THE DIRECT TRANSFER OF DNA BY EMBRYO MICROINJECTION

K.A. WARD, I.R. FRANKLIN, J.D. MURRAY, C.D. NANCARROW, K.A. RAPHAEL, N.W. RIGBY, C.R. BYRNE, B.W. WILSON and C.L. HUNT, AUSTRALIA
C.S.I.R.O Division of Animal Production,
PO Box 239, Blacktown, Australia, 2148

SUMMARY

The transfer of genes by microinjection of recombinant DNA into one-cell embryos has been amply demonstrated in mice. Its application to domestic animals offers a novel approach to their genetic improvement. Currently, there are limitations to the general application of the technique to commercial species such as sheep. This paper identifies these limitations and discusses possible solutions.

Microinjection in sheep is more difficult than in mice. The pronuclei are more difficult to see, and the survival of embryos immediately following injection, and at later stages, is reduced. We have yet to achieve gene transfer into sheep, and published reports support the view that the efficiency of integration may be low in this species.

The judicious selection of gene sequences is of paramount importance for the successful application of the technique to domestic livestock. Four potential areas for gene transfer are discussed.

INTRODUCTION

Animal breeding, like most disciplines in biology, faces its greatest challenge since the development of quantitative genetics theory in the 1930s and 40s. Recent developments in DNA manipulation and embryo handling allow genetic information to be transferred directly between organisms. The genetic improvement of domestic livestock by this procedure has considerable potential (Jones, 1980; Ward, 1982; Smith and Hespall, 1983; Ward et al., 1984; Franklin, 1985; Church et al. 1985; Wagner et al., 1985) and consequently has attracted attention both within and without scientific circles. Such attention has been speculative because of the paucity of results in domestic species. Recently, successful transfers in animal species other than mice have been reported, and these results permit a better evaluation of the potential for domestic animal improvement. This paper describes the current technology, discusses recent results and considers the potential and the limitations of the approach.

The Integration of Foreign DNA into Eukaryotic Cells

A number of fundamental requirements must be satisfied to achieve the transfer of functional genetic information into an animal. First, the recipient cells must be capable of stable integration at an efficiency high enough to allow the production of practicable numbers of transgenic animals. The ability of eukaryotic cells to take up and integrate foreign DNA was first shown in tissue culture, using fragments of adenovirus DNA as a source of genetic information (Graham et al. 1973, 1974). This important finding demonstrated that eukaryotic cells contain the enzymes necessary for the ligation of foreign DNA into their genome. The technique has been refined during the past decade so that it is now a relatively simple technical exercise to introduce DNA to cells in culture, but the efficiency of integration is only of the order of one in 10^5 to one in 10^6. This is too low to be useful for the study of gene transfer to whole organisms.

To improve efficiency, direct injection was investigated. Capecchi et al. (1980) showed that integration into cell culture could be enhanced by direct injection into cells and, previously, Jaenisch and Mintz (1974) had shown the integration of foreign DNA injected into the blastocoel cavity of developing mouse embryos. Several laboratories embarked upon microinjection into the pronuclei of one-cell stage mouse embryos, and this proved to be singularly successful. Gordon et al. (1980) were the first to report the successful integration of a recombinant DNA into mice by this procedure. The DNA consisted of a recombinant molecule containing the Herpes simplex virus thymidine kinase gene, the SV40 virus origin of replication and the plasmid pBR322. Immediately following the announcement of these experiments, successful transfers were reported from four other laboratories, in which the rabbit β-globin gene (Costantini and Lacy, 1981; Wagner et al.,1981b), the human β-globin and the Herpes simplex virus thymidine kinase genes (Wagner et al., 1981a) and the human insulin gene (Burki and Ullrich, 1982) were all integrated into the mouse genome.

The second requirement for practical gene transfer is that the introduced DNA be heritable. Costantini and Lacy (1981), in their study of the integration of the rabbit β-globin gene, showed that the new DNA was inherited by offspring of the original transgenic mice in a normal mendelian fashion, and a number of independent laboratories have since confirmed this finding. In the majority of cases the foreign DNA integrates in a single chromosomal site, which differs for each transgenic mouse line examined (Costantini and Lacy, 1981; Lacy et al. 1983).

The third requirement is that the introduced gene be functional. Despite the relatively low efficiency with which transgenic mice are produced by microinjection (about 0.2% to 0.5% of the embryos injected), this was adequate for the study of the expression of introduced genes. Thus, Brinster et al. (1981) constructed a plasmid consisting of the Herpes simplex virus thymidine kinase gene and the mouse metallothionein-I gene 5' regulatory sequences, and found that the thymidine kinase was expressed at moderate levels in transgenic mice. Subsequently, Brinster et al. (1982) injected a series of plasmids in which various portions of the metallothionein promoter sequence were deleted, and studied the effects of these deletions on gene expression. An important conclusion from these experiments was that regulatory sequences, such as the metallothionein gene promoter, can control a wide range of structural gene sequences, and furthermore, that such hybrid genes can be efficiently expressed when they are inserted into apparently random positions within the genomes of the transgenic animals.

The expression of recombinant DNA in transgenic animals opened the way for attempts to alter the phenotype of an animal by gene transfer. Palmiter et al. (1982) fused the structural portion of the rat growth hormone gene to the promoter sequences of the mouse metallothionein-I gene and introduced the recombinant molecule into mice. The fusion gene was expressed in the transgenic animals, resulting in elevated levels of circulating growth hormone, and the animals responded by growing more rapidly and for a longer period of time. Similar results were subsequently achieved when a fusion gene containing the human growth hormone structural sequence was substituted for the rat sequence (Palmiter et al. 1983).

The mechanism by which foreign DNA is integrated into the genome of a eukaryotic cell is largely unknown. However, Brinster et al. (1985) have provided some information. First, linear molecules integrate more efficiently than circular molecules, and are also readily circularised by the mouse pronucleus.

