL IMPLICATIONS

Should we use genetic engineering? A frequently asked question is "Do we have the right to change the genetics of a species?" and each member of these research teams had to answer that question to his, or her, own satisfaction. As agricultural and basic research scientists, we realized that this question had already been answered thousands of years ago. Soon after mankind first domesticated plants and animals selective breeding began to be practiced, thus leading to man-made changes in the genomes of all domesticated species.

Wild cattle were bred to become Herefords, Angus and Holsteins. The original domesticated wild dogs have been selected for different purposes and bred until today there are a multitude of breeds. All of our domesticated animals, both those used in agriculture and the companion species, are now represented by a wide variety of breeds or types. The same is true for plants, although for plants mankind has gone much further. Using currently acceptable breeding techniques plant breeders have already produced entirely new species, such as the grain triticale, for use in agriculture.

Traditional breeding techniques rely on hybridization between breeds or species to yield genetic variation upon which we can impose selective breeding to fix desirable gene combinations. Essentially this involves manipulating and introducing changes into an animal's entire genome, containing perhaps as many as a hundred thousand genes. By comparison, genetic engineering involves introducing one or two characterized genes into one animal and assessing the effect of the expression of that gene on that animal and its immediate progeny. Thus, to us, transgenic technology is a more precise extension of the genetic manipulations characterized by selective breeding which farmers and agricultural scientists commonly practice.

TECHNICAL DEVELOPMENTS

Gene transfer in mice had been achieved by microinjecting a DNA containing solution directly into one of the two pronuclei of the zygote (for technical details see Hogan, *et al.*, 1986). In the mouse the optimal time for collecting pronuclear embryos suitable for microinjection is approxi-

mately 15 hours after fertilization. Following microinjection, mouse embryos are transferred immediately back into the oviduct of pseudopregnant females. Alternatively, microinjected mouse embryos can be cultured overnight to the two-cell stage and then transferred into a recipient.

Superovulation protocols were well worked out for the production and collection of sheep embryos around seven or eight days old, but protocols had to be developed for the collection of fertilized sheep eggs at the pronuclear stage. The techniques required for the transfer of day six to eight blastocysts to a recipient ewe's uterus were established, but again there was a need to develop the most optimal protocol for the transfer of day one embryos. Lastly, sheep zygotes had not previously been studied to determine if pronuclei were visible during the one-cell stage or, if they were visible, at what time were they optimum for microinjection. Thus, when work was started to produce the first transgenic sheep, there was considerable background information which needed to be established empirically before standardized protocols could be established.

The first transgenic sheep was reported in 1985 by Hammer, e *t al.* This single animal demonstrated that it was at least possible to transfer a foreign gene, in this case a mouse metallothionein human growth hormone (mMThGH) fusion gene, into sheep albeit at a very low efficiency. To date there are reports in the literature from three groups that have successfully, and routinely, produced transgenic sheep resulting in sheep carrying nine different transgenes (Table 1). However, as reviewed by Rexroad and Pursel (1988) the efficiency of producing genetically engineered sheep is still low, with only about 0.75 to 1 percent of the microinjected eggs transferred into recipient ewes resulting in the birth of transgenic lambs. An even lower percentage of embryos transferred result in lambs expressing the incorporated transgene, as not all transgenic animals express the new gene.

To reach this point essentially required a better understanding of the timing of the early development of the sheep embryo, rather than new technological breakthroughs. A treatment step requiring the administration of gonadotropin releasing hormone (GnRH) to the superovulation protocol was added to ensure that ovulation in the donor ewes was more nearly synchronous, thus greatly increasing the number of fertilized eggs collected at the pronuclear stage (Nancarrow, *et al,* 1984). Centrifugation

Table 1. Efficiency of production of transgenic sheep

Transgene	Number of injected embryos transferred	Lambs born	Lambs transgenic*	Transgenic lambs expressing Number	%
mMT-hGH*	1032	73	1	—	—
mMT-bGH*	842	47	2	2	100
mMT-hGRF*	435	63	9	1/7	14
mMT-bGH*	*M7*	*42*	11	3	27
mAL-hGRF*	171	*16*	4	2	50
mMT-Tk[b]	150	*29*	1	–	–
BLG-FIX[b]	307	52	4	2/2	100
BLG-alpha lATh	49	11	1	1	100
Mtla-oGH5[c]	1089	83	4	0	0
Mtla-oGH9[c]	409	23	3	3	100
Totals	3699	439 (n.8)	40 (1.1)	14	35

