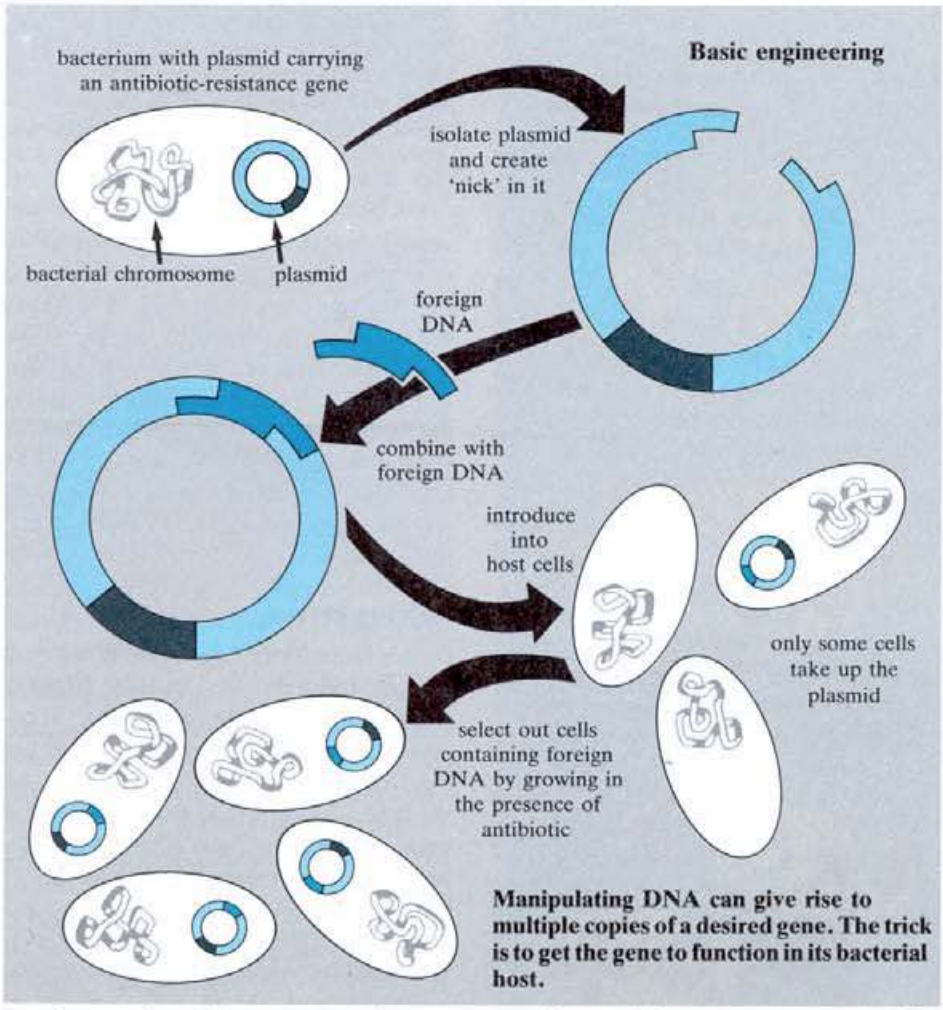
Genetic engineering: the state of the art



Manipulating DNA can give rise to multiple copies of a desired gene. The trick is to get the gene to function in its bacterial host.

It is only 43 years since Dr Oswald Avery and his colleagues at Rockefeller University in New York showed that DNA had some role in bacterial genetics and heredity.

At the time it seemed a fairly unremarkable discovery, but in the intervening years our knowledge about the pivotal role of DNA (deoxyribonucleic acid) in the development of all organisms has accelerated relentlessly.

To achieve the current understanding, scientists had to develop techniques for manipulating DNA. In 1958 the first enzyme capable of the test-tube synthesis of DNA was isolated, and in 1967 the enzyme DNA-ligase, capable of joining two DNA chains, was discovered. These 'tools' were soon complemented as scientists isolated the first of the 'restriction' enzymes that cut the DNA chain at specific points.

With this battery of enzymes it was only a matter of time before a bit of cutting and stitching and tampering with the genetic code occurred and, in 1973, Dr Herbert Boyer, Dr Stanley Cohen, and collaborators at Stanford University and the University of California reported that they had been able to isolate, and artificially recombine, DNA from one strain of that workhorse of modern molecular biology *Escherichia coli* and then transfer it into another strain.

This opened up the possibility of breaching the species barrier and constructing completely new organisms that would never have existed without the intervention of man and his test-tubes. And soon it came about, with *E. coli* being the recipient of a range of plant, animal, and viral genes. The science of genetic engineering had been born.

