J. Reprod. Fert., Suppl. **34** (1987), 237–250

Printed in Great Britain

Transgenic livestock

J. P. Simons and R. B. Land

AFRC Institute of Animal Physiology and Genetics Research, King's Buildings, West Mains Road, Edinburgh EH9 3JQ*

Summary. Single genes can now be added routinely to the genome of mice by molecular manipulation as simple Mendelian dominants; this complements the normal process of reproduction to give 'transgenic' animals. Success in ruminants is limited to a few examples in sheep and although gene expression has yet to be documented, there is every reason to expect that it will be achieved. The application of this technology to livestock improvement depends on the identification of circumstances in which the phenotype is limited by the deficiency of a single protein. While there is little evidence to indicate that single dominant genes are in general likely to have favourable effects, it is argued that there are likely to be exceptions. These include particular combinations of promoter and structural gene sequences to alter feedback control, for example through a change in tissue specificity, and the alteration of definitive proteins such as those of milk. A mouse model has been established to study the molecular manipulation of sheep milk proteins. The sheep beta lactoglobulin gene has been incorporated and the sheep whey protein is secreted by the mammary gland of transgenic mice.

For the future, means to delete or reduce the expression of existing genes are likely to be important, as are more effective means of incorporation such as retroviral based methods and the incorporation of multigene constructs. The resources required to test transgenic livestock will, however, be greater than those required to create them.

Introduction

Reproduction in domestic ruminants is sexual. Both parents transmit one half of their genes to each offspring and it is these genes inherited by the new individual which are largely responsible for the determination of its characteristics. The independent segregation of chromosomes at meiosis and their recombination in the new individual ensures genetic variation among individuals within a species, even within full sib families. Sexual reproduction therefore accommodates the need for both resemblance and variation. Natural selection among this limited variation is the basis of evolution; artificial selection enables the characteristics of livestock to be changed to better meet the needs of society.

The rate of response to selection is affected directly by the genetic variation in the population in question. This is illustrated dramatically in general terms by the much greater response to artificial selection in populations of *Drosophila* with transposing elements than in those without (McKay, 1985). Cytoplasmic inhibitors prevent the movement of P transposable elements in P but not M type stocks. New variation is induced in a cross in which the female parent is M type and the male parent is P type. The result is a 2-fold greater response to selection in this, dysgenic, population over the reciprocal, non-dysgenic, cross (Fig. 1). By contrast, asexual reproduction (cloning) would

*Formerly Animal Breeding Research Organisation.

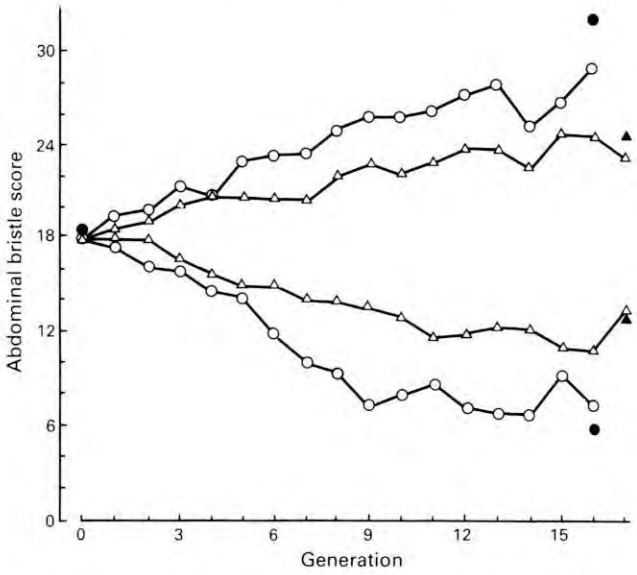

Fig. 1. Increased response to selection on dysgenic *Drosophila melanogaster*. Generation means of abdominal bristle score in lines selected for bristle number. ○, Lines derived from dysgenic flies (M♀♀ × P♂♂); □, non-dysgenic (P♀♀ × M♂♂) control lines. ●, ■, end points of the second replicates of the dysgenic and non-dysgenic selection lines respectively. (After Mackay, 1985.)

remove the genetic variation among individuals. This would both prevent further change and enable favourable existing genotypes to be replicated, as with plants such as fruit trees and potatoes.

Several procedures to introduce genetic variation have been considered. There is evidence from plants that genetic material from irradiated male gametes may be introduced at fertilization with a normal gamete following pollination with a mixture of normal and irradiated pollen (Powell *et al.*, 1983). The evidence for success in animals, however, is equivocal (McKay & Wishart, 1984) and furthermore there is no control over which genes are introduced.

Recombinant DNA techniques and the technology of embryo manipulation and transfer opened the way for specific genes to be introduced into animals (Gordon *et al.*, 1980) such that they are expressed (Brinster *et al.*, 1981; Wagner *et al.*, 1981a, b) and transmitted through the germ line (Costantini & Lacy, 1981; Gordon & Ruddle, 1981; Wagner *et al.*, 1981a); animals carrying newly introduced genes are termed 'transgenic'. Recent advances in the understanding of gene structure and expression, together with the ability to restructure genes, make it possible to target the additional variation to meet particular requirements. Such transgenic reproduction would cause animals to be quantitatively or qualitatively different, increasing the rate or extent to which livestock can adapt to meet the requirements of the community. Before considering transgenic reproduction, the subject of this paper, it is relevant to put this new technology in the context of other reproductive methods.