[a] Rexroad, *et al.,* 1990.
mMT = mouse metallotheionein 1
mTF = mouse transferrin
mAL = mouse albumin
hGH = human growth hormone
bGH = bovine growth hormone
hGRF = human GH releasing factor

[b]Simons, *et al,* 1988
BLG = ovine beta-lactoglobulin
Tk = thymidine kinase
FIX = human blood clotting factor IX
alpha iAT=human alpha i-antitrypsin

[c]Murray, *etal,* 1989
Mtla = ovine metallothionein ta
oGH = ovine growth hormone
Mtla-oGHs transgene incorporated SV40 viral enhancer sequences, while the Mtla-oGH9 construct did not.

* Nancarrow, *etal.,* (1991) have since produced 1 additional transgenic sheep with each of the Mtla-oGH constructs, with the Mtla-pGH9 individual expressing the transgene. However, the numbers of injected embryos transferred and number of lambs born were not reported.

of the zygotes was tried to improve the visualization of the pronuclei (Nancarrow, *etal,* 1984), but in contrast to pig embryos (Wall, *etal, 1985)* this was not helpful. Suitable visualization of pronuclei in sheep zygotes can be obtained by the critical use of differential interference contrast optics, whereby over 90 percent of pronuclear stage eggs can be successfully microinjected (Simons, *etal,* 1988; Murray, *etal,* 1989; Rexroad, *et al.,* 1989).

Part of the inefficiency in the production of transgenic farm animals results from imperfect handling techniques. Immediate transfer of embryos derived from superovulation resulted in 47 percent continuing to develop compared to 88 percent of the embryos left *in situ* (Rexroad and Powell, 1991). Embryo survival after collection is no better when a complex medium is used (Medium 199 plus 0.10 percent fetal bovine serum) than for a simple phosphate buffered saline with serum medium. Microinjection further reduces viability (Rexroad and Wall, 1987; Walton, *etal.* 1987) resulting in approximately 12 percent of embryos surviving to lambing (Table 1).

Selection of embryos for transfer that were known to be viable and to have incorporated the transgene would reduce the costs associated with maintaining pools of recipients. Sheep embryos can be co-cultured for three days on oviductal cells with only about a 15 percent loss in viability if transferred to recipients that came into estrus 24 hours after the donors. Delayed recipients are necessary because co-culture somewhat retards embryonic development (Rexroad, *etal,* 1990). However, as the early cleavage divisions are largely programmed by maternal gene products, this does not allow an adequate test of the embryos long-term viability. Longer periods of co-culture are possible, but only modest results with respect to long term development have been reported thus far (Gandolfi and Moor, 1987). If longer culture intervals of up to five to seven days can be reliably achieved, development in culture may become useful for predicting long-term viability.

A further benefit of a longer culture period would be the ability to remove a few cells from an embryo and use PCR technology to ascertain whether or not the microinjected DNA had been incorporated into the embryo's genome. Thus we would be in the position of transferring only those embryos to recipients that were known to be both viable and transgenic.

Although there are at present a number of inefficiencies in the technology used to produce transgenic livestock, they are likely to be overcome during this decade. However, the long-term application of this technology in animal agriculture is presently limited by our lack of knowledge about the genetic basis of rate-limiting steps that affect production traits. In most cases we do not know what genes to transfer in order to gain the maximum benefit to production efficiency.

GROWTH PROMOTING TRANSGENES TRANSFERRED INTO SHEEP

A number of fusion genes encoding growth promoting hormones have been transferred into sheep, including fusion genes encoding the human (Hammer, ***etal.,*** 1985), bovine (Rexroad, ***etal.,*** 1989) and ovine growth hormone (Ward, ***etal.,*** 1988; Murray, ***et al,*** 1989) genes (GH) and the human growth hormone releasing factor (hGRF) (Rexroad, ***etal.,*** 1989). A number of these transgenes have used the mouse metallothionein I (mMT) promoter. Other promoters used include the control sequences from the sheep metallothionein la (Mtla) gene (Ward, ***et al.,*** 1988) and the mouse albumin and transferrin genes (Rexroad, ***etal.,*** 1990).