Announcing the birth, the world's media trumpeted to a bewildered public all the possibilities inherent in the miraculous new technology: super-plants, super-cows, a cure for cancer, vast riches. Very little was left to the imagination.

So far the miracle hasn't eventuated. While some animal products have entered the market-place, only one genetically engineered product with a significant impact on human welfare has come onto the market: human insulin, which has an unusual amino acid composition that makes it easy to produce (see the box). Other proteins — and proteins are the major concern of genetic engineers — have more complex structures that are not so amenable to manipulation.

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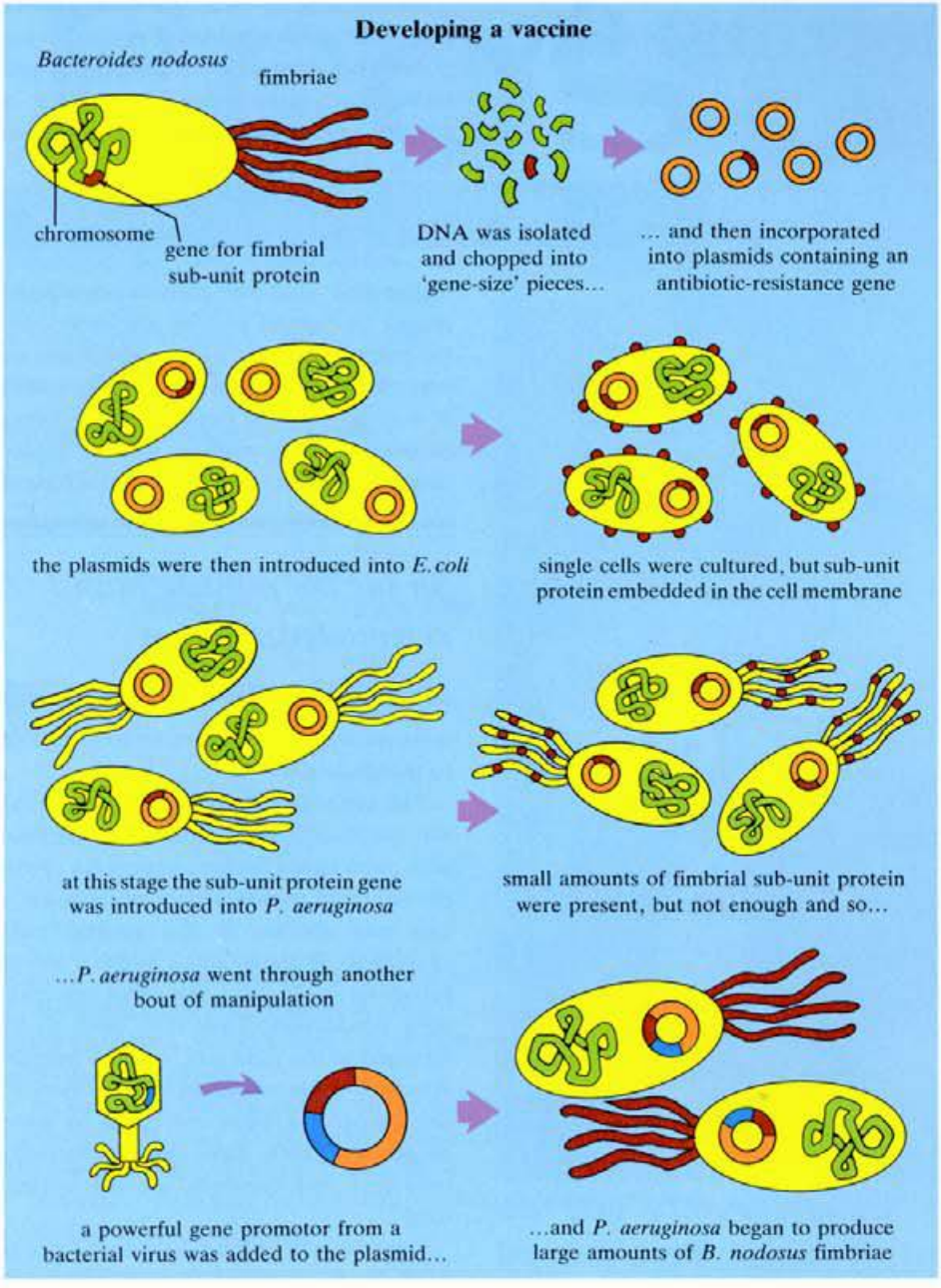
The molecular biology of DNA and the way the proteins it codes for are synthesised and packaged within organisms have proved more complicated than those early cuts and stitches in the genetic code suggested. Proteins often need a lot of follow-up work after the DNA specifies their production. They may need to be trimmed to the right size, or their internal structure tightened up by the addition of, for example, a sugar molecule; in some cases they then have to be correctly packaged and presented to the outside world.

