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# From animals in the service of nutrition . . . to the potential of biotechnology

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In 1986, in her paper, 'Animals in the service of human nutrition', celebrating the award of the E. V. McCollum International Lectureship in Nutrition, Dr Elsie Widdowson observed: 'Animals have served human nutrition well over the past century .... They are still of great service in human nutrition and may be more essential in the future as proper animal models for human diseases are discovered'. Ten years on, those animal models are an integral part of nutrition research and are providing fundamental tools to study the effects of diet on many of the major diseases of the Western world, including cardiovascular disease, obesity and cancer. Many of these models have been developed through the use of recombinant DNA technology and the expression of normal or mutated genes in the genome of transgenic mice.

Recombinant DNA technology originates from the elucidation of the structure of DNA in 1953 by two scientists, like Dr Widdowson, working at the University of Cambridge (Watson & Crick, 1953). Their discovery of the double helix held together by adenine–thymine and guanine–cytosine base pairs, opened up a whole new technology in which scientists are able to manipulate genetic material. In simple terms, individual genes can now be excised from the chromosomal DNA of bacteria, plants or animals using specific restriction enzymes and introduced into specialized DNA cloning vectors. These vectors not only allow the gene to be expressed at high levels resulting in copious amounts of protein, but also allow the gene sequence to be manipulated to change, delete or insert nucleotides. Genetic manipulation knows few boundaries. The technology allows us to express mammalian genes in bacteria and bacterial genes in mammals. Animals expressing 'introduced' or 'foreign' genes are called transgenic animals and the introduced or foreign gene is called a transgene.

## TRANSGENIC TECHNOLOGY

### *Gene expression directed to a specific cell type*

Transgenic technology is a relatively precise science for it allows us to direct the expression of a transgene to a specific animal tissue or cell type. This is achieved by using specific DNA regulatory sequences that control the expression of a particular gene. If, for example, transgene expression is required only in the exocrine pancreas of a transgenic animal, e.g. the mouse, DNA regulatory sequences are used from a gene which is only expressed in the exocrine pancreas, for example the elastase 1 gene which encodes a pancreatic protease. By fusing the elastase 1 regulatory sequences to a structural gene, e.g. human growth hormone (hGH), expression of hGH is limited to the exocrine pancreas of the transgenic animal (Hammer *et al.* 1987). Similarly the technology can be used to

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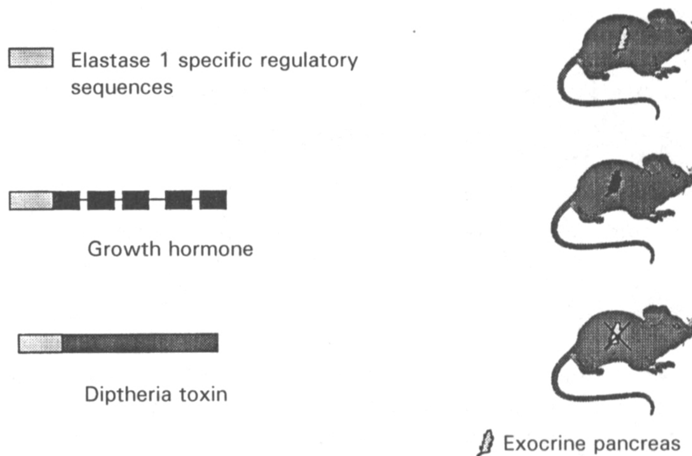


Fig. 1. Directing gene expression to the exocrine pancreas. Fusion of the rat elastase 1 regulatory sequences, −205 to +8, to a structural gene e.g. human growth hormone, directs the expression and synthesis of growth hormone to the exocrine pancreas of a transgenic animal. If the structural gene is a 'toxigene', e.g. diptheria toxin, then this results in the specific ablation of the exocrine pancreatic tissue.

ablate specific tissues or cell types. In this case the regulatory sequences are fused to a 'toxigene', e.g. the gene encoding diptheria toxin A polypeptide. If the elastase 1 regulatory sequences are fused to the diptheria toxin gene then the exocrine pancreas is destroyed (Palmiter *et al.* 1987) (Fig. 1).