Multiple ovulation and embryo transfer would affect the rate of response to artificial selection of beef cattle dramatically and for dairy cattle would enable a small closed herd to achieve similar rates of response to those which might be attained in programmes based on progeny testing (Land & Hill, 1975; Nicholas & Smith, 1983). The importance of achieving similar rates of response to that with progeny testing should not be underestimated. With a single herd it is possible to focus decision making to ensure that selection is conducted to achieve breeding objectives. Even for sheep and goats the technique would have a favourable effect (Smith, 1986). Cloning, by contrast, would

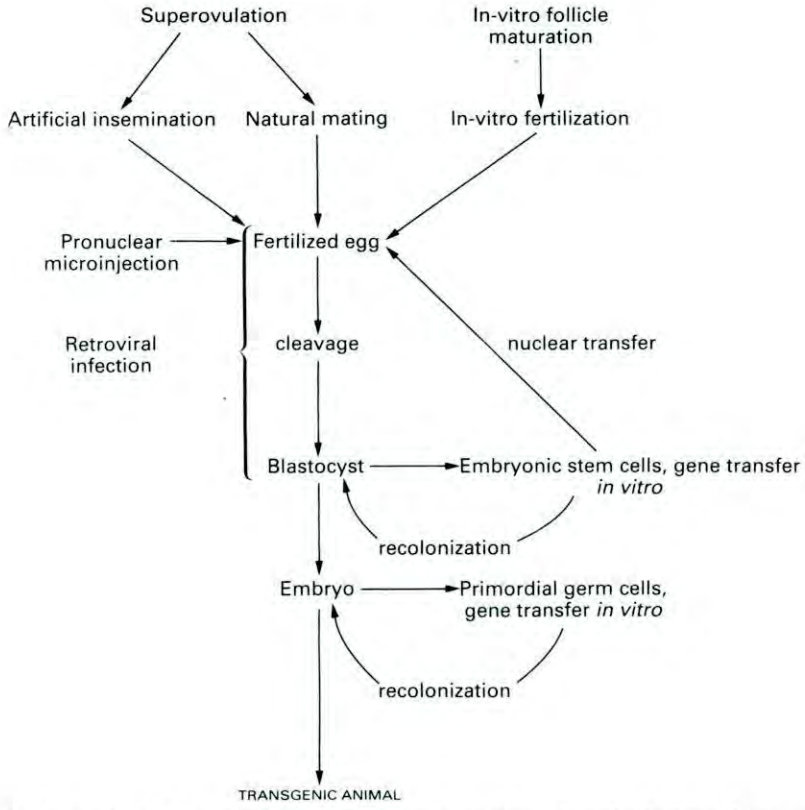

Fig. 2. Methods of making transgenic animals. A variety of methods have been used to make transgenic animals. These and others (not yet proven) are shown together with allied reproductive technologies.

have little impact (Gibson & Smith, 1986). It is proposed to discuss the present and likely future opportunities to transfer genes and then to consider the implications of transgenic reproduction.

Methods of gene transfer

The techniques for the transfer of genes fall into three broad classes: microinjection, infection by retroviral vectors and stem cell transfer. They are not all independent and the relationships between these and other relevant technologies are outlined in Fig. 2. Indeed it might be expected that the combination of these approaches may lead to significant increases in the rate of incorporation. Nevertheless, it is useful to consider them one by one.

Microinjection

The most widely used method for the generation of transgenic mammals is microinjection of DNA into one pronucleus of fertilized eggs. The eggs are usually obtained by superovulation and natural mating of the donor animals. They are immobilized by suction onto a blunt holding pipette, a fine injection pipette is inserted into one of the pronuclei and about 2 pl of the solution containing a few tens to a few hundred molecules of DNA are injected (Fig. 3). Surviving eggs are implanted into foster mothers, in which their development continues. A proportion of the animals which

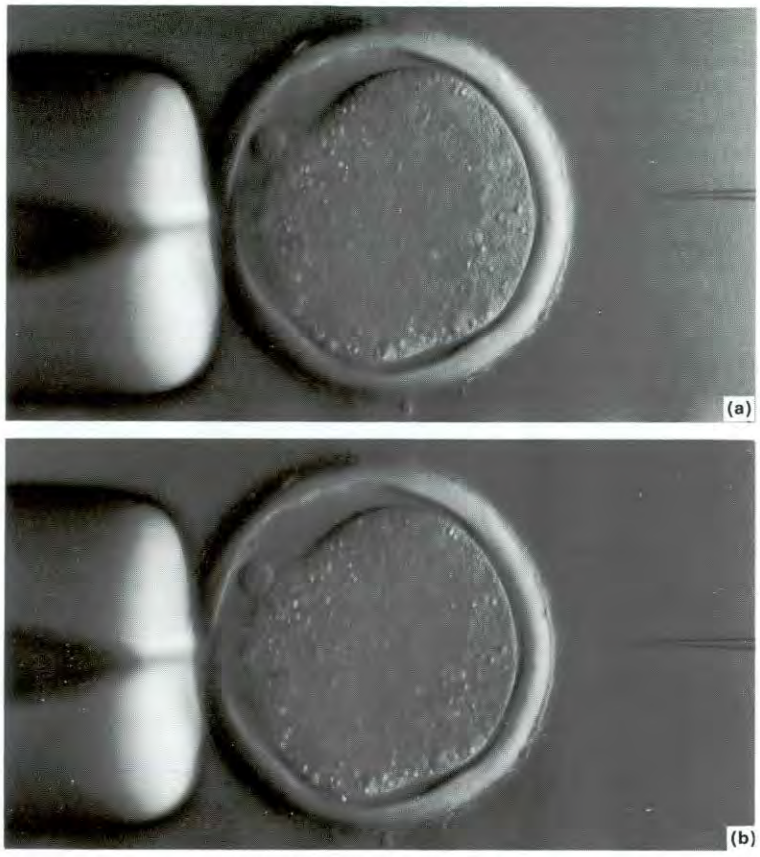

Fig. 3. A sheep egg (a) before and (b) after microinjection of DNA into a pronucleus, visualized by differential interference-contrast microscopy. The egg was held on the holding pipette (left) and injected using the injection pipette (right). The pronuclei can be seen to the right of centre and above and right of centre.

derive from the injected eggs will carry the new gene. These transgenic animals usually contain the injected gene in all cells, integrated into a chromosome. The integrated DNA is usually present in multiple copies, as a tandem repeat, integrated into a single site.

The presence of the gene in all cells is presumably due to integration before the first cleavage. The practical consequence of this is that transmission to the next generation is efficient, whereas if the recipients of the gene are chimaeric, the gene would be inherited by only a proportion of the offspring. This approach has been extremely successful for mice for which an average of 20% of eggs survive injection, transfer and development into young, and about 25% of the young carry the new gene (Brinster *et al.*, 1985). Microinjection has also been used to generate transgenic rabbits, pigs and sheep (Hammer *et al.*, 1985c), but the proportion of injected embryos which develop as transgenic young may be lower (Table 1). The success rates in mice are in general not dependent on the gene introduced. The loss of embryos is likely to arise from lethal physical damage at the time of injection, inefficiency of egg transfer or possibly lethal genetic damage due to the site of incorporation.