Second, when two different DNA molecules are co-injected, they usually integrate together. Third, it is clear both from Palmiter and Brinster's work and from a number of earlier studies that the injected DNA integrates at a single, randomly-selected chromosomal location as a tandem, head-to-tail array of multiple copies of the gene, with the exact number of copies being highly variable. Brinster et al. (1985) have suggested, as a working hypothesis, that integration of linear DNA molecules may occur at spontaneously-generated breaks in the chromosome. Other molecules that have circularised after injection then recombine with the integrated DNA to generate tandem arrays.

There is evidence that the insertion site influences gene expression. Thus, a rabbit β-globin gene was expressed in inappropriate tissues in two transgenic mouse lines (Lacy et al. 1983). This was attributed to the influence of surrounding chromatin at the site of integration. In another example, only about 70% of transgenic animals, created using thymidine kinase or growth hormone genes fused to the mouse metallothionein promoter, expressed the introduced gene (Palmiter et al. 1983), despite the fact that most contained an apparently functional DNA sequence. Furthermore, transgenic lines, all containing the same fusion gene, differ in their level of expression, apparently independently of the number of gene copies. A possible explanation is that regardless of the actual copy number, only those genes located at the extremities of the tandem gene array are actually expressed (Palmiter et al. 1983), this expression being the result of the influence of surrounding chromatin. Taken together, these results suggest that the chromatin immediately surrounding the inserted gene influences the level of gene expression. It is also probable that different fusion genes differ in their susceptibility to influence by the surrounding chromatin. Thus, metallothionein-thymidine kinase recombinants appear to be more susceptible to local influence than metallothionein-growth hormone recombinants (Palmiter et al. 1984).

APPLICATION OF GENE TRANSFER TO DOMESTIC ANIMALS

The major difficulty in applying genetic engineering techniques to commercial livestock is our lack of knowledge of the genetics of production traits. Even if we could recognise the existence of major genes, and knew their chromosomal location, this would help us little, for we would have to develop a method to identify the presence of these genes in a genomic library. Also, there is no way, at present, to deduce the function of a gene from its DNA sequence. It is now possible to recognise potential start and stop signals for transcription and translation of sequences. Hence an amino acid sequence coded by an unknown gene may be guessed and inferences can be made about the tertiary structure of such sequences. However, gene function can be determined only from ancillary information derived from biochemistry, physiology, or genetics.

In addition, the regulation of a gene cannot presently be deduced from its structure. While much of the control of gene expression is encoded in regions in the immediate vicinity of the gene (cis-regulation), the initiation of transcription may depend on the products of other loci (trans-regulation). Consequently, the development of an organism cannot be predicted from gene sequence data alone, and it is doubtful that this will ever be possible. Biological knowledge can only proceed by an analysis of the whole animal, in conjunction with studies of gene structure and function.

Aside from these basic complexities, there are specific areas of research to be developed for routine gene transfer into livestock. Some of these are essentially technological goals; others require fundamental biological research.

Embryo culture and in-vitro fertilisation.

The pronuclei of newly fertilised mouse eggs are easily seen, but in most domestic species (sheep, cattle, swine) the egg contains numerous lipid particles which obscure the nuclei, making injection difficult. The collection of newly fertilised eggs through superovulation and surgical removal is expensive and sometimes yields erratic results. Greater control could be achieved if oocytes could be cultured and fertilised in vitro. Both of these techniques are being developed for species such as the sheep. The culture of embryos following fertilisation is also important so that eggs which fail to develop may be rejected.

Vectors.

Direct injection has a number of disadvantages. One is physical damage to the egg. Another is the control of the number of gene copies which become incorporated into the host genome. These problems may be overcome by the development of vectors for gene transfer. Many species, such as bacteria, yeast, maize, and Drosophila, contain transposable elements (transposons) which can be induced to mobilise within the genome. The use of transposable elements as vectors, where the gene of interest is inserted within the transposon, is now important in Drosophila for the study of the control of gene expression.

The class of RNA viruses known as retroviruses are structurally similar to transposable elements; these replicate by making double stranded DNA copies which are integrated into the host genome. Retroviruses are known to carry foreign genes and to express them in the host; oncogenic retroviruses such as bovine leukemia and Rous sarcoma are examples. Retroviruses are common in vertebrate species and are potential vectors for gene transfer.

Selection in cell culture.

The transformation of livestock would be considerably simplified if it were possible to transform cells in culture, to screen these for successful integration and expression, and then to grow an entire organism from a single cell. (This is one of the advantages in working with plant tissue.) Certain embryonic cells can be grown in culture and, when aggregated with other embryonic tissue, apparently participate in normal development. If these cells were to develop into germ tissue any genetic changes induced in these cells would be inherited. This is a technique explored, so far, only in the mouse.

Isolation of promoters.

As indicated above, transfer experiments involving the growth hormone gene have used a fusion gene constructed from a gene from man or rat coupled with the mouse metallothionein promoter. This promoter has proven satisfactory for the systemic expression of growth hormone, but there are more potent promoters of transcription, such as those isolated from SV40, or the LTR's (long terminal repeats) of retroviruses. However, in almost all practical circumstances we would wish some control of both the timing and site of expression of a particular gene. The above promoters are not highly tissue specific, and much work is needed in the isolation and characterisation of more precise promoters for future gene transfer experiments.

The selective replacement of genes

It is now quite feasible to increase the level of production of an important protein. However, it is much more likely that we will wish to alter it, reduce

the level of its production, or modify its regulatory control. In order to do this we need techniques for the selective replacement of portions of the genome, not simply the random insertion of extra copies of a particular gene. One possibility is the construction of "anti-sense" genes, that is, genes which produce an mRNA complementary to the messenger produced by the normal reading strand, in the expectation that the mRNA's will bind and interfere with translation. This solution still falls short of our requirement for the replacement of specific genes.

WHICH GENES?

Given our current level of understanding of gene regulation and development, the choice of gene sequences which might reasonably be expected to improve economic efficiency is limited. There are classes of genes which we can exclude for the time being. For example, morphogenetic loci, those which determine cell commitment and hence organogenesis, appear to act combinatorially. Any modification of these controlling genes is likely to have far-reaching and unforeseen consequences. The 'housekeeping' genes, those coding for the enzymes of intermediary metabolism, are expressed in most cells of the body. Because of their ubiquity, these are unlikely candidates for genetic modification directed at a change in a specific productive process.