These promoter elements were chosen in an attempt to direct the expression of the transgenes to specific organs, e.g. the liver, or to try to maintain a degree of external control over the level of expression of the transgenes. The mMT promoter had previously been shown to direct the expression of growth hormone to the liver, kidney and other organs in transgenic mice (Palmiter, ***et al.*** 1982,1983). The promoter elements of the various metallothionein genes can be stimulated to increase the level of expression of a linked coding region by the addition of heavy metals to the diet or drinking water. The mMt promoter had the disadvantage of being leaky, that is there was always sufficient expression of the growth hormone transgenes in the absence of heavy metal stimulation to promote growth of the mice to approximately 1.5X the size of non-transgenic littermates (Palmiter, ***etal.,*** 1982,1983). However, in the absence of direct experimental evidence, the pattern of expression of the various mMT-GH and GRF transgenes in sheep could not be predicted.

The sheep Mtla promoter was selected by the Australian group for basically three reasons. First, ideally the expression of a transgene affecting growth should be under external control so that transgenic animals only express the transgene when it is economically advantageous to have them

respond. Secondly, we thought that a more tightly controlled a transgene would be, the less likely to have adverse effects on the individuals carrying it. Thirdly, it was felt that transgenes composed entirely of sheep gene sequences would be more acceptable to lay persons, in particular consumers. For these reasons, we elected to use the sheep Mtla promoter as it was known to have a lower basal level of expression than the mouse MT promoter, while still retaining its heavy metal inducibility (Peterson and Mercer, 1986).

The mouse albumin and transferrin promoters were selected to try to limit the expression of the transgenes to liver tissue in transgenic animals. These promoters were tried to attempt to limit the degree of undesirable side effects that had been observed in transgenic pigs carrying mMT based GH transgenes (Pursel, *et al, 1987).*

THE CONSEQUENCES OF GROWTH PROMOTING TRANSGENES EXPRESSION IN SHEEP

Thirty-six lambs have been produced by microinjecting fusion genes encoding either growth hormone or growth hormone releasing factor (Table 1). Nine of these transgenic sheep carried transgenes that expressed high levels of growth hormone, while three lambs expressed growth hormone releasing factor. Many of the 24 non-expressing sheep have transmitted the transgene to their progeny, which also failed to express the transgene (Rexroad, *etal.,* 1989,1990; Murray, *etal.,* 1989; Nancarrow, *et al,* 1991).

The transgenic lambs expressing either GH or GRF grew at approximately the same rate as non-transgenic controls, even though they had circulating plasma GH levels from 3 to >1,000 times higher than found in control animals (Rexroad, *etal,* 1989; Murray, *etal,* 1989*).* The expressing transgenic animals also had elevated levels of circulating IGF-I and insulin (Rexroad, *et al,* 1990; Nancarrow, *et al,* 1991). Additionally, we have observed that plasma levels of prolactin and the thyroid hormones were depressed in expressing females, but were elevated and not different from controls in a single expressing transgenic male. Lower levels of thyroid hormone are normally associated with a reduced basal metabolic rate (BMR), but in the case of two of the GH expressing females, BMR was increased about 30 percent and metabolic heat production by 20-50 percent (Nancarrow, *etal, 1991).*

The secretion of FSH and LH from the anterior pituitary gland was normal in both males and females, as were the serum levels of the sex steroids. However, both expressing males and females appear to have delayed sexual development when compared to controls (Nancarrow, *etal.,* 1991). This is perhaps similar to the situation observed in expressing growth hormone transgenic pigs where females remain anestrous and males lack libido, but produce viable spermatozoa (Pursel, *et al.,* 1990).

One of the goals of this research was to determine if GH transgenic sheep were more feed efficient and produced less fat than currently available animals, as has been observed in GH transgenic pigs (Pursel, *etal.,* 1989). Expressing transgenic sheep do not appear to have increased feed efficiency (Rexroad, *et al.,* 1989*),* but they do have significantly reduced amounts of body fat (Ward, *et al.,* 1990; Nancarrow, *et al.,* 1991).

The continuously high levels of circulating growth hormone observed in the expressing transgenic sheep has led to severe health problems (Nancarrow, *et al,* 1991). None of the twelve expressing animals has attained puberty, with all of them dying before one year of age. The cause of death has varied, but there are clear data that the over-expression of GH adversely affects liver, kidney and cardiac function (Nancarrow, *et al.,* 1991). Plasma levels of insulin and glucose are also abnormal, indicating an inability to maintain serum sugar levels that results in a diabetic condition (Rexroad, *etal.,* 1990).