Foreign genetic material can be introduced into experimental animals by several methods of which DNA microinjection is the most widely used (Allen *et al.* 1987). For this procedure, which has been developed in mice, 1 d fertilized eggs are flushed from a superovulated mouse and injected with a solution (1–2 pl) containing about 200–500 copies of DNA. The injected eggs are allowed to divide *in vitro* to the two or four cell stage and then surgically introduced into pseudopregnant female mice. The resulting offspring are screened for the transgene by Southern analysis and those containing the foreign DNA (about 1 : 3 for mice transgenics) bred to establish transgenic lines (Fig. 2).

Microinjection, however, allows no control over the position of the foreign DNA in the mouse chromosome, with integration being a completely random event. Control of the integration site can be achieved by the use of embryonic stem cells. These cells are derived from the inner cell mass of a 3-d-old blastocyst and are cultured under *in vitro* conditions which allow them to grow and divide but not differentiate. These cells can then be used to target foreign DNA to specific chromosomal sites by homologous recombination.

## *Gene knockout*

Embryonic stem (ES) cells are routinely used to inactivate or 'knockout' genes or replace defective genes. Gene knockout has also been used to create transgenic models which simulate human diseases. The target gene to be manipulated is first isolated *in vitro* and cloned into a vector where it is easily modified. In general the gene sequence is disrupted by the introduction of DNA sequences encoding resistance to the antibiotic neomycin ($neo^{R}$) and sensitivity ($tk^{HSV}$) to the drug gancyclovir. The modified DNA is then introduced into the ES cells were it recombines with the chromosomal DNA. The DNA can

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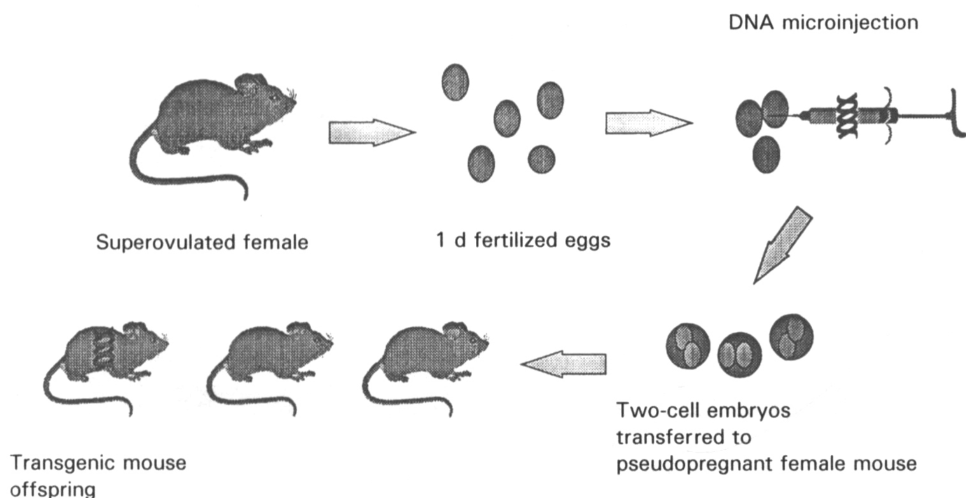


Fig. 2. DNA microinjection in mice. Fertilized eggs (1-d-old) collected from superovulated mice are each microinjected with 1–2 pl foreign DNA (200–500 copies). Following cell division the embryos are transferred to pseudopregnant mice. Offspring are analysed for the transgene by Southern hybridization. In mice approximately 30 % of the offspring born are transgenic.

undergo either homologous or non-homologous recombination. Following homologous recombination, where the gene recombines with the target gene, the integrated DNA loses its sensitivity to gancyclovir. In contrast DNA that randomly integrates into the chromosome retains its sensitivity to the drug (Fig. 3). The ES cells in which the target gene has been modified can then be selected using neomycin and gancyclovir, and microinjected into a recipient mouse blastocyst which is subsequently transferred into a pseudopregnant mouse. Mating mice heterozygous for the knockout allele will result in progeny homozygous for the knockout mutation.