Scientists within CSIRO have been actively exploring the potential of genetic engineering, and some of their experiences provide insights into the sorts of problems that have to date stymied the full development of the new technology. The problems they have encountered emphasise the complexity of genetic expression and reveal how a better understanding and very clever manipulation of the system will be necessary if molecular biology is ever to reach its full commercial potential in, for example, vaccine production.

Developing a vaccine

Many bacterial and viral pathogens have a protein that they use to attach themselves to the cell of the organism they infect. This protein meshes with a receptor on the host cell and, after attachment, the pathogen brings into operation a fresh battery of proteins — enzymes — that complete the penetration of the cell and produce the full-blown disease.



Basic genetic engineering techniques were used to isolate the gene responsible for the fimbrial protein sub-unit. However, *E. coli* could not produce the protein in the correct form and, since *Pseudomonas aeruginosa* seemed to be a more appropriate host, the gene was transferred again. The initial yield of the protein was poor, and to overcome this problem the gene was combined with a powerful promoter from a virus. As a result, pure *B. nodosus* fimbriae were produced by the *Pseudomonas* bacterium.

A new generation of vaccines is being developed for a wide range of human and animal diseases, and one that has reached a fairly advanced stage of development is for footrot — a crippling, debilitating disease of sheep caused by the bacterium *Bacteroides nodosus*. A conventional vaccine against footrot is available, but its production and quality are beset by the sorts of problems mentioned above, and its high cost — about 80 cents a dose, with two doses being necessary — deters graziers from using it.

Fighting footrot

Dr David Stewart of the CSIRO Division of Animal Health, Dr John Mattick, Dr Brian Dalrymple, and Ms Margaret Bills, of the CSIRO Division of Molecular Biology, and Dr Tom Elleman, Dr Neil McKern, and Mr Peter Hoyne, of the CSIRO Division of Protein Chemistry, along with Ms Beau Anderson and Professor John Egerton, of the Faculty of Veterinary Science at the University of Sydney, have made considerable progress in developing a new footrot vaccine through the use of recombinant-DNA technology.

Dr Stewart and his colleagues at the Division of Animal Health showed that the important footrot protein occurs in the fine hair-like filaments covering the surface of the *B. nodosus* cell. Although the exact function of these 'hairs', termed fimbriae, in the footrot organism is uncertain, it seems likely that they are involved in attachment to, or colonisation of, the tissues of the hoof by the bacterium. Once attached, the invader then produces an array of enzymes that break down the protein in hoof tissues and produce the footrot syndrome.

The fimbrial proteins are built up from protein sub-units, and their production through genetic engineering could simplify vaccine production. The first step in the construction of such a vaccine is the isolation of the genes responsible for the fimbrial protein and its assembly.

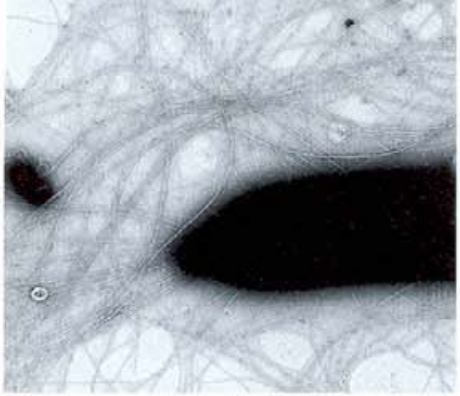
The scientists achieved this by breaking up the *B. nodosus* DNA with a restriction enzyme and then placing individual frag-

Attachment is of fundamental importance to the pathogen's colonisation of the host and continuing survival, but in the never-ending battle between host and pathogen this is often thwarted by the activities of the host's immune system.

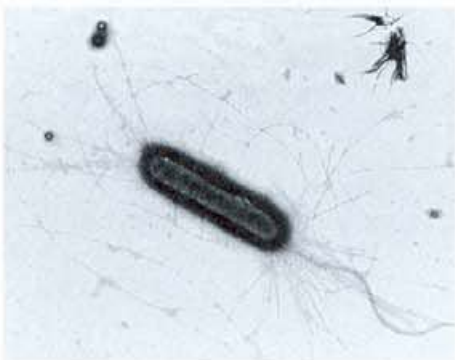
Circulating white blood cells focus on molecules on the surface of the pathogen, including the attachment protein (immunologists call these molecules antigens), and this leads to the host synthesising a matching protein (or antibody) that, just like the receptor on a vulnerable cell, binds to the invading protein, effectively immobilising the pathogen. Soon after, the invader is devoured by the scavenging cells that form another part of the immune system's armoury.