There are four potential areas which, if altered by gene transfer, might result in enhanced production. The most obvious group are the genes for circulating peptide hormones and growth factors. Another set of genes of interest are those conferring resistance or tolerance to parasites, particularly the major histocompatibility complex. There are also the structural proteins, such as the keratins, caseins, ovalbumin, and the proteins of muscle. Finally, and perhaps most important, is the possibility for adding new metabolic pathways and novel promoters through the transfer of genes across wide taxonomic boundaries.

Hormones and growth factors

There are numerous peptide growth factors. The modification of the level and timing of their expression is an important target of genetic engineering. Here, we need to identify a trait whose expression is hormone-controlled, to isolate the gene encoding either the hormone itself or a factor which regulates the hormone, to alter the regulatory sequences of this gene, and insert it into the genome of the chosen animal species. One of the main advantages in manipulating hormone levels is that tissue-specific expression is not necessary because the gene product is carried in the circulation to its target tissues, largely independent of the source of its production. The use of this approach has been pioneered by Palmiter et al. (1982, 1983) in their transgenic mouse studies using metallothionein-growth hormone fusion genes, and the potential application of these genes to domestic animals was clearly recognised by these authors. More recently, the phenotype of transgenic mice has been altered by the use of a modified gene encoding the releasing factor for growth hormone, rather than the gene for the hormone itself (Hammer et al. 1985a). The production of supraphysiological levels of the releasing factor in the transgenic animals resulted in elevated levels of circulating growth hormone and increased growth rate. Increasing body size *per se* is probably of little importance in improved production efficiency, particularly in the extensive industries, but other effects, such as growth hormone's role in nutrient partitioning, are more promising. However, in transgenic mice, the high levels of circulating growth hormone in transgenic mice are associated with reduced female fertility (Hammer et al. 1984); this would counteract any of the beneficial effects if repeated in commercial livestock.

Despite the wealth of knowledge which has been accumulated within the past decades in our understanding of domestic animal physiology, there remains a paucity of information concerning the involvement of hormones in the control of many areas of animal production. Nevertheless, there are several areas which are well-understood and hence might be manipulated by this approach. For example, skeletal growth is clearly influenced by growth hormone itself, its releasing factor, and insulin-like growth factor-I (Daughaday et al. 1972). Milk production is dependent on prolactin for initial induction and can be increased substantially by increasing the concentration of growth hormone (McCutcheon and Bauman, 1985). Reproductive performance is under complex hormonal control, but nevertheless, can be greatly enhanced by altering the concentration of one or more of the hormones involved (Cox et al. 1985). In other areas our knowledge is insufficient to permit gene transfer manipulation at present. The intake, absorption and digestion of food, for example, is believed to be under hormonal control, although few details are available. Wool growth is probably influenced by a number of hormones, thyroxine, growth hormone, steroids and melatonin being a few of those implicated. However, physiological knowledge is currently insufficient for this area to be useful in gene transfer.

Hormones and growth factors do not appear to have any intrinsic enzymatic function; they are messengers which act by binding to receptors which, in turn, trigger a response within the cell. The characteristics of an organ or tissue are presumably determined by the distribution of cell types within that tissue, and the density of cell surface receptors on the constituent cells. Modification of hormone levels is a crude tool in the manipulation of body composition and tissue function; much greater flexibility could be achieved if receptor distribution and density could be manipulated directly.

Modification of structural proteins

A second category are those genes which encode particular animal products. Thus, by altering the regulation of one or more of the wool keratin genes (Ward et al. 1982) it may be possible to increase wool production. By altering a casein gene, milk production might be influenced. There are several difficulties associated with this. First, in most instances it would be essential to preserve the tissue-specific expression of the modified gene. The expression of wool keratin genes, for instance, in the liver or kidney of a transgenic animal would almost certainly be lethal. The regulatory sequences of the gene must be identified and their mechanism of control over the gene understood in detail. Modification of the sequences must preserve the inherent tissue specificity while altering the rate of transcription. Second, if it is the aim of the experiments to increase the amount of a specific product, it is necessary that the level of gene transcription be rate-limiting for its production. This may not be the case. For example, wool production is limited by the availability of substrate, and all available evidence indicates that keratin messenger RNA is present in wool follicles in excess of requirements.

Disease Resistance

Alleles of the histocompatibility locus are known to be associated with resistance or susceptibility to certain diseases. This complex of loci is therefore a candidate for gene transfer or modification by genetic engineering. Another possibility is the creation of a transgenic animal which possesses innate, rather than acquired, resistance to a specific disease. A functional immunoglobulin gene could be isolated that encodes resistance to the disease of interest and transferred back into the host species. Immunoglobulin genes are

expressed in transgenic mice, and produce specific, functional antibodies (Brinster et al. 1983; Grosschedl et al. 1984). In our laboratory, a cell line which expresses a specific sheep immunoglobulin has been created by fusing antigenically-stimulated sheep lymphocytes with a mouse myeloma cell line (K. Beh, S. Haynes, K.A. Ward, unpublished), and we are currently isolating the sheep immunoglobulin gene from these cells. We intend to introduce this gene into sheep and progeny test for specific antibody production.

Novel metabolic pathways

A major area for gene transfer is the introduction to the animal of new enzymic pathways. We might wish to repair a pathway which is present in most mammals but has been rendered inoperable by a mutational event in a particular species, or attempt to introduce a pathway which either has never existed in the lineage or has been lost during the course of evolution. In the latter case, the genes which encode this pathway must be isolated from an appropriate source, modified for their expression in the selected mammalian environment, and then introduced into the target species.

An example currently being pursued is the introduction to sheep of a pathway for the biosynthesis of cysteine from serine (Ward et al. 1984). The purpose is to increase the production of wool. It is known that the concentration of the amino acid cysteine circulating in the blood of sheep is the limiting factor in wool growth under many nutritional regimes (Reis and Schinkel, 1963; Reis et al. 1973 a,b). Attempts to increase the level of cysteine by simple dietary supplementation do not succeed, however, because the bulk of the additional cysteine is degraded by bacteria in the rumen of the sheep. Cysteine is an essential amino acid in all mammals because they lack the pathway of synthesis. The key elements of this pathway are shown below.