Although the efficiency of producing transgenic sheep is low, it is apparent that foreign genes can be reliably transferred into sheep and expressed. The work with growth hormone transgenic sheep and pigs referred to here clearly shows that basic research is still required in a number of areas. The efficiency of the procedure needs to be improved to reduce the cost of producing genetically engineered animals.

More importantly, further work is required to identify promoter elements that, when used in transgene construction, will give a sufficient degree of control over the tissue specificity, developmental timing and level of expression of the transgene. Clearly, the uncontrolled over-production of genes encoding hormones will mostly likely be detrimental to the animal as observed in these studies.

Although there is probably no limit on the types of genes that can be transferred into livestock species, further research is required to identify

the genetic basis of the rate-limiting steps affecting economically important traits. If genetic variation exists in a species that can positively affect the trait of interest, then selective breeding can be practiced. If variation does not exist and a gene from another species can be identified that will positively affect the trait, then gene transfer becomes the technique of choice. To date, there have only been a few candidate genes identified for transfer into livestock, such as the two genes of the cysteine biosynthetic pathway for wool production in sheep (Ward, *et al,* 1990), that can reasonably be expected to significantly improve a production trait. The one exception may be for the dairy industry, where there are a number of potential ways genetic engineering techniques maybe used to alter or improve milk (Wilmut, *et al.,* 1990).

FUTURE CONSIDERATIONS

The demonstration that genetic engineering techniques can be successfully applied to livestock animals raises a number of questions in such diverse areas as ethics and animal rights, economics and the future directions and applications of this research. As scientists involved in this work, we are satisfied that the information collected and the potential applications will be valuable to mankind. As our population increases and the resources of the Earth decrease, agricultural production must become more efficient. Does the non-scientist accept this point of view? Do we have the right to alter the genetics of our farm animals to increase production efficiency, or for that matter, will the public accept the agricultural use of transgenic animals?

Apart from these questions on the morality and ethics of modifying a species' genome, a number of other of questions remain to be answered. Who should pay for this research, which is clearly long-term and expensive, and who will benefit? At present, most of the significant work has been carried out in laboratories with a high level of direct government funding, for example the USDA/ARS laboratory at Beltsville, Maryland or the CSIRO's Division of Animal Production in Australia.

Transgenic experiments using livestock species requires long-term research projects, in part due to the reproductive cycles of the animals and in part because of the need to carry out a careful examination of the consequences of transgene expression in at least two or three generations of

animals. Because the work is long-term with little or no prospect of early returns, only a few companies are currently prepared to contribute financially to this work. At a time when federal government granting agencies are experiencing a drop in the level of projects they can support, they are unwilling to commit support for the eight to ten years that these types of projects require.

It is not sufficient to conclude that the basic research should be done in less costly laboratory animals like the mouse and only scaled up to large animals after the transgene is proven. The Mtla-oGH transgene acts as a completely controllable gene in the mouse (Shanahan, *et ah,* 1989), yet was totally uncontrollable in transgenic sheep (Murray, *etal,* 1989). Furthermore, Mtla-oGH transgenic mice, when switched on, grow to about 1.5X larger than controls, are more feed efficient with respect to lean production, deposit as much fat as controls (Pomp, *etal,* submitted) and do not suffer any apparent health problems. Thus, each transgene construct intended for use in farm animals will ultimately need to be tried in farm animals during the research phase. It is only after the basic patterns of expression and consequences of a transgene's expression have been assessed in a livestock species can valid predictions of the worth of that transgene to agriculture be confidently made.

Thus, if this work is deemed important, who will pay for the research? Additionally, how will a suitable transgene be bred into the national herds? Who will control the flow of a valuable transgenic germplasm into the national herds? And how will this dissemination be paid for?

The production of transgenic animals also raises a number of legal or paralegal questions, both in the United States and in the international arena. For example, in the United States it is legal to patent a transgenic animal, but in Europe it is not. This raises a number of issues. How different must two lines of transgenic animals be for separate patents to be issued? How are patents on livestock going to be enforced in a loosely regulated industry such as farming? What are the consequences in terms of international trade?

The broad scientific questions are clear. We need to identify the rate limiting steps affecting production traits and appropriate genes that, when transferred, will overcome these limiting steps. In addition, we need to build up a library of tissue and developmentally specific promoter elements that can be used to drive the appropriate level of expression of a

transgene. However, the societal answers to the questions we have raised here, and the setting of clear priorities and directions for the application of transgenic technology in animal agriculture have not yet been addressed.

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