Microinjection is technically demanding, especially for farm animals which have almost opaque eggs, making visualization of nuclei difficult. In sheep, careful microscopy with differential interference-contrast optics is required (Hammer *et al.*, 1985c; J. P. Simons & I. Wilmut,

Table 1. Comparison of mice and sheep for efficiency of transgenic animal production by microinjection

	Mice[a]	Sheep[b]	Sheep[c]
No. of eggs injected and transferred	299	92	1032
No. of young	78[d]	23	73[e]
No. transgenic	23	1	1

(a) J. P. Simons, unpublished; sheep beta lactoglobulin gene.
(b) J. P. Simons, unpublished; pMK injected into 1- and 2-cell eggs.
(c) Hammer *et al.* (1985c); MT-hGH injected into 1-, 2- and 4-cell eggs.
(d) Surviving to weaning.
(e) Fetuses and lambs.

unpublished), and pig pronuclei may be seen after centrifugation of eggs (Wall *et al.*, 1985). Transfer of genes by microinjection of eggs requires large numbers of eggs at a precise stage. Such large numbers of eggs with pronuclei are easy to obtain from mice, but very expensive and time consuming to collect from large animals. For these reasons other possible routes which might increase the efficiency of incorporation would be very advantageous.

Retroviral vectors

Retroviruses have a genome of RNA which, on infection of a cell, is copied into DNA which is then integrated into a chromosome. Infection and integration can be extremely efficient (King *et al.*, 1985). Retroviral vectors have been constructed which carry non-viral sequences and are capable of infecting cells and integrating, but are defective in the production of new virus. Such vectors have the potential to carry new genes into the germ line of animals with minimal manipulation of embryos and with simultaneous treatment of large numbers of embryos. Experiments with mice have shown that this approach does work, but is inefficient as yet (van der Putten *et al.*, 1985; Jaehner *et al.*, 1985). The proportion of animals produced which are transgenic was no better than by microinjection and even this required co-infection with infectious helper virus; integration was less frequent in the absence of helper. An additional problem is that these experiments used eggs in the cleavage stages; transgenic animals derived in this way would be mosaic, the germ line would be chimaeric and genetic transmission less efficient. By contrast with microinjection, genes transferred using retroviral vectors will be integrated as single copies.

Embryo-derived stem cells

Embryo-derived stem cells are pluripotential cells which have been isolated from the inner cell masses of blastocysts (Evans & Kaufman, 1981; Martin, 1981). These cells may be grown *in vitro* for long periods and subsequently reintroduced into blastocysts. The introduced cells efficiently colonize the inner cell mass and contribute to the developing animal, which will be a mosaic of cells derived from the blastocyst and the reintroduced stem cells (Bradley *et al.*, 1984). The introduced stem cells contribute both to the soma and the germ line. A particular advantage of this route is that the fusion gene may be introduced into the stem cells *in vitro*. The genetically manipulated cells can then be selected and characterized directly, and only those with desirable characteristics subsequently introduced into blastocysts. Mosaic transgenic animals have been produced by this route (Lovell-Badge *et al.*, 1985; Wagner *et al.*, 1985). Expression of genes transferred by this route has been demonstrated (Lovell-Badge *et al.*, 1985), although germ-line transmission has not yet been reported. To date, there have been no reports of embryo-derived stem-cell lines isolated from species other than mice.

Achievements

The potential of gene transfer for the gross manipulation of animal phenotypes was dramatically demonstrated by the 'giant' mice of Palmiter *et al.* (1982). These animals carried a hybrid metallothionein/rat growth hormone gene, and were up to 1·87 times the weight of control non-transgenic littermates at 74 days of age. In these mice, rat growth hormone was synthesized under the control of the metallothionein promoter in the liver, an ectopic site for growth hormone synthesis. This ectopic expression of the growth hormone gene is insensitive to the feedback mechanisms which normally operate to determine the concentration of circulating growth hormone. Consequently, the levels of growth hormone in these mice were up to 700 times those of control animals. Similar results have been obtained with mice expressing high levels of human (Palmiter *et al.*, 1983) or bovine (Hammer *et al.*, 1985a) growth hormone. One secondary effect of abnormally high levels of growth hormone is frequent female infertility (Hammer *et al.*, 1984, 1985a). Mice have been generated which express human growth hormone-releasing factor under the control of the metallothionein promoter (Hammer *et al.*, 1985b). They also grow faster, although not to the same extent. The elevated concentrations of growth hormone-releasing factor stimulate increased growth hormone synthesis by the pituitary gland, the normal site of growth hormone synthesis. This manipulation did not lead to female sterility.

Another clear example of the impact of an introduced gene on the phenotype is the restoration of immune competence to respond to particular polypeptides. Some strains of mice are unable to make the I-E antigen, one of the class II major histocompatibility antigens, which are involved in the immune response. This deficiency results in an inability of these mice to mount an immune response to a synthetic polypeptide: poly(glutamic acid-lysine-phenylalanine). The deficient mice have no functional gene for the alpha subunit of the I–E antigen; introduction of functional genes for E alpha has been shown to restore the response to poly(glu-lys-phe) (Le Meur *et al.*, 1985; Yamamura *et al.*, 1985).

In addition to the examples given above, which have clear relevance to applications in ruminants, a great deal of information of less direct relevance has been obtained from the use of transgenic mice. Areas to which transgenic animal studies have been particularly fruitful are regulation of gene expression, oncogenesis and the regulation of immunoglobulin gene rearrangements. The field has been extensively reviewed by Palmiter & Brinster (1986).

The genes of choice

The evidence from large animals, laboratory studies and from theory all indicate that single genes in general are unlikely to have a significant favourable effect on commercial traits and it is concluded that successful genetic manipulation will depend on the identification of exceptions (Land & Wilmut, 1987). Genes code for proteins, additional genes will potentially lead to the synthesis of additional protein. Favourable effects of gene transfer will therefore be dependent upon circumstances in which a single protein is deficient except when the deficiency of that protein arises from an inadequacy of one or more amino acids. The options now available are to introduce genes for novel proteins or for the production of existing proteins at higher levels in different tissues or at different times.