(1) serine + acetyl-CoA ---(serine transacetylase)--- O-acetylserine + CoA

(2) O-acetylserine + H_2S ---(O-acetylserine sulphydrylase)--- cysteine + acetate

Recently, the genes which encode the two enzymes which catalyse this pathway have been isolated from the the bacterium E.coli, (Boronat et al., 1984; Ingle and Loughlin, 1980; J.E. Cronan, unpublished; C.L. Hunt, R.E. Loughlin, K.A. Ward, unpublished), and are currently being prepared for transfer to sheep. Underlying these experiments is the hypothesis that these genes, when expressed in rumen epithelial cells, will enable the synthesis of cysteine from serine, hydrogen sulphide and acetyl-CoA, and that cysteine will be absorbed and transported to the wool follicles of the sheep.

Similarly, it should be possible to increase the supply of lysine in pigs or dairy cattle in order to increase growth rate or milk production respectively. Specific nutrients such as vitamins could be made non-essential by introducing their biosynthetic pathways into domestic species. However, most nutrient pathways are complex, involve many enzymic steps and are frequently subject to complex regulation by their products and intermediates, and as a result, the modification of an animal's biochemistry by gene transfer is a formidable challenge.

THE TECHNIQUES OF GENE TRANSFER TO DOMESTIC ANIMALS

The application of the mouse one-cell embryo microinjection technique to domestic animals has met with a number of technical problems. The most serious of these is the difficulty experienced in visualizing the pronuclei because of the heavy concentration of optically dense cytoplasmic vesicles in the cytoplasm of the embryos. Recently, techniques have been developed which enable adequate visualization in sheep, rabbit, pig, goat and cattle embryos (Hammer et al. 1985b). In general, these depend on the use of differential interference contrast microscopy, although in the case of pig embryos, a short period of centrifugation of the embryos is also necessary. Utilising these techniques, Hammer et al. (1985b) have recently reported the successful integration of a metallothionein-human growth hormone gene into rabbits, pigs and sheep. The integration efficiencies for rabbits (12.8%), pigs (10.4%) and sheep (1.3%)) are lower than that for the mouse (approx. 30%). The figure for sheep, 1.3%, is particularly low, and may reflect some unique difference in the properties of the genome of this species. The expression of the fusion gene was detected in both rabbits and pigs, and resulted in elevated levels of circulating human growth hormone. However, no detectable growth response was obtained in these transgenic animals, suggesting that the response observed in mice may not be as easily obtained in domestic species.

In our own laboratory, we have produced a number of transgenic mouse lines, and are attempting to produce transgenic sheep. The following summarises our progress to date.

Production of Zygotes and Microinjection Techniques

Single-cell sheep embryos are produced by methods similar to those outlined previously (Nancarrow et al. 1984). The protocol is summarised in Table 1 which includes details of the timing of procedures relative to reproductive events.

Table 1. Timing of procedures designed for optimal collection, injection[+] and transfer of zygotes for production of transgenic sheep.

Day of pregnancy	Time	Event	Time (h) elapsed after (a) PMSG	(b)sponge removal	Age of embryo(h)
-2	0800	PMSG	0	-	-
-1	1700	sponge removal	33	0	-
0	1700	GnRH	57	24	-
1	0900	A.I.	73	40	-
1	1700	ovulation	81	48	0
2	0900	embryo recovery	97	64	14
2	1500	embryo transfer	103	70	20

[+] Pronuclear injection of DNA occurs between embryo recovery and transfer.

Oestrus-synchronised ewes were superovulated with 1200-1500 I.U. PMSG, and ovulation synchronised by the injection of gonadotrophin releasing hormone (GnRH). The animals were artificially inseminated using fresh diluted semen deposited into each uterine horn by laparoscopy. Zygotes were flushed from the oviducts at about 14h after fertilisation and transferred to the laboratory for microinjection.

All eggs were initially sorted into groups of fertilized one cell eggs with and without visible pronuclei (Figure 1a). The presence of sperm on the zona pellucida, two polar bodies or pronuclei are considered evidence of fertilisation. Zygotes without visible pronuclei are treated in medium with cytochalasin B and then centrifuged. This procedure developed by Dr P. Simons (ABRO, Edinburgh) is effective in allowing pronuclear visualization (Figure 1b) while still maintaining viability (pers. com. Drs P. Simons and I. Wilmut). We have observed pronuclei in 58.5% of the fertilized one cell eggs collected (1617/2762) prior to treatment. Pronuclei were visible in 39.5% of the eggs treated with cytochalasin B/centrifugation (367/930), yielding an overall figure of 72% of the collected zygotes having visible pronuclei (1984/2762) suitable for microinjection.

Eggs are transferred to a depression slide containing medium overlaid with light paraffin oil. Untreated eggs with visible pronuclei are injected in the presence of cytochalasin B, while treated eggs are not further exposed to cytochalasin B. The eggs are immobilized by gentle suction using a fluid filled, blunt ended holding pipette. Injections are carried out using a fine needle connected to a 20 or 30ml ground glass syringe. The injection system is air filled in contrast to the fluid filled system used by Brinster et al. (1985). The injection pipette is rapidly withdrawn as soon as signs of pronuclear swelling are observed, resulting in approximately 2 pl of DNA solution being injected.

In our hands, 51% (960/1883) of the injected sheep eggs survived the injection procedure as assessed by failure to lyse by 1 hour after injection. There is no difference in the survival rates of non-treated or cytochalasin B/centrifugation treated eggs (705/1425 vs 255/458 respectively). This survival rate is lower than we experience with mouse eggs in our laboratory (61%).