Vaccination is a way of accelerating the host–pathogen interplay. Killed or attenuated pathogens, incapable of causing a full-blown infection, are introduced into the potential host. The host's immune system responds as if it has been assaulted by the fully infectious pathogen and produces antibodies, which continue circulating in the body, protecting the host from any fresh challenge by the pathogen.

The production of vaccines is a sophisticated process with high standards that need to be maintained: major public health problems have arisen when people were dosed with 'killed' pathogens that still retained their pathogenicity. In addition, the process is often difficult and/or expensive. For example, production of the influenza vaccine involves growing the virus in fertilised hen's eggs. Many of these problems could quickly be overcome if the pathogen DNA (or the related ribonucleic acid, RNA) coding for the attachment protein, or other relevant antigens, could be isolated and then synthesised in a friendly bacterium.



***B. nodosus* under the microscope, showing the fimbriae projecting from the cell surface.**



Fimbriae of *B. nodosus* — but produced in *P. aeruginosa*.

ments into a plasmid — a short piece of bacterial DNA — that also contained a gene coding for antibiotic resistance. After these 'recombinant' DNA molecules were transferred into *E. coli*, they cultured the bacteria on a medium amended with antibiotic.

A combination of genetic tricks enabled the scientists to determine which bacterial colonies (or clones) contained the *B. nodosus* DNA; and to find out which ones contained the fimbrial sub-unit gene, the team challenged the bacteria with antibodies against the fimbrial protein. Out of the two thousand clones prepared, eight were found to be producing the sub-unit.

But it's not so simple as that: while these genetically engineered *E. coli* could be induced to produce copious quantities of the fimbrial protein sub-unit, no mature fimbriae were formed. A closer look at individual bacteria revealed why: the sub-unit protein was embedded in the cell membrane and had gone no further. The group then tried the same trick with a strain of *E. coli* that possesses fimbriae but, again, mature fimbriae refused to form.

From other studies on the fimbriated *E. coli*, the Australian group knew that a cluster of five or six genes is involved in the construction of fimbriae. One codes for the fimbrial sub-unit, another for a larger protein that anchors the fimbriae to the cell wall, and the remainder are apparently involved in the assembly of the mature fimbriae.

A similar assembly system probably operates in *B. nodosus*; presumably the other genes involved were not transferred to *E. coli* along with the sub-unit protein gene and this may explain their failure to produce typical fimbriae. However, attempts to transfer a larger part of the *B. nodosus* genome, or to use *E. coli*'s fimbrial assembly genes, have thus far provided no solution.

Evidently there is a basic incompatibility between the fimbrial systems of these two bacteria. However, when Dr McKern sequenced the *B. nodosus* protein sub-unit it became clear that this had a great many similarities with those occurring in the fimbriae of *Neisseria gonorrhoeae* (one of the venereal disease organisms), *Moraxella bovis* (the cause of pink eye in cattle), and *Pseudomonas aeruginosa* (a common saprophyte.)

P. aeruginosa, in particular, is easy to grow and its genetics are well understood. More importantly its fimbrial assembly machinery is compatible with that of *B. nodosus* because, when the scientists transferred the footrot bacterium's protein sub-unit genes into it, the new host produced bulk quantities of intact mature *B. nodosus* fimbriae. A patent application has been lodged for this process and preliminary tests on the fimbriae suggest that they are at least as good as the conventionally produced vaccine. And, because *P. aeruginosa* is easier to grow than the footrot bacterium, the protein yield is much higher, suggesting that production may be much simpler.

At present the footrot vaccine is being put through further trials involving the various CSIRO Divisions and the University of Sydney, and an agreement between CSIRO and two animal health companies for its commercial production is being negotiated. There are still some technical problems to be overcome — one, a rather common complaint in genetic engineering, is that the recombinant bacteria tend to be unstable in culture — but if all goes well Australian graziers should be able to make use of one of the first genetically engineered vaccines in the near future.

Engineering plants and animals

Bacteria have made such an enormous contribution to the science of molecular biology because of their simplicity. As members of the group of organisms known as prokaryotes, they lack a membrane-bound nucleus where the DNA is found. The great bulk of the bacterial DNA occurs in a single long chromosome floating around the cell's interior; as such, it is easily accessible compared with the DNA found in the eukaryotic organisms — plants and animals — that have a nucleus.