Proteins act as signals (e.g. hormones), enzymes or definitive products such as wool, body structure or milk. The basis for the argument that individual genes are unlikely to have large effects and for the identification of circumstances in which message is likely to be limiting are best considered for each in turn.

Increased levels of signal proteins such as hormones do affect the phenotype. Follicle-stimulating hormone (FSH) is well known to increase the number of eggs shed by the ovary, and growth hormone increases the rate of milk production by the mammary gland. Natural genetic

variation in either trait is not, however, necessarily associated with variation in concentrations of the trophic hormone. Equally, the intrinsic synthetic capacity of the trophic system is not limited by the structural gene itself. In the case of FSH, for example, castration of the male and ovariectomy of the female both lead to a several-fold increase in the concentration of the hormone in peripheral plasma. The intrinsic synthesis potential for the hormone is not limiting, plasma concentrations are limited by the feedback control of gene expression. Any application of gene transfer to increase the output of signal proteins such as hormones would therefore require uncoupling of the synthesis of signal protein from the normal feedback control. The previously described giant mice of Palmiter *et al.* (1982) demonstrate well that this approach is effective. A refinement would be to express the structural gene from a promoter which is activated in the desired circumstances. Increased growth hormone synthesis during lactation, for example, would be expected to increase milk yield; such fine control will allow any deleterious effects of the manipulations to be minimized.

Enzymes affect the flux through pathways, but most pathways are made up of several steps, each dependent upon the effects of different enzymes. Kacser & Burns (1979) show theoretically that flux is insensitive to changes in individual enzymes and this is supported by experimental evidence, particularly in micro-organisms. Conversely, selection for the series of enzymes in a pathway does increase the flux and the phenotype, e.g. enzymes in NADP synthesis pathways in which high levels are positively correlative with the thickness of backfat in the pig (Muller, 1986). The implication is that it would be necessary to extend the present technology to constructs coding for several enzymes and this has recently been confirmed experimentally for tryptophan synthesis in yeast. Even many copies of genes for the individual enzymes had little impact on the flux but the introduction of a vector carrying genes for all 5 enzymes in the pathway showed a response in synthesis nearly proportional to the number of plasmids in the cell (P. Niederberger, quoted by Kacser, 1987). The application of transgenic technology to increase the rate of conversion of substrate to product through existing pathways would therefore depend on the development of multi-gene constructs. In addition, as described above, feedback control will often, if not always, need to be subverted.

In the third class of gene products are definitive proteins such as those in muscle, wool and milk. The rate of protein synthesis relates to the level of messenger RNA. The introduction and expression of an additional gene will change the message available for translation and hence, for definitive proteins, gene transfer affects the product directly. Of this group of products, only milk and wool proteins are truly definitive because muscle proteins are continually recycled. (The regulation of protein turnover is likely to have as important an effect on the rate of accretion of muscle as the expression of the structural genes for the muscle proteins themselves.) The keratin genes have been studied extensively, but the evidence indicates that the keratin messenger RNA is abundant in wool follicles and that the rate of wool production is limited more by the availability of the required amino acids than the availability of message (Ward *et al.*, 1986). The qualitative nature and the proportions of wool and milk proteins could, however, potentially be changed by introducing genes expressed in the wool follicle and mammary gland respectively, and the molecular biology programme in our laboratory has focussed on alteration of the composition of milk. In 1982 Palmiter *et al.*, on finding very high levels of circulating growth hormone in some of their transgenic mice, suggested that transgenic animals may be useful as production systems for valuable proteins. Lathe *et al.* (1986) suggested that the mammary gland of the lactating ewe would be the system of choice for such applications, being so prodigious in protein synthesis and secretion, and milk being so easily harvested. There is the opportunity to introduce genes coding for novel proteins of high value such as medicines or industrial enzymes, and to modify the composition and hence the nutritional value of milk as a food.

The molecular manipulation of milk

Milk is composed largely of water, fats, lactose and proteins. The most abundant proteins in the

milk of ruminants are the caseins, beta lactoglobulin and alpha lactalbumin. The most abundant single protein is beta casein, at up to 16 g/litre. Each of these proteins is synthesized in the mammary gland; other more minor components, including proteins such as albumin, enter the milk from the blood stream.

Various considerations, however, make it desirable to have a model system for the molecular manipulation of ruminant milk. First, as already outlined, the production of transgenic ruminants is time consuming, expensive and not yet routine. Second, the timescale for experiments with ruminants is very long: in sheep, it may take 2–3 years from the time of injection of the DNA into an egg until milk can be harvested from a transgenic ewe. Two models were considered, primary mammary culture systems in which the effectiveness of alternative constructs could be evaluated after transfection, and transgenic mice. Given that fully differentiated mammary tissue cultures are not available and that transgenic mice will be a closer model, the latter was the option of choice: for a transgenic mouse the interval from egg injection to milking can in principle be 3 months.

Fig. 4. SDS polyacrylamide gel electrophoresis of milk proteins to show sheep beta lactoglobulin in milk of a transgenic mouse: (A) control mouse whey, (B) control sheep whey, (C) whey from a mouse transgenic for the sheep beta lactoglobulin gene. The arrow shows the position of sheep beta lactoglobulin.

In our first experiments, the sheep gene which codes for beta lactoglobulin was isolated and introduced into mice, which do not normally make beta lactoglobulin. Figure 4 shows an electrophoretic analysis of the whey proteins from one such mouse. A major band which comigrates with sheep beta lactoglobulin is present, and this has now been shown to be genuine sheep beta lactoglobulin by western blotting. The concentration of beta lactoglobulin in this milk is approximately 20 mg/ml. Several other lines of mice transgenic for the sheep beta lactoglobulin gene similarly secrete this protein.

These results demonstrate, first, that the sheep milk protein gene can be incorporated into the genome of the mouse in such a way that it is expressed in the homologous tissue. Breeding experiments also show that the gene and its expression are inherited. The next questions are whether the expression is limited to the mammary gland and whether the milk protein genes can be used as vehicles for the control of structural genes for specific non-milk proteins. A schematic construct and its expression are illustrated in Fig. 5. The mouse model system will allow the assessment of a variety of combinations of components of constructs designed for the production of foreign proteins, allowing relatively rapid optimization of construct design. Having shown that beta lactoglobulin expression is not species specific, the results obtained in mice should be directly relevant to ruminants.