Eggs which have not lysed are then transferred into the oviducts of synchronised, recipient ewes. Recipient ewes were treated identically to the donor ewes with the exception that only 200-400 I.U. PMSG were injected. This was necessary because without PMSG treatment, the ewes take 24h longer to display oestrus than do the superovulated animals, indicating that adequate ovarian stimulation will not have occurred by the time that GnRH is given. This treatment regime gives good synchronisation between donors and recipients. Between 1 and 6 embryos were transferred into the exposed oviducts of laparotomised, anaesthetised recipient ewes, with occasionally embryos being transferred to both oviducts. However, we have now chosen to transfer 3 or 4 embryos to a single oviduct for each ewe. Pregnancy was assessed both by progesterone radioimmunoassays on blood collected 16 and 19 days after embryo transfer, and by ultrasound at 26 days of gestation, using an intracorporeal probe.

Approximately 24% of recipient ewes became pregnant (Table 2). Ewes which received centrifuged or non-centrifuged injected embryos did not differ in pregnancy rate. However, there were significantly more fetuses recovered from ewes which had received non-centrifuged, injected eggs than from those ewes into which treated eggs had been transferred ($X^2(1)=5.2$, $P<0.025$). Degenerating fetal remains and membranes were recovered from recipient ewes, but this appears to be unrelated to the centrifugation treatment. It is not known if the observed fetal mortality is related to the microinjection procedure.

Table 2 Pregnancy rates of recipient ewes following transfer of non-treated or cytochalasin B/centrifugation treated, injected embryos.

experiment		embryos transferred	No. ewes	No. preg.	embryos implanted	fetuses normal	degen.
85/10-85/33	NT	250	78	26	35	31	4
	CC	162	49	11	11	9	2
85/34-85/54	NT	418	123	24	-	-	-
	CC	22	7	1	-	-	-
sub-totals	NT	668	201	50	-	-	-
	CC	184	56	12	-	-	-
total		852	257	62	-	-	-

NT = not treated; CC = cytochalasin B/centrifugation treated.
Experiments 85/34-85/54, pregnancies are being carried to term.

Progeny analysis following microinjection

(a) Mice. In parallel with the gene transfer studies carried out on sheep, we have conducted a series of experiments to create transgenic mice. The microinjection technique used for the mouse experiments is essentially that described by Costantini and Lacy (1981). Seven different recombinant DNA species have been used (see legend to Table 3), and four of these have resulted in the production of transgenic mouse lines. Our results are summarised in Table 3, and show that there is a considerable variation in the efficiency with which mice are born from injected eggs transferred, and in the number of mice which integrate the donor DNA. This latter figure in our hands ranges from 18% down to 0%. We have yet to determine the reasons for the poor integration of certain DNA species.

Table 3. Summary of transgenic mice from various recombinant DNAs.

DNA	pregnancies/ recipients	eggs transferred	mice born	mice positive
mouse-human β-globin	10/16	199	48	7
H-2 L^d	12/25	519	21	1
MT-Factor IX	6/7	158	40	7
$p\mu$	6/11	170	14	2
HSV106	10/21	281	38	0
MT-ovine GH	10/16	334	49	0

Sources of recombinant DNA molecules as follows: hybrid mouse/human β-globin, Chada et al. 1985; H-2 L^d, Raphael and Costantini(unpublished); MT-Factor IX, Choo and Raphael(unpublished); $p\mu$, Grosschedl et al. 1984; HSV106, McKnight and Gavis 1980; MT-ovine GH, Ward and Byrne, unpublished.

(b) Sheep. Three series of experiments have been conducted in attempts to transfer DNA to sheep. In the first two series, foetuses were collected after 28 to 90 days of gestation and examined for integration of the donor DNA. In the last set of experiments, which have yet to be analysed, pregnancies are being allowed to proceed to term.

The first series of experiments used a 3.5kb BamH1 fragment of the plasmid HSV106 which encodes the Herpes simplex virus thymidine kinase (McKnight and Gavis, 1980). Thirty-one embryos were collected, liver DNA extracted by conventional procedures and examined for the presence of the injected DNA by Southern blot hybridisation. None of the embryos contained the recombinant fragment. The second series of experiments utilised a sheep growth hormone cDNA linked to the regulatory sequences of a sheep metallothionein-I gene (Figure 2a). Forty embryos were examined for the presence of this sequence but, again, no embryos contained the recombinant DNA.

The DNA used in the most recent experiments consists of the sheep growth hormone genomic sequence with the 5'-regulatory sequence replaced by the sheep metallothionein-I gene regulatory sequence (Figure 2b). This DNA has been tested extensively in a mouse cell line and in transgenic mice, and has been shown to express the sheep growth hormone protein, this expression being heavy-metal inducible (M.J. Sleigh and K.A. Ward, unpublished). Prior to its injection into sheep, the fragment indicated in Figure 2b was purified on Elutip-d columns (Schleicher and Schull, W. Germany). A total of 25 recipient ewes are currently pregnant from embryos which have been injected with this DNA, and analysis of these offspring is to commence during February, 1986.

The results obtained so far support the conclusions of Hammer et al. (1985) that the integration efficiency of microinjected DNA by sheep may be substantially lower than that in mice. To date, 71 sheep embryos have been examined for the possible integration of recombinant DNA, and all have been shown not to contain such sequences. We do not consider our numbers to be sufficiently large to be certain of a lower sheep integration efficiency, however, because many of these embryos represent some of our early experiments in which our methods of embryo injection were still undergoing technical evolvement. The most recent series of microinjections, utilising the sheep growth hormone gene, will produce more definitive conclusions, because our technique has been standardised, and the sheep injections have been run in parallel with a series of mouse injections using the same DNA. If it is true that the integration efficiency of sheep is low, this will constitute a substantial barrier to the production of transgenic sheep. Because the mechanism of integration of foreign DNA into the eukaryotic genome is not known, it is difficult to determine why sheep might be more recalcitrant to the uptake of recombinant DNA than other species. It will be necessary to carry out a systematic investigation of the manner in which the DNA is presented to the sheep embryo during the microinjection procedure, compared to that used for our mouse experiments. Because of the long lead time involved in sheep experiments, the evaluation of such an investigation would take a considerable length of time. It may also be necessary to consider DNA transfer by routes other than microinjection, although no such mechanisms are yet available for use in sheep.