The eukaryotic cells of plants and animals contain much more DNA, packaged away in the nucleus. Any introduced foreign DNA has to traverse the cell membrane

A crown gall infection of tobacco.

The cereals tend to form a mass of undifferentiated cells — or callus — when cultured.

(and with plants a substantial cellulose cell wall before the membrane) and then the nuclear membrane, before it can possibly be integrated into the host genome. Such a tortuous path presents problems to biologists attempting to manipulate the genetics of plants and animals.

Large numbers of bacteria can easily be grown from a single cell using only relatively simple media containing carbon and nitrogen sources, some minerals, and possibly some growth factors such as the B-group vitamins; but plant and animal cells are much more demanding and this creates further complications.

Individual eukaryotic cells are difficult to manipulate and they demand extra growth factors, such as those found in blood serum or an array of hormones, if they are ever to grow in culture. Even then they are still very refractory.

For example, members of the cereal family — including the rice, maize, and wheat that provide the bulk of the world's calories — refuse to form complete plantlets, capable of growing on in the wider world after removal from their test-tube residence. Animal cells, despite the best hormones and blood serum factors, are resolute in their refusal to go any further than a mass of cells covering the surface of a culture dish.

A final major hurdle that has to be overcome in the genetic engineering of the higher organisms is perfection of the delivery systems needed to introduce new DNA sequences into the genome. Bacteria have their plasmids, but the viruses, bacteria, and physical methods used to introduce genes into plants and animals have yet to reach the same level of refinement.

Transforming plants

The most promising gene-delivery system for the plant world comes by courtesy of a common plant pathogen, the crown gall bacterium *Agrobacterium tumefaciens*, which infects a wide variety of species. It contains plasmids, and one of these — the Ti (or tumour-inducing) plasmid — gets incorporated into the host plant's genome and takes over the genetic machinery, diverting the plant's supply of the amino acid arginine into amino acids that only *A. tumefaciens* can use.

The tumour, or gall, that forms on the plant is outside the normal controls on growth and development — just like a cancer — and serves to further enhance the bacterium's success because the uncontrolled growth creates a 'sink' to draw nutrients away from more productive uninfected parts of the plant.

Engineering insulin

Insulin, so essential in the control of blood sugar levels, illustrates the complexity involved in protein synthesis and packaging within an organism.

Before it can ever enter the blood-stream insulin undergoes some remarkable transformations. The first protein formed is preproinsulin, which has a special sequence that allows the protein to be transported across cell membranes. The 'pre-' is lost during transport to give the storage form of insulin — proinsulin.

The proinsulin molecule consists of a sequence of 84 amino acids arranged in a complicated loop. To convert it to insulin, enzymes within the pancreatic cells chop off 33 amino acids to leave the two chains, connected by disulfide bridges, of the active insulin molecule.

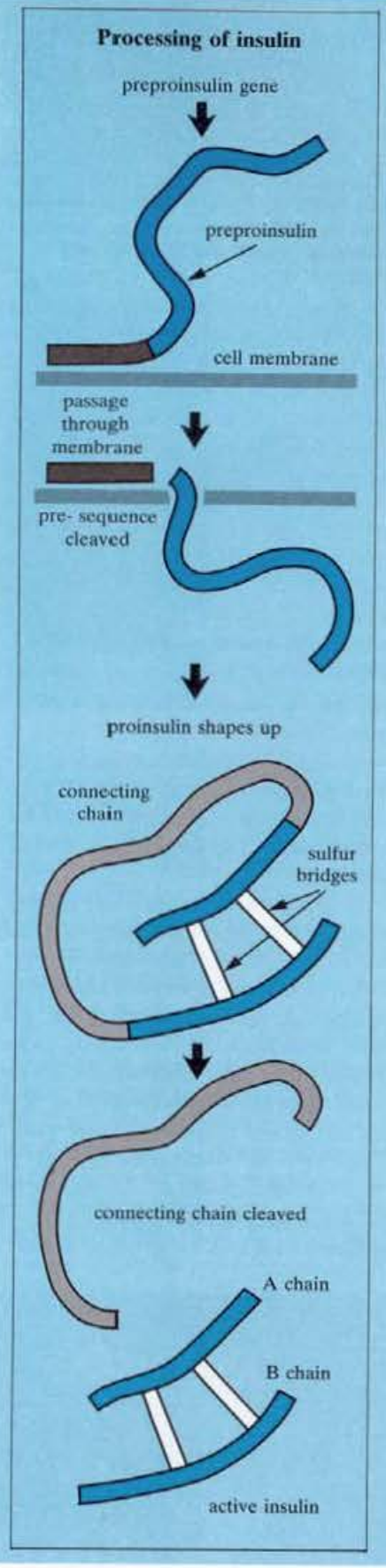
Clearly, starting with the preproinsulin gene would be a very inefficient way to produce insulin using genetic engineering techniques; far too much secondary processing is involved.