The production of high value proteins such as medicines and industrial enzymes is a simple application of transgenic technology in ruminants, whether they be cattle, goats or sheep. The vigorous expression of the sheep beta lactoglobulin gene in the mouse also indicates that it might well be possible to increase the proportion of protein in ruminant milk by the introduction of additional structural genes for the milk protein required. Such manipulations could be aimed simply to improve the nutritional value of milk (for human consumption or to increase the growth

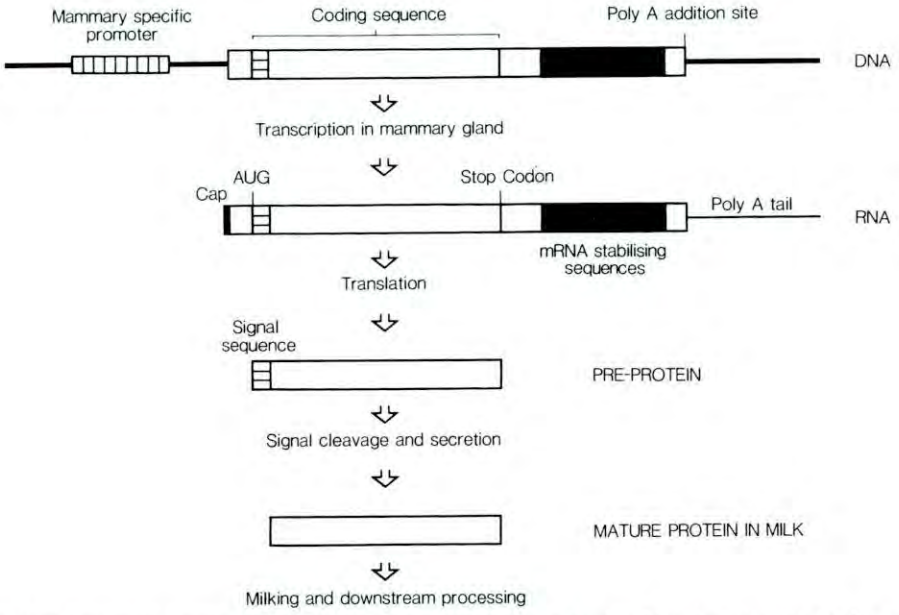

Fig. 5. Diagram of a generalized gene designed to direct synthesis of foreign proteins in the milk of transgenic animals, and steps in its expression. The mammary-specific promoter directs the synthesis of a messenger RNA. Sequences in the RNA ensure that the mRNA is stable in the mammary gland. The coding sequence is translated into a precursor protein from which the signal peptide is cleaved when the protein is secreted. The mature protein is harvested in the milk.

rate of suckled young), or to alter milk qualitatively to make it more suitable for processing by the dairy industry.

Other options would be to reduce the lactose content of milk. Not only is lactose the least valuable of the milk solids, the vast majority of humans lose the ability to digest lactose at weaning. These people cannot ingest milk or dairy products without severe discomfort resulting. The production of lactose-free milk is thus an attractive possibility. One route to this end would be to construct animals which secrete lactase, the enzyme which hydrolyses lactose, in their milk. The lactose would be broken down to glucose and galactose *in situ*. A second method of producing lactose-free milk would be to prevent the synthesis of lactose, which occurs within the mammary gland and which requires alpha lactalbumin as a factor in the combination of glucose and galactose to form the disaccharide. This, however, would require the extinction of alpha lactalbumin expression. While this is presently beyond existing transgenic technology, possible methods are discussed later.

Application to livestock breeding

Novel products are an intermediate term application of new molecular biological knowledge to the livestock industry. The product can be readily identified and harvested. Even if the fertility or another component of normal performance were depressed, the high value of the product would outweigh such a disadvantage. In conventional agriculture, however, it would be necessary for the transgenic process to increase the economic merit of the strain in which it is practised. Therefore, not only must the addition of a single gene have a desirable effect on a biological component of merit, but the benefits of this effect must be greater than any disadvantageous side effects.

Possible strategies for the use of transgenic stock are discussed by Smith *et al.* (1987) who draw attention to two points of particular importance. First, there is at present no control of the site of incorporation; while the direct effects of a particular fusion gene might be similar each time it is introduced, the indirect effects would be expected to be unique through the effects of incorporation on the function of adjacent genes. Each transgenic introduction would therefore have to be evaluated separately. Second, transgenics cannot be assessed accurately as individuals and it will be necessary to establish populations both hemi- and homozygous for the new gene for the effects to be measured. Indeed, the difficulty of assessment is such that it might well be more cost effective simply to introduce potentially useful transgenics to the population and allow the genes to find their own frequency under the effects of selection. In any case, but with such an approach in particular, it would be important to work with the best populations.

To put the question of testing into perspective, resources have never been allocated for the accurate comparison of existing populations so it is not reasonable to assume that they will be available for new ones. It must be remembered that the numbers required are considerable; 50 offspring would be required to give a 95% chance of detecting a gene which increased the mean by one standard deviation; 16 would be required even when the effect was as large as 2 standard deviations. Traditional selection would be expected to change the mean by 2% per generation so that the mean could be increased by 10% in the duration of the 5 or so generations needed to introduce and evaluate a transgenic. A threshold of useful effects might then be 10% of the mean. For typical growth traits with a coefficient of variation of 10%, the 50 offspring given above would be required for testing. For reproductive traits with coefficients of variation of 25%, 250 animals would be required!

On occasion, integration of a new gene disrupts an endogenous gene, termed 'insertional mutation' (see Palmiter & Brinster, 1986). One consequence of disruption of endogenous genes at the site of incorporation is that the adverse side effects of transgenesis may be fully recessive and hence it is important to test homozygous as well as hemizygous populations. If hemizygous favourable transgenics are introduced, half of the population will be hemizygous before the proportion of

homozygotes would be expected to reach 10%. Most insertional mutations are recessive lethals, and so retrospective culling could have to be very severe.