## *Animal cloning by nuclear transfer*

It has not yet proven possible to culture ES cells from large animals such as sheep and cattle. In addition, microinjection is very inefficient, < 0·5–2 %, in these species and as a result large-animal transgenics has lagged behind that of mice although there have been notable successes (see pp. S131–S132). In 1996, a technique to create transgenic animals, involving nuclear transfer, was published by scientists at Roslin, Edinburgh (Campbell *et al.* 1996). In this system embryonic disc cells of a 9 d sheep embryo are dissected and cultured *in vitro* to produce an epithelium of thousands of identical cells, designated TNT (totipotent for nuclear transfer) cells. Oocytes are then taken from a second donor animal and enucleated so that all the genetic material is lost. For embryo reconstruction, a single TNT cell is fused to the enucleated oocyte by electroporation (high voltage) and the cell, under the influence of the donor cytoplasm, develops into an embryo. The reconstructed embryos are then transferred to recipient animals for development to term. All the offspring arising from the procedure are derived from a single cell population and are thus clones of one another (Fig. 4). The advantage of this technique is that the TNT cells allow the genome to be easily manipulated and provide a route for the efficient production of large-animal transgenics.

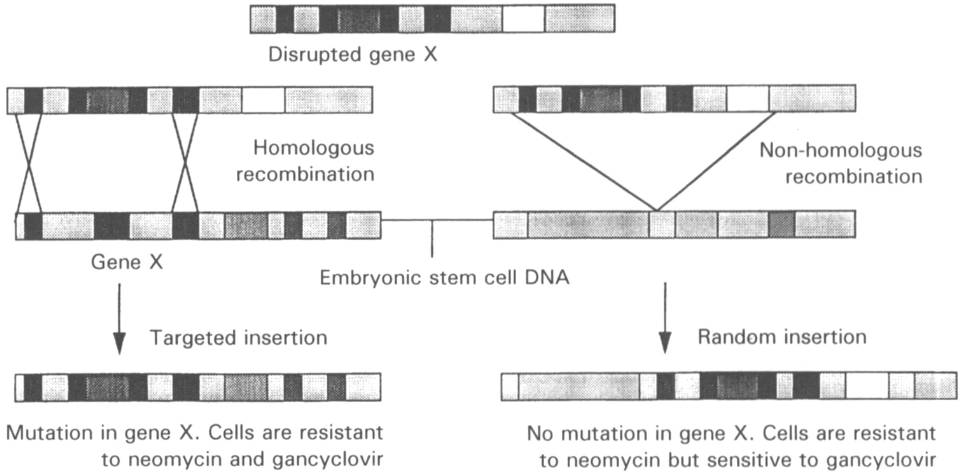


Fig. 3. Homologous recombination. The target gene X, to be manipulated in the chromosomal DNA is first isolated *in vitro*. It is modified to contain genes encoding resistance to neomycin (*neo*$^R$, ■;), and sensitivity to the drug gancyclovir (*tk*, □). The modified DNA is introduced into embryonic stem cells where it recombines with the chromosomal DNA. Following homologous recombination the embryonic stem cell DNA contains the target gene X, disrupted by the neomycin resistance gene. Following non-homologous recombination the embryonic stem cell DNA contains both the *neo*$^R$ and *tk* genes. The embryonic stem cells undergoing homologous recombination are thus selected by treatment with neomycin and gancyclovir. (Adapted from Lodish *et al.* 1995.)