To get around this difficulty, scientists chemically synthesised DNA chains for the two insulin chains — insulin is a relatively small protein and its amino acid sequence and corresponding DNA sequence were well known. They then attached these chains, separately, to a bacterial gene, but with the addition of a nucleotide sequence specifying the amino acid methionine.

Both chains, plus the attached bacterial protein, were produced by the bacterium. Then came the trick that ensured the commercial success of the new insulin.

Insulin does not contain methionine in its amino acid sequence, and through the use of cyanogen bromide (a reagent that selectively destroys methionine) the insulin chains were released from the larger protein. After purification the two chains were mixed and reconnected in a reaction that formed the disulfide bridges.

As most commercially useful proteins contain methionine, the cyanogen bromide trick has few applications.



Insulin undergoes some elaborate processing in the pancreas. Genetic engineering circumvents this by working backwards from the amino acid sequence of the two insulin chains and constructing synthetic genes for each chain, which can then be introduced into bacteria.

Scientists have disarmed and domesticated the Ti plasmid and used it to transfer simple genes — usually for some character such as antibiotic resistance — into tobacco and petunia plants; most recently the more agriculturally important plants soybean and cotton have also been induced to accept the Ti plasmid. But, unfortunately,

this is still a long way removed from transforming the yield potential of our crop plants. The reason is that crop yield is under multi-gene control.

For example, that fine idea of wheat plants being made capable of fixing their own nitrogen — like the clovers do — remains no more than a fine idea. The interaction between the clovers and their *Rhizobium* bacterial partners involves some 10–15 genes in trapping the atmosphere's nitrogen and making it available for enhanced plant growth, and a delicate two-way flow of genetic information passes between the plant and its partner. At the moment we have only the most superficial knowledge of the structure and interactions of these genes.

However, maize is one crop plant whose genetic structure has proved amenable to study. The male and female parts of the individual plant (the tassel at the top and the corncob on the stem) are well separated and provide ideal tools for geneticists. Many maize mutants have been identified, and some genes — for example, those involved in the plant's response to high temperatures and waterlogging — have been transferred to bacteria, where they can be studied more closely.

Maize was chosen by a group within the CSIRO Division of Plant Industry, including Dr Jim Peacock, Dr Tony Pryor, and Dr Liz Dennis, for intensive study of its molecular biology. The group has had to confront two major problems: the lack of a gene-delivery system (*A. tumefaciens* does not infect cereals); and the difficulty of getting single maize cells to develop into complete plants in the test-tube.

The problem of culturing maize cells is fundamentally one of developing the 'right' recipe for growth and development. This problem has arisen with many other plant species, but has been overcome by getting the right balance of ingredients or by introducing an entirely new compound; previously intractable species, such as lucerne and the clovers, are now routinely cultured in test-tubes. However, maize remains as refractory as ever.

A number of techniques for introducing foreign DNA into maize, involving either physical shocks or chemical treatment, have been tested, but with limited success. However, recently the CSIRO group and two of their colleagues in the Division, Dr Liz Howard and Dr John Walker, have shown that one method works. Called electroporation, it involves subjecting individual cells to a minor electric current while they are in a solution containing the foreign DNA. The current encourages small holes, or pores, to develop within the cell membrane, allowing the extra-cellular DNA to pass through into the interior.

Using electroporation, the scientists introduced an antibiotic-resistance gene, derived from a bacterium, into maize. In their first trials a bacterial gene coding for antibiotic resistance was spliced into a plasmid containing regulatory sequences — or part of the DNA chain that encourages or promotes the translation of nearby gene(s) — from the cauliflower mosaic virus genome. This promoter enabled the foreign gene to be expressed because the scientists detected the breakdown products of the antibiotic in the maize cells. However, this is still far removed from the development of a transformed maize plant and its eventual appearance in a farmer's field.

The race was soon on to transform animals more commercially useful than mice.

In cases where the crown gall bacterium is capable of infecting a particular plant, and that plant is adapted to life in a test-tube, the prospects for genetic manipulation look much better. Other scientists in the Division of Plant Industry, including Dr T. J. Higgins, Dr Don Spencer, and Dr Danny Llewellyn, have recently exploited tobacco's susceptibility to the bacterium, and the ease with which it can be grown in test-tubes, to transfer into it a gene coding for a protein found in pea seeds.