DISCUSSION

While there remain numerous technical obstacles, the results obtained so far indicate that all animal breeders must seriously consider the potential for gene transfer into domestic livestock. The animal geneticist has an important role to

play in paving the way for this revolution. Molecular biology offers more than the tools for gene transfer; it provides methods for increasing our knowledge of the genetics of commercial species. RFLP mapping, and the use of mouse or human probes to identify loci of potential importance are two examples. However, the aspiring genetic engineer has much to prove.

Attention is continually drawn to the promise of genetic engineering, often overlooking the proven efficacy of traditional methods. We know from the intensive industries that gains of 2% to 3% per annum are possible, and these gains are the yardstick against which genetic engineering must be judged. As in the case of new mutations, the larger the change which the genetic engineer attempts to introduce the greater will be the chance of undesirable pleiotropic effects on other aspects of production efficiency. Hence, the genetic engineer is faced with the task of making a series of changes and adjustments to the organism, probably in parallel.

If genetic engineering is to become a reality, the animal breeder will be required to work more closely with molecular and developmental biologists. The procedures for defining breeding objectives will remain unchanged. The major difference between the new techniques and the old is that a greater level of understanding of the biology underlying those objectives will be required. For the first time we shall need to know what limits production - in metabolic terms, not simply in economic terms. Then we shall have to identify a simple genetic change which removes or ameliorates this limit, but which has no disadvantageous pleiotropic effects on overall production efficiency. If he is to meet this challenge, the animal breeder will have to add to his repertoire a detailed knowledge of the formal genetics of domestic species.

Currently, the animal breeder is expected to be a competent statistician and have a solid understanding of the industry he serves. In addition, he should possess a working knowledge of agricultural economics, genetics, reproduction, physiology, nutrition, and perhaps a little sociology. Very few animal breeders meet these criteria; to add to this list molecular, cellular, and developmental genetics is asking much of a single individual. We cannot simply dispense with the traditional skills. Even if it becomes possible to select new breeding stock in the laboratory, the task of evaluation and promulgation of improved genotypes throughout the industry will remain. It may be that two kinds of individual are necessary; one primarily a biometrician trained in the definition of objectives and the comparative evaluation of productivity, the other a physiological geneticist with specialist knowledge of the genetics of development and intermediary metabolism in livestock species. It appears that the animal breeding component of animal production will, in the future, require a team effort.

REFERENCES

BORONAT, A., BRITTON, P., JONES-MORTIMER, M.C., KORNBERG, H.L., LEE, L.G., MURFITT, D. and PARRA, F. 1984. Location on the Escherichia coli genome of a gene specifying O-acetylserine (thiol)-lyase. J. Gen. Microbiol., 130, 673-685.

BRINSTER, R.L., CHEN, H.Y., TRUMBAUER, M., SENEAR, A.W., WARREN, R. and PALMITER, R.D., 1981. Somatic expression of Herpes thymidine kinase in mice following injection of a fusion gene into eggs. Cell, 27, 223-231.

BRINSTER, R.L., CHEN, H.Y., WARREN, R., SARTHY, A. and PALMITER, R.D., 1982. Regulation of metallothionein-thymidine kinase fusion plasmids injected into mouse eggs. Nature, 296, 39-42.

BRINSTER, R.L., RITCHIE, K.A., HAMMER, R.E., O'BRIEN, R.L., ARP, B. and STORB, V. 1983. Expression of a microinjected immunoglobulin gene in the spleen of transgenic mice. Nature, 306, 332-336.

BRINSTER, R.L., CHEN, H.Y., TRUMBAUER, M.E., YAGLE, M.K. and PALMITER, R.D. 1985. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc. Natl. Acad. Sci. USA, 82, 4438-4442.

BURKI, K. and ULLRICH, A. 1982. Transplantation of the human insulin gene into fertilized mouse eggs. The EMBO Journal, 1, 127-131.

CAPECCHI, M. (1980). High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. Cell, 22, 479-488.

CHADA, K., MAGRAM, J., RAPHAEL, K., RADICE, G., LACY, E. and COSTANTINI, F. 1985. A foreign β-globin gene expressed specifically in erythroid cells of transgenic mice. Nature, 314, 377-380.

CHURCH, R.B., SCHAUFELE, F.J. and MECKLING, K. 1985. Embryo manipulation and gene transfer in livestock. Can. J. Anim. Sci. 65, 527-537.

COSTANTINI, F. and LACY, E. 1981. Introduction of a rabbit β-globin gene into the mouse germ line. Nature, 294, 92-94.

COX, R.I., WILSON, P.A. and WONG, M.S.F. 1985. Manipulation of endocrine systems through the stimulation of specific immune responses. in Reviews in Rural Science, "Biotechnology and Genetic Improvement of Animals", vol. 6, eds. R.A. Leng, J.S.F. Barker, D.B. Adams and K.J. Hutchinson. University of New England, Australia. pp 150-160.

DAUGHADAY, W.H., HALL, K., RABEN, M.S.., SALMON, W.D.Jr., VAN den BRANDE, J.L. and VAN WYK, J.J. 1972. Somatomedin: proposed designation for sulphation factor. Nature, 235, 107.

FRANKLIN, I.R. 1985. Genetic engineering and animal improvement. Proceedings of 2nd World Congress on Sheep and Beef Cattle Breeding, Pretoria, South Africa., pp 300-306.

GORDON, J.W., SCANGOS, G.A., PLOTKIN, D.J. BARBOSA, J.A. and RUDDLE, F.H. 1980. Genetic transformation of mouse embryos by microinjection of purified DNA. Proc. Natl. Acad. Sci. USA, 77, 7380-7384.

GORDON, J.W. and RUDDLE, F.H. 1981. Integration and stable transmission of genes injected into mouse pronuclei. Science, 214, 1244-1246.

GRAHAM, F.L. and VAN der EB, A.J. 1973. Transformation of rat cells by DNA of human adenovirus 5. Virology, 54, 536-539.

GRAHAM, F.L., ABRAHAMS, P.J., MULDER, C., MEIJNEKER, H.L., WARNAAR, S., DE VRIES, F.A.J., FIERS, W. and VAN der EB, A.J. 1974. Studies on in vitro transformation by DNA and DNA fragments of human adenovirus and simian virus 40. Cold Spring Harbor Symp. Quant. Biol. 39, 637-650.

