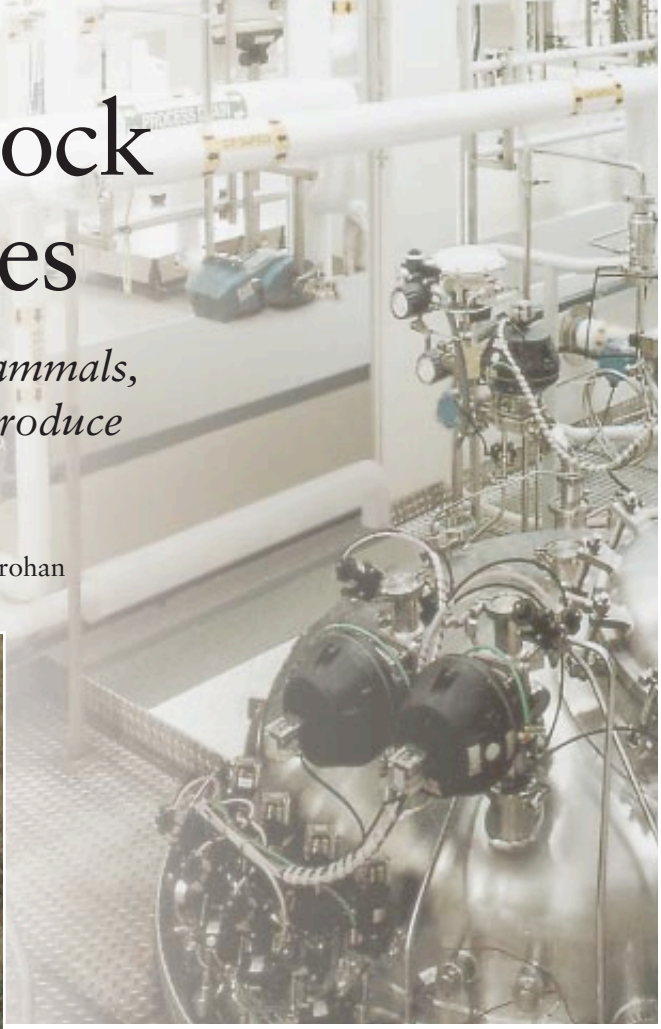
Transgenic Livestock as Drug Factories

By introducing key human genes into mammals, biologists can induce dairy animals to produce therapeutic proteins in their milk

by William H. Velander, Henryk Lubon and William N. Drohan



Exactly one year after her own birth, Genie, our experimental sow, was serenely nursing seven healthy piglets, her milk providing the many nutrients these offspring needed to survive and grow. But unlike other pigs, Genie's milk also contained a substance that some seriously ill people desperately need: human protein C. Traditional methods of obtaining such blood proteins for patients involve processing large quantities of donated human blood or culturing vast numbers of cells in giant stainless-steel reactor vessels. Yet Genie was producing copious amounts of protein C without visible assistance. She was the world's first pig to produce a human protein in her milk.

Genie's ability to manufacture a therapeutic drug in this way was the outcome of a research project conceived almost a decade ago. In collaboration with scientists from the American Red Cross who specialized in providing such blood proteins, we began to consider the possibility of changing the composition of an animal's milk to include some of these critically needed substances. In theory, this approach could generate any required quantity of the various therapeutic blood proteins that are regularly in short supply.

Demand for such drugs comes from many quarters. For instance, hemophiliacs may lack any of several different clotting agents, particularly blood proteins called Factor VIII and Factor IX. Certain people with an inborn deficiency require extra protein C (which acts to control clotting) to supplement their body's meager stores, and patients undergoing joint replacement surgery can benefit from this protein as well. Another important example of the need for therapeutic blood proteins involves people suffering strokes or heart attacks: these cases often demand quick treatment with a protein called tissue plasminogen activator, a substance that can dissolve blood clots. And some people suffering from a debilitating form of emphysema can breathe more easily with infusions of a protein called alpha-1-antitrypsin.

All these proteins are present in donated blood only in tiny amounts, and hence they are currently so difficult to produce that their expense precludes or severely limits their use as drugs. For example, treatment with purified Factor VIII (restricted to those times when someone with hemophilia is actually bleeding) typically costs the patient tens of thousands of dollars every year. The cost of continuous replacement of this blood protein for the same period—a desirable but rarely available option—would exceed $100,000.