As with the need to consider exceptions in the identification of genes which affect the biological components of economic merit, it is also perhaps relevant to consider exceptions in the identification of target biological traits for transgenic improvement. The addition of growth hormone genes could have some beneficial effects, but might also have both direct and indirect adverse effects. In other cases, the only adverse effects which might reasonably be anticipated are those resulting from insertional mutation. One example is disease resistance for which additional MHC loci could be introduced. More specifically, the expression of viral coat protein genes in animals normally susceptible to virus infection would competitively reduce the binding of infecting viruses to the receptor proteins on the cell surface and so confer resistance to infection. Exactly the same path to resistance has recently been demonstrated in plants: the introduction into tobacco plants of a gene which codes for the coat protein of the tobacco mosaic virus interfered with the normal progression of the viral infection (Abel *et al.*, 1986).

Knowledge for the future

The knowledge that gene products are in general unlikely to be limiting indicated the need to look for exceptions. Equally, the same knowledge indicates that the phenotype is susceptible to a reduction in the product of a single gene. The manipulation of single loci could therefore be highly relevant in circumstances such as those in which decreased levels of the product decrease negative feedback on the target system. One example would be the feedback control of reproduction where the inhibition of the production of ovarian hormones controlling the release of FSH would be likely to increase the ovulation rate. Active and passive immunization against ovarian steroids increases the ovulation of sheep (Scaramuzzi & Hoskinson, 1984; Webb *et al.*, 1984), but this technology has not been extended to cattle. The discovery of key feedback hormones would identify targets for immunization, and knowledge of steps in the biosynthesis of these hormones would indicate targets for gene neutralization.

Two potential methods for gene neutralization can be envisaged: anti-sense gene expression and site-directed mutation. The first step in expression of a gene is transcription, i.e. synthesis of a messenger RNA (mRNA) using one of the gene's strands, the coding strand, as a template. The mRNA is a 'sense' transcript. In normal circumstances the complementary strand is not transcribed. By rearranging the gene, however, expression of the complementary strand can be obtained. This transcribed anti-sense RNA and the mRNA from the coding strand are complementary with each other and so can hybridize. This will form a double-stranded RNA, interfering with one or more of the subsequent steps of gene expression: RNA processing, export from the nucleus and translation (Fig. 6). Experimental reduction of gene expression by anti-sense gene expression has been demonstrated (Izant & Weintraub, 1984; Kim & Wold, 1985; McGarry & Lindquist, 1986). One potential problem with this method of gene neutralization is that, due to the kinetics of hybridization, a large excess of anti-sense RNA over mRNA is required. In addition, if the gene being neutralized is subject to feedback control, the result may simply be to increase the rate of transcription. It is probable that anti-sense methods will result in a reduction of expression rather than total extinction; this may well prove to be a useful feature.

A second method for the removal of gene function may be in-vivo site-directed mutagenesis. It has been shown that genes introduced into cultured cells can integrate by homologous recombination (Smithies *et al.*, 1985; Thomas *et al.*, 1986). This could be used to extinguish gene expression by inserting DNA which disrupts the gene (Fig. 7). Such homologous recombination is infrequent, and as yet would not be applicable in transgenic animals for this reason. It is likely, however, that, as the mechanism of homologous recombination becomes elucidated, conditions which increase the frequency will be determined. The combination of the embryo-derived stem-cell route and

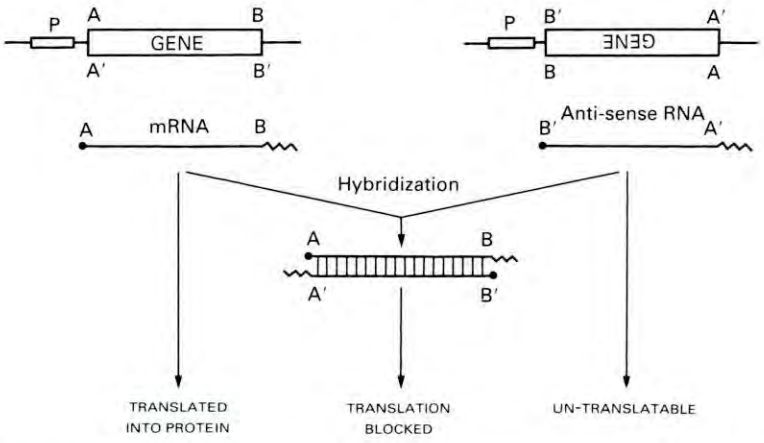

Fig. 6. Neutralization of gene expression by anti-sense gene expression. An anti-sense gene directs the synthesis of anti-sense RNA. This RNA hybridizes to the mRNA transcript of the gene to be neutralized. The hybridization removes the mRNA from the pool available for translation into protein resulting in reduced levels of the protein product.

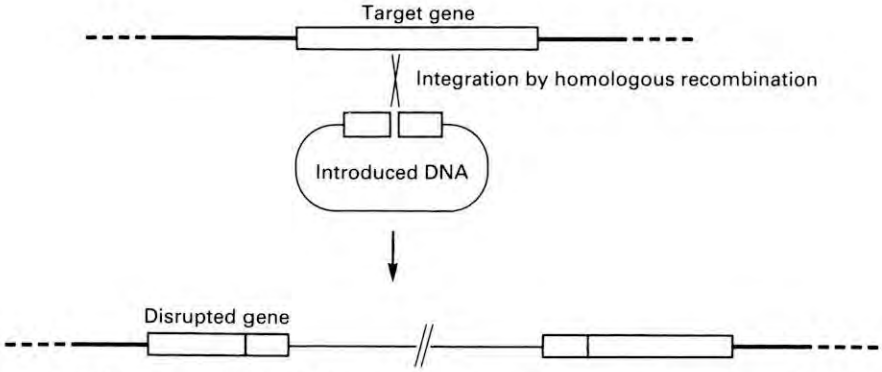

Fig. 7. Gene disruption. The DNA introduced has homology with the target gene (open boxed regions). On occasion, integration will be by homologous recombination; this results in a partial duplication of the gene sequences and an interruption of the gene. The interruption will prevent normal expression of the gene.

homologous recombination is attractive: it would be possible to select those cell lines which have the desired integration *in vitro*, before entering the more expensive phase of work with animals.