GROSSCHEDL, R., WEAVER, D., BALTIMORE, D. and COSTANTINI, F. 1984. Introduction of a μ-expressing immunoglobulin gene into the mouse germ line: specific expression in lymphoid cells and synthesis of functional antibody. Cell, 36, 647-658.

HAMMER, R.E., PALMITER, R.D. and BRINSTER, R.L. 1984. Partial correction of a murine hereditary growth disorder by germ-line incorporation of a new gene. Nature. 311, 65-67.

HAMMER, R.E., BRINSTER, R.L., ROSENFELD, M.G., EVANS, R.M. and MAYO, K.E. 1985a. Expression of human growth hormone releasing factor in transgenic mice results in increased somatic growth. Nature, 315, 413-416.

HAMMER, R.E., PURSEL, V.G., REXROAD Jr, C.E., WALL, R.J., BOLT, D.J., EBERT, K.M., PALMITER, R.D. and BRINSTER, R.L. 1985b. Production of transgenic rabbits, sheep and pigs by microinjection. Nature, 315, 680-683.

INGLE, C.P. and LOUGHLIN, R.E. 1980. Isolation and characterization of a λ-transducing bacteriophage carrying the cysE gene of Escherichia coli K-12. Aust. J. Biol. Sci. 33, 605-612.

JAENISCH, R. and MINTZ, B. 1974. Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. Proc. Natl. Acad. Sci. USA, 71, 1250-1254.

JONES, K.W. 1980. Muscle cell differentiation and the prospects for genetic engineering. Br. med. Bull. 36, 173-180.

LACY, E., ROBERTS, S., EVANS, E.P., BURTENSHAW, M.D. and COSTANTINI, F.D. 1983. A foreign β-globin gene in transgenic mice: integration at abnormal chromosomal positions and expression in inappropriate tissues. Cell, 34, 343-358.

MANIATIS, T., FRITSCH, E.F. and SAMBROOK, J. 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, USA.

McCUTCHEON, S.N. and BAUMAN, D.E. 1985. The application of hormones produced by recombinant DNA technology to increasing animal productivity. In: Reviews in Rural Science, "Biotechnology and Recombinant DNA Technology in the Animal Production Industries", eds. R.A. Leng, J.S.F. Barker, D.B. Adams and K.J. Hutchinson, vol.6, University of New England, Australia. pp 131-140.

McKNIGHT, S.L. and GAVIS, E.R. 1980. Expression of the herpes thymidine kinase gene in Xenopus laevis oocytes: an assay for the study of deletion mutants constructed in vitro. Nucleic Acids Res., 8, 5931-5948.

NANCARROW, C.D., MURRAY, J.D., BOLAND, M.P., SUTTON, R. and HAZELTON, I.G. 1984. Effects of gonadotrophin releasing hormone in the production of single-cell embryos for pronuclear injection of foreign genes. In: Reproduction in Sheep, eds. D.R. Lindsay and D.T. Pearce. Australian Academy of Science and Australian Wool Corporation, Canberra. pp 286-288.

PALMITER, R.D., BRINSTER, R.L., HAMMER, R.E., TRUMBAUER, M.E., ROSENFELD, M.G., BIRNBERG, N.C. and EVANS, R.M. 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature, 300, 611-615.

PALMITER, R.D., NORSTEDT, G., GELINAS, R.E., HAMMER, R.E. and BRINSTER, R.L. 1983. Metallothionein-human GH fusion genes stimulate growth in mice. Science, 222, 809-814.

PALMITER, R.D., WILKIE, T.M., CHEN, H.Y. and BRINSTER, R.L. 1984. Transmission distortion and mosaicism in an unusual transgenic mouse pedigree. Cell, 36, 869-877.

REIS, P.J. and SCHINKEL, P.G. 1963. Some effects of sulphur-containing amino acids on the growth and composition of wool. Aust. J. Biol. Sci., 16, 218-230.

REIS, P.J., TUNKS, D.A. and DOWNES, A.M. 1973a. The influence of abomasal and intravenous supplements of sulphur-containing amino acids on wool growth rate. Aust. J. Biol. Sci., 26, 249-258.

REIS, P.J., TUNKS, D.A. and SHARRY, L.F. 1973b. Plasma amino acid patterns in sheep receiving abomasal infusions of methionine and cysteine. Aust. J. Biol. Sci., 26, 635-644.

SMITH, C.J. and HESPELL, R.B. 1983. Prospects for development and use of recombinant deoxyribonucleic acid techniques with ruminal bacteria. J. Dairy Sci. 66, 1536-1546.

WAGNER, E.F., STEWART, T.A. and MINTZ, B. 1981a. The human β-globin gene and a functional viral thymidine kinase gene in developing mice. Proc. Natl. Acad. Sci. USA. 78, 5016-5020.

WAGNER, T.E., HOPPE, P.C., JOLLICK, J.D., SCHOLL, D.R., HODINKA, R.L. and GAULT, J.B. 1981b. Microinjection of a rabbit β-globin gene into zygotes and its subsequent expression in adult mice and their offspring. Proc. Natl. Acad. Sci. USA. 78, 6376-6380.

WAGNER, T.E. 1985. The role of gene transfer in animal agriculture and biotechnology. Can. J. Anim. Sci. 65, 539-552.

WARD, K.A. 1982. Possible contributions of molecular genetics to animal improvement. In 'Future Developments in the Genetic Improvement of Animals'. eds J.S.F. Barker, K. Hammond and A.E. McClintock. Academic Press Australia, Sydney. pp 17-39.

WARD, K.A., SLEIGH, M.J., POWELL, B.C. and ROGERS, G.E. 1982. The isolation and analysis of the major wool keratin gene families. Proc. of 2nd. World Congr. Genet. Appl. Livestock Product., 6, 146-156.

WARD, K.A., MURRAY, J.D., NANCARROW, C.D., SUTTON, R. and BOLAND, M.P. 1984. The role of embryo gene transfer in sheep breeding programmes. In 'Reproduction in Sheep'. eds. D.R. Lindsay and D.T. Pearce. Australian Academy of Science and Australian Wool Corporation, Canberra. pp 279-285.