Such enormous sums reflect the many problems involved in extracting these proteins from donated blood or establishing specialized production facilities using cultured cells—an enterprise that can require an investment of $25 million or more to supply even modest amounts of a single type of protein. Developing "transgenic" animals such as Genie (that is, creatures that carry genes from other species) demands only a small fraction of such costs. Yet the new breeds simplify procedures enormously and can produce vast quantities of human blood protein. Replacing conventional bioreactors with transgenic livestock thus offers immense economic benefits.

Creating blood proteins in this fashion also stands to better the other cur-

BARRY L. WILLIAMS (*pig*); JOHN HORNER ARCHITECTURAL PHOTOGRAPHY (*bioreactor*)

BIOREACTORS are typically large stainless-steel tanks with complicated controls for maintaining the broth in which countless individual cells are grown. But a new strategy for producing protein-based medicines circumvents the need for such elaborate, and often costly, machinery by using transgenic livestock, such as the pig (*inset*) engineered by the authors to produce one such protein in its milk.

rent practice—purifying them from donated blood—because it would circumvent the risk of contamination with infectious agents. Although blood proteins derived from pooled blood plasma are considered relatively safe now that donors are carefully screened and virus inactivation treatments are routinely applied, the threat from some pathogens always looms. For example, the fear of inadvertently spreading HIV (the AIDS-causing agent) and the hepatitis C virus is spurring researchers to seek substitutes for drugs now derived from human blood. Similarly, recent concerns about Creutzfeldt-Jakob disease (a degenerative disease of the nervous system) has caused some blood products to be withdrawn from the U.S. and Europe. Creating human blood proteins with transgenic livestock that are known to be free of such diseases would deftly sidestep these difficulties.

The many gains that would result from the use of transgenic animals as bioreactors gave us ample reason to pursue our vision of tidy stalls occupied by healthy livestock carrying a few key human genes. But at the outset of our work, we had many worries about the technical hurdles facing us in breeding such transgenic animals and garnering usable quantities of protein from their milk. Fortunately, we were able to progress rapidly, benefiting from a body of trailblazing research that had already been done.

Prior Mousing Around

As early as 1980, Jon W. Gordon and his colleagues at Yale University had determined that a fertilized mouse embryo could incorporate foreign genetic material (DNA) into its chromosomes—the cellular storehouses of genetic material. Shortly afterward, Thomas E. Wagner and his associates at the University of Ohio demonstrated that a gene (a segment of DNA that codes for a particular protein) taken from a rabbit could function in a mouse. Using a finely drawn glass tube of microscopic dimensions, these researchers devised a way to inject a specific fragment of rabbit DNA into a single-cell mouse embryo. Amazingly, that DNA would often become integrated into the mouse's chromosomes, perhaps because it was recognized by the cell as a broken bit of DNA that needed to be repaired.

These researchers then implanted the injected embryos in a surrogate mother mouse and found that some of the mice born to her contained the rabbit gene in all their tissues. These transgenic mice in turn passed the foreign gene on to their offspring in the normal manner, following Mendel's laws of inheritance. The added gene functioned normally in its new host, and these mice made rabbit hemoglobin in their blood.

Another milestone on the road to transgenic animal bioreactors was passed in 1987. Along with their respective colleagues, both Lothar Hennighausen of the National Institute for Kidney and Digestive Diseases and A. John Clark of the Institute of Animal Physiology and Genetics at the Edinburgh Research Station in Scotland established means for activating foreign genes in the mammary glands of mice. Foreign protein molecules created in this way were then secreted directly into a transgenic mouse's milk, where they could be easily collected. These researchers accomplished this feat by combining the foreign gene of interest with a short segment of DNA that normally serves to activate a gene for a mouse milk protein.

Whereas Hennighausen's mice produced the desired human protein (in that case, tissue plasminogen activator) at disappointingly low concentrations, Clark's mice produced 23 grams of a sheep milk protein (known as beta-lactoglobulin) in each liter of milk—approximately matching a mouse's own major milk proteins in abundance. But beta-lactoglobulin was not a human protein in short supply, nor were these tiny mice the proper vehicle to provide useful quantities of milk. So Clark and his colleagues went to work injecting sheep embryos with DNA that contained a medically important human gene.