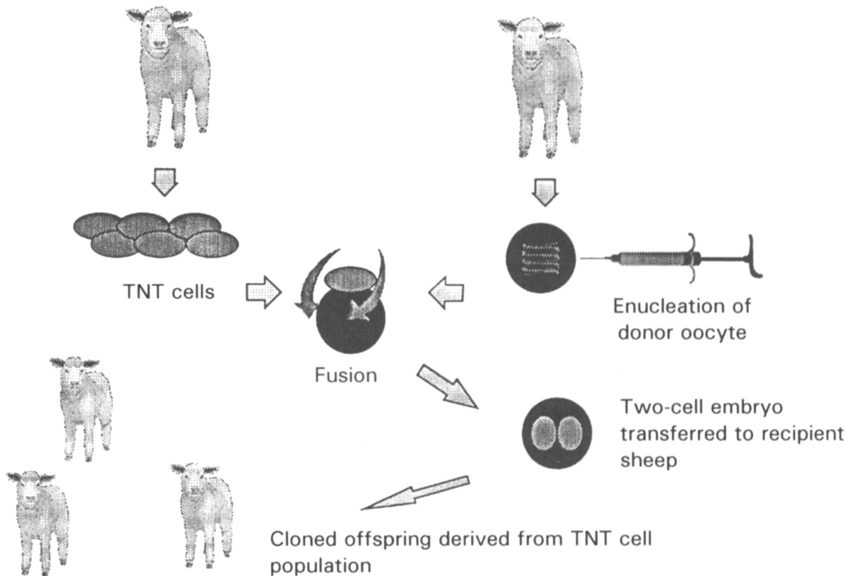


Fig. 4. Cloning by nuclear transfer. Embryonic disc cells taken from a 9-d-old sheep embryo are cultured *in vitro* to produce an epithelium. Fusion of one epithelial cell (termed TNT cell) with an enucleated sheep oocyte results in the development of an embryo. Embryos are transferred to a recipient sheep and allowed to develop to term. Offspring born using this technique are clones of one another having all been derived from the same cell line.

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## APPLICATIONS TO NUTRITION RESEARCH

### *Lipoproteins and heart disease*

Recombinant DNA technology allows the scientist to modify genetically animals in one generation, but how is this technology of use to nutrition research? One important area is the development of animal models to investigate cardiovascular disease and the contribution diet plays in the aetiology of the disease. For example, transgenic mice models have been developed to study the role of lipoproteins in the development of heart disease and atherosclerosis (Breslow, 1994). Included in these models is one in which the human apolipoprotein (Apo) gene, *Apo* CIII, encoding a protein involved in triacylglycerol hydrolysis has been expressed in mice. In one line of mice the expression of an extra single copy of the gene resulted in 30–40% more *Apo* CIII in plasma and triacylglycerol levels double the normal level (Reaven *et al.* 1994). In transgenic mice with many copies of the *Apo* CIII gene and high levels of *Apo* CIII expression, the plasma triacylglycerol concentrations were elevated by 700-fold compared with normal. The results of these studies indicate that overexpression of *Apo* CIII in humans may be a cause of hyper-triacylglycerolaemia with the increased risk of developing atherosclerosis.

Additional models involve the inactivation or 'knockout' of genes encoding proteins involved in the metabolism of triacylglycerol-rich particles. One model is the *Apo* E knockout mouse (Plump *et al.* 1992). Triacylglycerol-rich particles, chylomicrons (CM) and VLDL, are hydrolysed postprandially by lipoprotein lipases leaving CM remnants and VLDL remnants called intermediate density lipoproteins (IDL). Both lipoproteins are high in cholesterol esters and are thought to be atherogenic. In addition both contain Apo E on their surface which is involved in lipoprotein metabolism and clearance from plasma. When the gene encoding *Apo* E is inactivated by gene knockout, using a transgene in which the neomycin gene replaces the first two exons of the *Apo* E gene, then the CM remnants and IDL particles rich in cholesterol esters, accumulate in the plasma. This single genetic manipulation thereby transforms the mouse from a species highly resistant to atherosclerosis to one which is highly susceptible. Indeed, in contrast to normal mice fed on a chow (low-fat) diet with plasma cholesterol levels of 1·55 mmol/l the cholesterol levels of the 'knockout' mice show increases of up to eightfold. This mouse model has been used to investigate the effects of diet on the development of atherosclerosis. When fed on a 'Western-type diet' containing 1·5 g cholesterol/kg and 210 g fat/kg the normal wild-type animals increased their cholesterol levels twofold to 3·41 mmol/l. In the 'knockout' mouse the increase was 15-fold with levels reaching 51·72 mmol/l. Associated with the increase in cholesterol was the development of atherosclerotic lesions mimicking those seen in human subjects. Interestingly the mice showed the same sequence of lesions whether fed on a chow or Western-type diet, although with the chow diet the disease was less extensive and occurred later, indicating that while diet can attenuate it cannot deflect the disease. The question now arises as to whether there is variation in the expression of *Apo* E in the human population and whether levels of *Apo* E are involved in determining our susceptibility to atherosclerosis and cardiovascular disease.