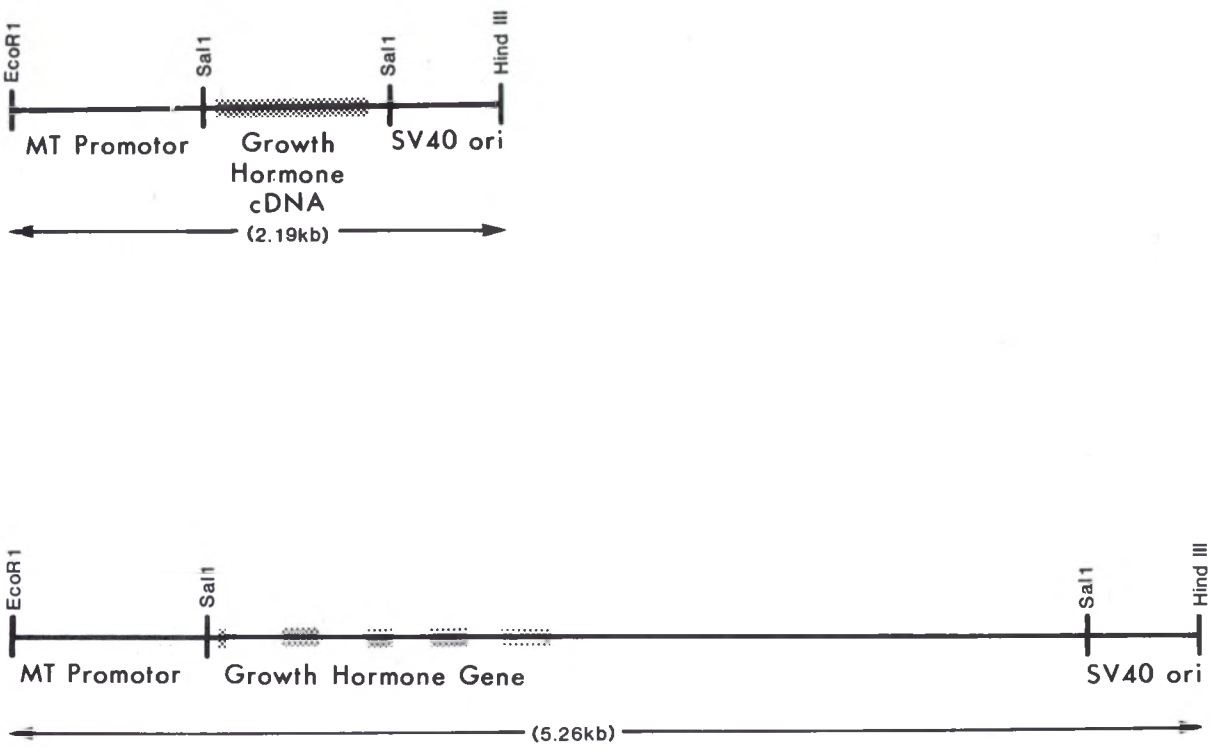

FIGURE 2:
DNA fusion genes for sheep embryo microinjection. (a) Sheep metallothionein promotor, full-length sheep growth hormone cDNA, SV40 origin of replication. (b) Same as (a), except that cDNA sequence replaced by 3.9 kb of sheep genomic DNA encoding growth hormone.

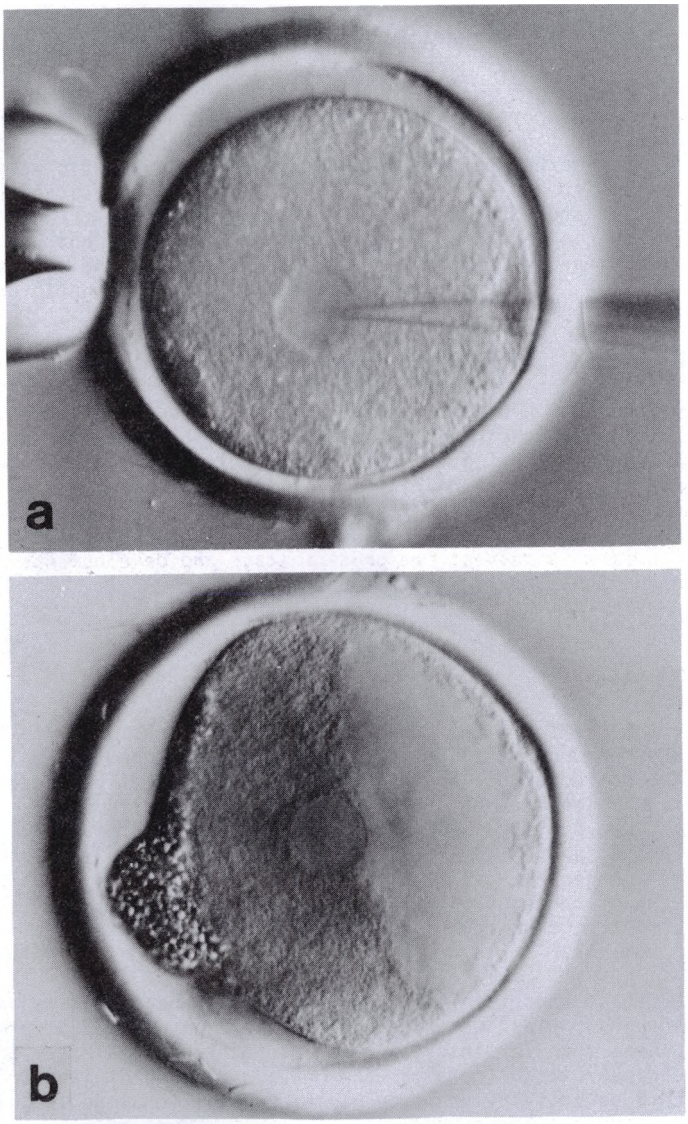

FIGURE 1. SHEEP ZYGOTES. A. PRONUCLEAR MICROINJECTION. B. PRONUCLEAR VISUALIZATION FOLLOWING CYTOCHALASIN B/CENTRIFUGATION.