They used the gene that codes for a blood-clotting factor (Factor IX), along with a segment of sheep DNA that normally switches on the production of beta-lactoglobulin in the mammary gland. Two years later Clark's transgenic sheep secreted Factor IX in their milk—but at barely detectable levels. It was at that juncture that we began our attempts to realize the potential of such pioneering work. But we decided to take a gamble and try a novel strategy.

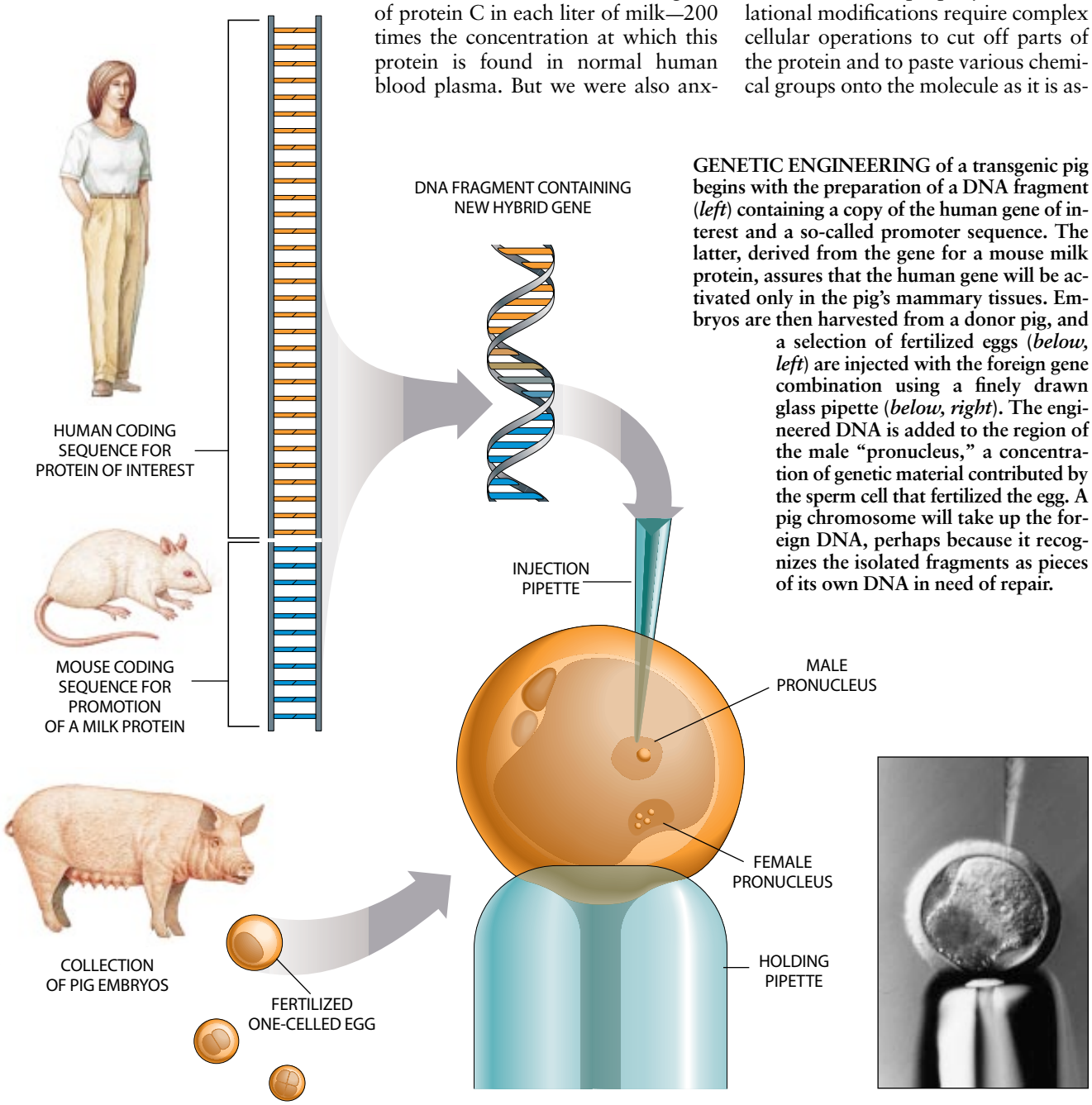
A Pig in a Poke

Whereas other research groups had picked sheep, goats or cows as suitable dairy animals for producing human proteins, we chose to work with pigs instead. Swine offer the advantages of short gestation periods (four months), short generational times (12 months) and large litter sizes (typically 10 to 12 piglets). Thus, producing transgenic pigs is relatively quick compared with transforming other types of livestock. And despite their lack of recognition as dairy animals, pigs do produce quite a lot of milk: a lactating sow generates about 300 liters in a year. The real question for us was whether this unconventional choice of transgenic animal could in fact be made to produce appreciable levels of human protein in its milk.