This is a high-sulfur protein that, if it could be introduced into lucerne or clover, would considerably boost wool production. The wool fibre — made from keratin proteins — is dominated by sulfur-rich amino acids, but pasture plants generally contain such a low level of sulfur that the sheep cannot reach their full wool production potential.

The transformed tobacco plant only produced the pea-seed proteins in its own seeds — the tobacco seed genes obviously recognising that this was where the protein belonged. To be of value the protein will have to be produced in other parts of the plant, and at the moment the scientists are attempting to couple the pea-seed protein gene to genetic signals that will specify its production in the leaf of a lucerne plant.

Lucerne can also be transformed by the crown gall organism and single cells can be grown into mature plants in the test-tube. The genetic surgery lucerne will experience within the next 12 months promises to make it an even more valuable plant for Australian graziers.

Transforming animals

Although animal cells are even more refractory than plant cells in artificial culture, animals have a marked advantage as targets for genetic engineering in that the embryos of many species can be surgically extracted, treated, and then re-implanted in the mother's womb.

In the early stages of fertilisation, just following penetration of the egg-cell wall by the sperm, the male and female DNA form separate nuclei, called pronuclei, that slowly move together and fuse. The two pronuclei are visible, and scientists have developed micro-injection techniques to introduce foreign DNA into one of them.

This involves very delicate manoeuvring of a hypodermic needle. The cell is only 150 microns across — ten of them would span the head of a pin — and the target nucleus is only about 20–30 microns wide. When that target is finally hit, a steady

Wheat — one of the prime candidates for genetic engineering.

The mouse on the left is larger because of the extra growth hormone circulating in its blood.

hand injects a miniscule amount — about one picolitre, or a billionth of a litre — into the pronucleus.

The first attempts at micro-injection were made on mouse embryos, a few of which went on to develop into baby mice. Animals, like plants, contain all the enzymes necessary to integrate foreign DNA into their nuclei and, provided the injection procedure hadn't damaged the embryo too much, once that DNA reached the vicinity of the mouse DNA it was soon incorporated.

Numerous genes, from such diverse sources as man, rats, chickens, and viruses, have been introduced into the mouse genome in this way, and been shown to be functional.

The big breakthrough, however, came in 1982, when Dr Richard Palmiter and Dr Ralph Brinster, and colleagues at the University of Washington and the University of Pennsylvania, introduced the rat growth-hormone gene into mouse embryos, which then went on to grow faster and bigger than they normally would.

The basis of this success was the linking of the rat growth-hormone gene to the promoter of a gene, active in the liver, that codes for the enzyme metallothionein, which is used to bind to potentially toxic heavy metals such as copper, zinc, lead, and cadmium.

Under normal circumstances growth hormone is produced only in the pituitary gland, with its production controlled by its own promoter sequence. Likewise metallothionein is only produced in the liver, with its production under the control of its promoter, which in turn responds to the level of heavy metals in the animal's diet.

By separating the genes from their respective promoters and linking the growth-hormone gene to the metallothionein promoter, the scientists circumvented the normal controls. Growth hormone was produced in every cell in the body when the animal was exposed to a low level of heavy metals.

The potential for the production of larger, faster-growing animals — which may make this extra growth by more efficient feed conversion — was not lost on animal scientists, and the race was soon on to transform animals more commercially useful than mice.

Given the importance of sheep in the Australian agricultural economy it was only natural that scientists in the CSIRO Division of Animal Production would join the race. The group, led by Dr Kevin Ward, includes Dr Jim Murray and Dr Colin Nancarrow. They are in the process of producing what they hope will be the world's first genetically transformed sheep.

Their approach resembles that employed in the mouse work. The sheep growth-hormone gene — normally under the control of its own promoter and only active in the pituitary gland — is being linked to the metallothionein promoter to ensure that the hormone is produced continuously throughout the animal's body. In a sense it is not 'true' genetic engineering, as the gene and the promoter involved are already in the sheep's genome; all they are attempting to do is rearrange the genes.