It should be pointed out that anti-sense extinction of expression will be a dominant effect since it acts on the RNA transcribed from both alleles of the gene in question. Gene disruption by homologous recombination will in most cases be a recessive mutation, and the effects can only be assessed by breeding to homozygosity. In the longer term we should hope to be able to change single nucleotides in a predetermined way; this would allow, in addition to complete extinction, very fine manipulation of genes potentially in both regulatory and coding regions.

Transgenic biology has clear implications for animal improvement. Although there are many potential pitfalls, the application to livestock improvement has considerable, widespread potential. Simple traits will be the easiest to manipulate. We have chosen to focus on manipulation of milk composition, and have demonstrated, in mice, that this is feasible. Success in these endeavours will

depend on careful design of the genes transferred. This design will be aided by an understanding of regulation at all levels: genetic, biochemical and physiological. In addition, the widespread successful application of transgenic technology will require improvement of existing and development of new techniques to increase the efficiency of generation of transgenic animals and to allow new types of manipulation.

The application of the technology will, however, depend on the establishment of a suitable social framework. The new gene is neither a drug nor an infectious agent and falls outside the legislation for either. The basic advantages of genetic improvement over direct manipulation of stock are strengthened by current moves against the use of hormones in commerce and the progressively strengthening preference for natural products. Transgenic practice could enhance these advantages by increasing the rate at which the characteristics of stock could be changed to meet the requirements of the community.

We thank many colleagues who have contributed to the establishment of molecular biology research at ABRO, particularly Dr Bishop, Dr Clark, Dr Lathe and Dr Wilmut and the farm staff who have made it possible.

References

Abel, P.P., Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T. & Beachy, R.N. (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science, N.Y.* **232,** 738–743.

Bradley, A., Evans, M., Kaufman, M.H. & Robertson, E. (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature, Lond.* **309,** 255–256.

Brinster, R.L., Chen, H.Y. & Trumbauer, M. (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., Trumbauer, M.E., Yagle, M.K. & Palmiter, R.D. (1985) Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc. nat. Acad. Sci. U.S.A.* **82,** 4438–4442.

Costantini, F. & Lacy, E. (1981) Introduction of a rabbit β-globin gene into the mouse germ line. *Nature, Lond.* **294,** 92–94.

Evans, M.J. & Kaufman, M.H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature, Lond.* **292,** 154–156.

Gibson, J.P. & Smith, C. (1986) Technology and animal breeding applications in livestock improvement. *Proc. 3rd Wld Congr. Genet. Appl. Livest. Prod., Lincoln, Nebraska,* Vol. 12, 96–105.

Gordon, J.W. & Ruddle, F.H. (1981) Integration and stable germ line transmission of genes injected into mouse pronuclei. *Science, N.Y.* **214,** 1244–1246.

Gordon, J.W., Scangos, G.A., Plotkin, D., Barbosa, J.A. & Ruddle, F.H. (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. natn. Acad. Sci. U.S.A.* **77,** 77380–77384.

Hammer, R.E., Palmiter, R.D. & Brinster, R.L. (1984) Partial correction of murine hereditary growth disorder by germ-line incorporation of a new gene. *Nature, Lond.* **311,** 65–67.

Hammer, R.E., Brinster, R.L. & Palmiter, R.D. (1985a) Use of gene transfer to increase animal growth. *Cold Spring Harbor Symp. Quant. Biol.* **50,** 379–387.

Hammer, R.E., Brinster, R.L., Rosenfeld, M.G., Evans, R.M. & Mayo, K.E. (1985b) Expression of human growth hormone-releasing factor in transgenic mice results in increased somatic growth. *Nature, Lond.* **315,** 413–416.

Hammer, R.E., Pursel, V.G., Rexroad, C.E., Jr, Wall, R.J., Bolt, D.J., Ebert, K.M., Palmiter, R.D. & Brinster, R.L. (1985c) Production of transgenic rabbits, sheep and pigs by microinjection. *Nature, Lond.* **315,** 680–683.

Izant, J.G. & Weintraub, H. (1984) Inhibition of thymidine kinase gene expression by anti-sense RNA: a molecular approach to genetic analysis. *Cell* **36,** 1007–1015.

Jaehner, D., Kirsten, H., Mulligan, R. & Jaenisch, R. (1985) Insertion of the bacterial *gpt* gene into the germ line of mice by retroviral infection. *Proc. natn. Acad. Sci. U.S.A.* **82,** 6927–6931.

Kacser, H. (1987) Biochemical pathways and their control. In *Mathematical Models in Microbiology:* 3. *Physiological Models* (in press). Eds M. J. Bazin & J. I. Prosser. CRC Press, Boca Raton.

Kacser, H. & Burns, J.A. (1979) Molecular democracy: who shares the controls? *Biochem. Soc. Trans.* **7,** 1149–1160.

Kim, S.K. & Wold, B.J. (1985) Stable reduction of thymidine kinase activity in cells expressing high levels of anti-sense RNA. *Cell* **42,** 129–138.

King, W., Patel, M.D., Lobel, L.I., Goff, S.P. & Chi Nguyen-Huu, M. (1985) Insertion mutagenesis of embryonal carcinoma cells by retroviruses. *Science, N.Y.* **228, 554–558.**

Land, R.B. & Hill, W.G. (1975) The possible use of superovulation and embryo transfer in cattle to increase response to selection. *Anim. Prod.* **21,** 1–12.

Land, R.B. & Wilmut, I. (1987) Gene transfer and animal breeding. *Theriogenology* **27,** 169–179.

Lathe, R., Clark, A.J., Archibald, A.L., Bishop, J.O., Simons, P. & Wilmut, I. (1986) Novel products from livestock. In *Exploiting New Technologies in Animal Breeding: Genetic Developments*, pp. 91–102. Eds C. Smith, J. W. B. King & J. C. McKay. Oxford University Press, Oxford.

Le Meur, M., Gerlinger, P., Benoist, C. & Mathis, D. (1985) Correcting an immune-response deficiency by creating E_α gene transgenic mice. *Nature, Lond.* **316,** 38–42.