Toward that end, we decided to use a DNA segment made up of a human gene and the so-called promoter for a major mouse milk protein (called whey acidic protein) that had been characterized by Hennighausen and his colleagues. By injecting this DNA combination into mouse embryos, those researchers were able to augment a mouse's chromosomes so that the creature would produce the desired human protein in its milk. To take advantage of this approach, we, too, fashioned a fragment of DNA that contained the human gene for the target protein (in our case, protein C) and the mouse promoter for whey acidic protein. But we injected this DNA into a set of pig embryos.

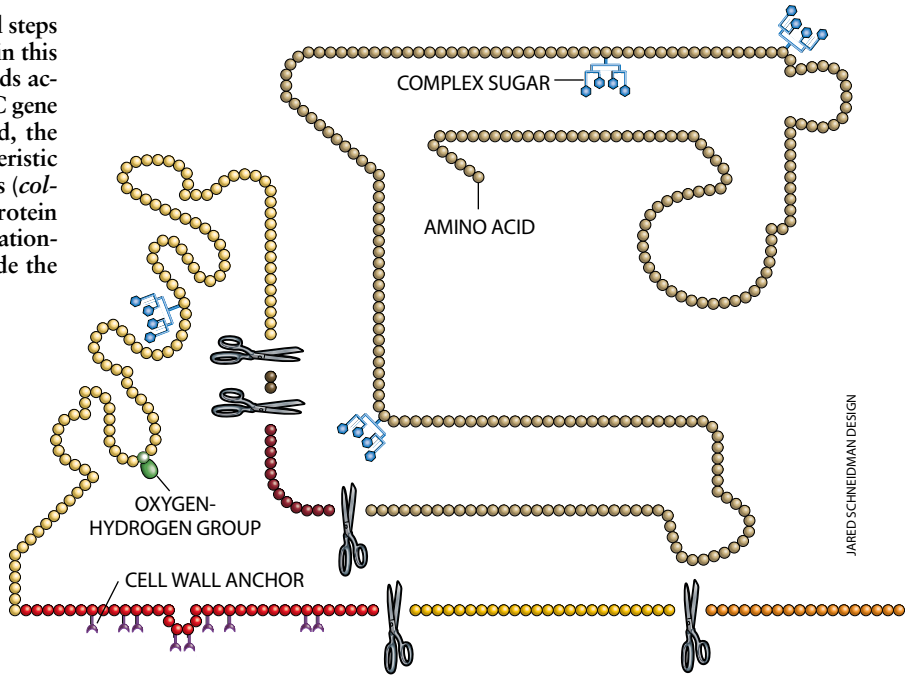
By implanting these fertilized cells in a surrogate mother pig, we could identify—after four months of nervous waiting—a newborn female piglet that carried the foreign DNA in all its cells. But even with this accomplishment, we had to remain patient for another year as our transgenic piglet, Genie, matured. Only then could we find out whether she would indeed produce the human protein in her milk. To our delight, Genie's milk contained protein C. Although the human protein was not as abundant as some of the pig's own milk proteins, it was nonetheless present in substantial amounts, with about one gram of protein C in each liter of milk—200 times the concentration at which this protein is found in normal human blood plasma. But we were also anxious to find out if this pig-made human protein would be biologically active.