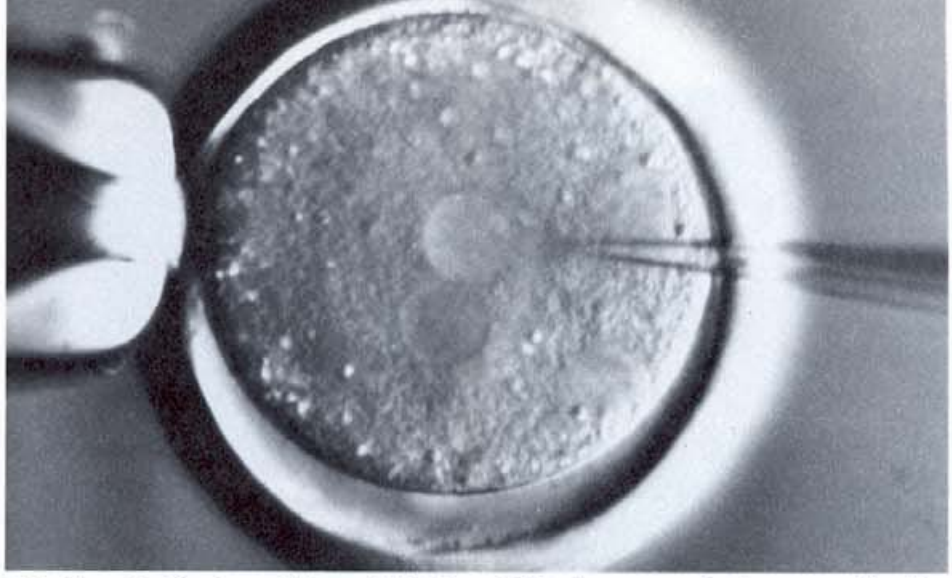
A new sheep

The development of an animal with a new arrangement to its genes involves a lot of expertise and time. About 30 people and 6 weeks of effort have to be co-ordinated in order to produce one batch of transformed embryos.

The first step is to prepare all the animals. Their health has to be checked, they have to be drenched and vaccinated, and they have to undergo a special feeding program. Two weeks before the actual transfer takes place the ewes have sponges saturated with the hormone progestagen inserted in their vaginas; the purpose is to synchronise the flock's oestrous cycle. Later on the ewes are injected with an extract from pregnant-mare serum, which encourages them to super-ovulate, or produce extra eggs.

While this is going on, other members of

A newborn lamb — soon to grow bigger and woollier?



Injecting a fertilised egg. The egg is held in place by a pipette, and the injection pipette introduces foreign DNA into one of the two pronuclei.

the team are culturing *E. coli* that has a plasmid containing the sheep growth-hormone gene and its new metallothionein promoter. Eventually they will extract bulk quantities of the plasmid from the bacteria.

Two days before the due day, the ewes are given an injection of luteinising hormone, which induces ovulation and helps ensure that they all ovulate within a very narrow time-frame. Ova are fertilised by artificial insemination, but not in the normal fashion. In these experiments sperm are introduced directly into the uterus in order to avoid the often tortuous and time-consuming path followed by the individual sperm.

The fertilised embryo is flushed out of the womb and, under a microscope, a steady hand guided by a fine eye inserts the DNA into one of the pronuclei. The transformed embryo is then whisked away to be implanted in the uterus of a surrogate mother. The surrogate ewes have undergone their own course of hormones to make their uteri receptive to the embryo.

On the basis of recent experiments, about half of the treated embryos can be expected to be non-viable. Of those that survive only 1–2% will actually have the gene, and about 70% of these will express it. With these figures less than 10 lambs, arising from the injection of 1000 embryos, can be expected to have the growth-hormone gene attached to the metallothionein promoter. But there is no guarantee that even those containing the gene will be viable or useful new animals.

If they do have elevated levels of the growth hormone in their circulation and actually show a growth response, they will still need to be carefully assessed. Any transfer of foreign genes — whether it be plant or animal — is plagued by the fact that the experimenters have no control over where the gene finally ends up.

Coupling the microscope to a video screen helps Dr Murray control the injection.

In the sheep experiments, 100–500 copies of the gene are injected into the embryo and these are randomly inserted into the embryo's genome. If one of the copies ends up in the middle of an important gene — for example, the gene coding for the skin protein, collagen — normal development cannot proceed and the embryo, without its very important skin, aborts. These unfortunate inserts are probably responsible for many of the early losses in the transfer program.

But even an apparently normal lamb may have hidden problems. Another random insert could conceivably affect its immune system and the animal may be more susceptible to disease. Among the transgenic mice infertility has been a problem, and if the same thing happens in the sheep those animals will have little commercial value.

To date, the CSIRO group has 'conceived' 96 lambs, but not one has shown any signs of producing extra growth hormone. As with plants, there are still many technical hurdles to be overcome and a lot of testing and breeding to be performed before a transformed animal will be seen grazing peacefully in a grazier's paddock.

It is now more than 10 years since the first claims about the unimaginable potential of genetic engineering were trumpeted throughout the world's media. And occasionally, albeit faintly now, those same notes can still be heard. However, the lessons of recent years are that the genetic machinery is far more complicated than first imagined and that many more years of research effort will be necessary before the new technology gets anywhere near reaching its full potential.

Wayne Ralph

More about the topic

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