Lovell-Badge, R.H., Bygrave, A.E., Bradley, A., Robertson, E., Evans, M.J. & Cheah, K.S.E. (1985) Transformation of embryonic stem cells with the human type-II collagen gene and its expression in chimeric mice. *Cold Spring Harbor Symp. Quant. Biol.* **50,** 707–711.

Mackay, T.F.C. (1985) Transposable element-induced response to artificial selection in drosophila melanogaster. *Genetics, Princeton* **111,** 351–374.

Martin, G.R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. natn. Acad. Sci. U.S.A.* **78,** 7634–7638.

McGarry, T.J. & Lindquist, S. (1986) Inhibition of heat shock protein synthesis by heat-inducible antisense RNA. *Proc. natn. Acad. Sci. U.S.A.* **83,** 399–403.

McKay, J.C. & Wishart, G.J.H. (1984) Genetic and physiological effects of irradiation of fowl semen. *Proc. 26th British Poultry Breeders' Roundtable, 1984, Norwich.*

Muller, E. (1986) Physiological and biochemical indicators of growth and composition. In *Exploiting New Technologies in Animal Breeding*, pp. 132–139. Eds C. Smith, J. W. B. King & J. C. McKay. Oxford University Press, Oxford.

Nicholas, F.W. & Smith, C. (1983) Increased rate of genetic change in dairy cattle by embryo transfer and splitting. *Anim. Prod.* **36,** 341–353.

Palmiter, R.D. & Brinster, R.L. (1986) Germline transformation of mice. *Ann. Rev. Genetics* 465–499.

Palmiter, R.D., Brinster, R.L., Hammer, R.E., Trumbauer, M.E., Rosenfeld, M.G., Birnberg, N.C. & Evans, R.M. (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature, Lond.* **300,** 611–615.

Palmiter, R.D., Norstedt, G., Gellnas, R.E., Hammer, R.E. & Brinster, R.L. (1983) Metallothionein-human GH fusion genes stimulate growth of mice. *Science, N.Y.* **222,** 809–814.

Powell, W., Caligari, P.D.S. & Hayter, A.M. (1983) The use of pollen irradiation in barley breeding. *Theor. appl. Gen.* **65,** 73–76.

Scaramuzzi, R.J. & Hoskinson, R.M. (1984) Active immunisation against steroid hormones for increasing fecundity. In *Immunological Aspects of Reproduction in Mammals*, pp. 445–474. Ed. D. B. Crighton. Butterworths, London.

Smith, C. (1986) Use of embryo transfer in genetic inprovement of sheep. *Anim. Prod.* **42,** 81–88.

Smith, C., Meuwissen, T. & Gibson, J.P. (1987) On the use of transgenes in livestock improvement. *Anim. Breed. Abstr.* **55,** 1–10.

Smithies, O., Gregg, R.G., Boggs, S.S., Koralewski, M.A. & Kucherlapati, R.S. (1985) Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination. *Nature, Lond.* **317,** 230–234.

Thomas, K.R., Folger, K.R. & Capecchi, M.R. (1986) High frequency targeting of genes to specific sites in the mammalian genome. *Cell* **44,** 419–428.

van der Putten, H., Botteri, F.M., Miller, A.D., Rosenfeld, M.G., Fan, H., Evans, R.M. & Verma, I.M. (1985) Efficient insertion of genes into the mouse germ line via retroviral vectors. *Proc. natn. Acad. Sci. U.S.A.* **82,** 6148–6152.

Wagner, T.E., Hoppe, P.C., Jollick, J.D., School, D.R., Hodinka, R.L. & Gault, J.B. (1981a) Microinjection of a rabbit β-globin gene into zygotes and its subsequent expression in adult mice and their offspring. *Proc. natn. Acad. Sci. U.S.A.* **78,** 6376–6380.

Wagner, E.F., Stewart, T.A. & Mintz, B. (1981b) The human β-globin gene and a functional viral thymidine kinase gene in developing mice. *Proc. natn. Acad. Sci. U.S.A.* **78,** 5016–5020.

Wagner, E.F., Keller, G., Gilboa, E., Ruther, U. & Stewart, C. (1985) Gene transfer into murine stem cells and mice using retroviral vectors. *Cold Spring Harbor Symp. Quant. Biol.* **50,** 691–700.

Wall, R.J., Pursel, V.G., Hammer, R.E. & Brinster, R.L. (1985) Development of porcine ova that were centrifuged to permit visualization of pronuclei and nuclei. *Biol. Reprod.* **32,** 645–651.

Ward, K.A., Franklin, I.R., Murray, J.D., Nancarrow, C.D., Raphael, K.A., Rigby, N.W., Byrne, C.R., Wilson, B.W. & Hunt, C.L. (1986) The direct transfer of DNA by embryo microinjection. *Proc. 3rd Wld Congr. Genet. Appl. Livest. Prod., Lincoln, Nebraska,* Vol. 12, 6–21.

Webb, R., Land, R.B. & Pathiraja, N. (1984) Passive immunisation against steroid hormones in the female. In *Immunological Aspects of Reproduction in Mammals*, pp. 475–499. Ed. D. B. Crighton. Butterworths, London.

Yamamura, K., Kikutani, H., Folsom, V., Clayton, L.K., Kimoto, M., Akira, S., Kashiwamura, S., Tonegawa, S. & Kishimoto, T. (1985) Functional expression of a microinjected E_α^d gene in C57BL/6 transgenic mice. *Nature, Lond.* **316,** 67–69.

Note added in proof. Since submission of this paper, we have obtained a further 5 transgenic sheep.

Germ-line transmission of genes transferred into mice via embryo-derived stem-cells has been reported. The gene transfer into stem-cells was performed by repeated retroviral vector infection, without selection (Robertson *et al.*, 1986), or by calcium phosphate-mediated transfection with biochemical selection for the introduced gene (Gossler *et al.*, 1986).

Gossler, A., Doetschman, T., Korn, R., Serfling, E. & Kemler, R. (1986) Transgenesis by means of blastocyst-derived embryonic stem cell lines. *Proc. natn. Acad. Sci U.S.A.* **83,** 9065–9069.

Robertson, E., Bradley, A., Kuehn, M. & Evans, M. (1986) Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. *Nature, Lond.* **323,** 445–448.