### *Adenomas and cancer*

A knockout approach has also been taken to develop an animal model which simulates the disease familial adenomatous polyposis, a dominantly inherited autosomal disorder characterized by the growth of polyps in the large bowel and a predisposition to developing colorectal cancer. Epidemiological studies have shown that aspirin and dietary fibre, in

particular resistant starch, can protect against colonic cancer. Research in mice has shown that mutating the *Apc* gene, which encodes a protein involved in the transmission of cell signals, by introducing a termination mutation into exon 15 results in the development of colonic polyps, which progress into adenomas and carcinomas along the gastrointestinal tract (Fodde *et al.* 1994). This mouse model has been used to investigate effects of diet on tumour formation. Surprisingly, the nutritional studies in mice did not support the epidemiological findings (Burn *et al.* 1996). Aspirin was not found to be protective and resistant starch actually caused an increase in the number of tumours. While these results may not apply to human subjects, they emphasize the need for caution in extrapolating from animals to man, a fact well recognized by Dr Widdowson who noted in her 1986 paper: 'there are of course important differences between human beings and animals, and for those of us who are interested in comparative nutrition – these provide the fun'.

## *Obesity*

Being too fat or too thin is almost a national obsession and as a result generations of nutritionists and physiologists have tried to unravel the complex networks that control body weight and fat content. Biotechnologists have now turned their attention to this area and engineered several transgenic animal models to assist in the study of obesity. One of these models has used cell ablation to investigate the role of brown adipose tissue in preventing obesity (Lowell *et al.* 1993). Transgenic mice have been established which carry a transgene containing the uncoupling protein regulatory sequences (uncoupling protein is a unique protein found only in brown adipose tissue where it is involved in thermoregulation), fused to the diptheria toxin coding sequence. At 16 d transgenic mice expressing this gene show obesity and less brown fat than their non-transgenic siblings. By 19 weeks the mice are very obese with more than 50 % of their body weight being fat. Metabolic changes accompany the obesity and these include insulin resistance as evidenced by hyperglycaemia, hyperinsulinaemia, hypertriacylglycerolaemia and hypercholesterolaemia. This animal model indicates that brown adipose tissue dysfunction can lead to obesity and provides a new model, not only to study aspects of obesity, but also insulin resistant diabetes.

## *Improvements in animal welfare*

Animals not only provide models of human diseases to study the effects of diet but also provide an important source of nutrition. As we move into the next millennium it is highly likely that genetically engineered animals will enter our food chain. One aim of biotechnologists is to attempt to increase the efficiency of animal production without affecting, or indeed, by improving animal welfare.

A group of researchers in Canada have achieved this aim with the development of transgenic salmon (Hew *et al.* 1995). At present it takes approximately 14–16 months of sea-cage culture to produce Atlantic salmon. If the growth rate of these fish could be doubled, then the time the fish are in sea-cages could be reduced by a season, which would increase fish production and reduce costs to the producer and consumer. The Canadian transgenic salmon contain transgenes, either cDNA or genomic sequences, encoding piscine growth hormone controlled by the ocean pout anti-freeze protein gene promoter. Expression is primarily confined to the liver of the salmon, and growth hormone levels are on average increased by 1·4-fold compared with non-transgenic siblings. The transgenic salmon grow five to ten times faster than their non-transgenic siblings, although some

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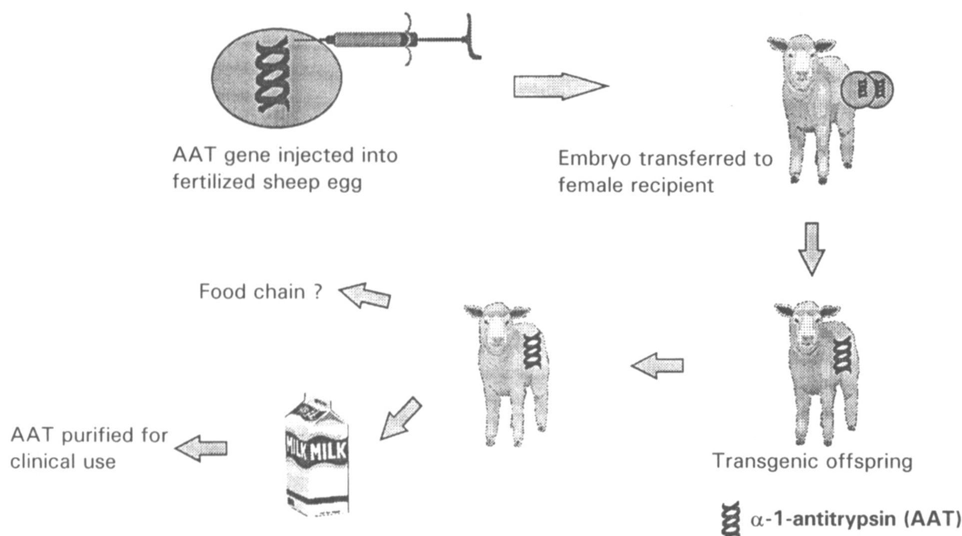


Fig. 5. Gene 'pharming'. The human gene encoding α-1-antitrypsin (AAT) under the control of regulatory sequences from a milk protein has been incorporated, using DNA micro-injection, into the chromosomal DNA of sheep. Milk taken from the transgenic sheep provides a supply of AAT for clinical use. In future the transgenic sheep may also provide a source of meat protein.

individuals show up to thirty times faster growth. The transgenic genotype is inherited and despite the increase in growth hormone there is no apparent impairment of reproductive ability.

Animal welfare is a very important issue not only to the consumer but also to the producer. One particular area of concern is the poultry industry. To provide poultry meat at the price that we the consumers demand, the poultry industry is continually looking for cheaper feed materials. Today, layers and broilers are increasingly fed on cereal-based diets which may lead to significant gastrointestinal problems for the livestock. These problems occur because the birds are unable to digest the NSP, e.g. β-glucans and arabinoxylans, in the cereal cell walls, which in turn form viscous gels in the guts of the birds. These gels not only inhibit the efficient absorption of nutrients but cause major welfare problems such as sticky faeces. If the birds could be genetically modified to synthesize and secrete enzymes that break down NSP, then the birds would be able to digest efficiently the cereal-based diets, allowing increased absorption of nutrients and improved welfare. The enzymes which degrade these NSP are of bacterial origin. Our laboratory in Newcastle has expressed a bacterial gene encoding a β-glucanase in the pancreas and intestinal enterocytes of a simple-stomached animal model, the mouse (Hall *et al.* 1993; Ali *et al.* 1996). The bacterial protein is synthesized, secreted and catalytically active. The aim is now to engineer transgenic chickens which express this bacterial gene, leading to both improved animal health and nutrition.

## *'Pharming'*

In addition to providing our meat and milk, livestock may, as we approach the next millennium, also supply our medicines with 'pharming' the new flagship in biotechnology. Scientists working at Roslin in Edinburgh have already engineered sheep which contain the

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human gene encoding $\alpha$-1-antitrypsin controlled by regulatory sequences from the milk protein $\beta$-lactoglobulin (Wright *et al.* 1991; Carver *et al.* 1992) (Fig. 5). The transgenic sheep produce the human protein, used in the treatment of emphysema, in their milk and at present this protein is undergoing clinical trials. So will our future 'pharms' supply our milk and meat, engineered to contain reduced fat of course, and our pharmaceuticals? Will our 'pharmers' of the future really be biotechnologists in disguise?

## CONCLUSIONS

Dr Widdowson (1986) observed that: 'nutrition is a subject that lends itself to work with animals'. As we move into the 21st century biotechnology will provide more transgenic models to allow the effects of nutrition and diet on human diseases to be investigated, provide for the more efficient production of animal protein while maintaining and improving animal welfare, and probably allow for the low cost production of pharmaceuticals. As to the potential of biotechnology in the next millennium? I again quote from Dr Widdowson's paper in which she so wisely states: 'experiments must go on, for who knows what discoveries lie ahead?'

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