We were concerned because the details of protein synthesis inside cells remain somewhat mysterious. The workings of the cellular machinery for reading the genetic code and translating that information into a sequence of amino acids—the building blocks for protein molecules—is, for the most part, well understood by biologists. But there are some subtle manipulations that need to be done by cells after the amino acids are joined together. These so-called post-translational modifications give a newly constructed protein molecule the final shape and chemical composition it needs to function properly. Post-translational modifications require complex cellular operations to cut off parts of the protein and to paste various chemical groups onto the molecule as it is as-



GENETIC ENGINEERING of a transgenic pig begins with the preparation of a DNA fragment (*left*) containing a copy of the human gene of interest and a so-called promoter sequence. The latter, derived from the gene for a mouse milk protein, assures that the human gene will be activated only in the pig's mammary tissues. Embryos are then harvested from a donor pig, and a selection of fertilized eggs (*below, left*) are injected with the foreign gene combination using a finely drawn glass pipette (*below, right*). The engineered DNA is added to the region of the male "pronucleus," a concentration of genetic material contributed by the sperm cell that fertilized the egg. A pig chromosome will take up the foreign DNA, perhaps because it recognizes the isolated fragments as pieces of its own DNA in need of repair.

ROBERTO OSTI (*animals*); JARED SCHNEIDMAN DESIGN (*diagram*); STEPHEN P. BUTLER (*photomicrograph*)



HUMAN PROTEIN C is synthesized in several steps within a cell. The cellular machinery involved in this task starts by stringing together 461 amino acids according to a prescription coded by the protein C gene (a step known as translation). As it is created, the nascent protein molecule folds into a characteristic configuration, forming several distinct domains (*colored regions*). But to function properly, the protein must also undergo several so-called post-translational modifications. These additional steps include the cleaving and removal of certain sections of the protein, as well as the addition of particular chemical groups to specific sites on the amino acid chain.

sembled. Would the cells of Genie's mammary tissue be able to carry out those modifications well enough to make a working version of the human blood protein?

To determine the answer, we had to tackle the new problem of isolating a human blood protein from pig milk. First we removed the milk fat by centrifugation. Then we purified the remaining whey using a procedure that would extract only the biologically active part of the human protein. To our amazement, this component amounted to about one third of the total complement of protein C present. Never before had functional protein C been produced and harvested at such high levels from a transgenic animal—or from a conventional bioreactor. Genie had passed a major test, providing the first practical demonstration that a complex human protein could be produced in the milk of livestock.

Next Year's Model?

We devoted several years to studying Genie and many of her extant offspring and then began to focus our efforts on increasing the concentration of active human protein in the milk. Our intent was to overcome the limitations of mammary tissue in making the needed post-translational modifications. In principle, breaking through those final barriers could triple the output of useful protein molecules produced.

With some painstaking research into the problem, we discovered that most of the protein C remained in an immature, inactive form because there were insufficient amounts of a key processing enzyme named furin—itself a complex protein—within these cells. Hence, we immediately asked ourselves whether we could improve the situation by introducing another foreign gene, one that would allow more of the needed processing enzyme to be made.

To test this possibility quickly, we switched our efforts temporarily from pig to mouse, the fast-breeding mainstay of most transgenic mammal experiments. In 1995 we succeeded in engineering a line of transgenic mice that contained two human genes—one for protein C and one for furin. We arranged for both of these transgenes to switch on in the mammary gland by attaching them to the DNA promoter we had previously incorporated in Genie.

After months of tedious effort in the lab, we were ecstatic to find that these mice were able to secrete the mature form of protein C in their milk. We have thus started development of a new and improved transgenic pig that contains human genes for both protein C and furin. We expect soon to see a pig that produces three times more active protein C than Genie did, and we anticipate that other researchers working with transgenic livestock will also be able to fashion genetic modifications that cause the manufacture of processing enzymes along with the target protein.

Chimerical Visions

The notion of obtaining essentially unlimited quantities of scarce human blood proteins at reasonable cost would have seemed pure fantasy just a short time ago. For more than two decades, molecular biologists and biochemical engineers have labored to overcome the problems of producing even modest amounts of human proteins from large-scale cell culture facilities. Yet making biological pharmaceuticals in huge stainless-steel vats of genetically engineered cells seemed destined to remain an awkward and expensive undertaking.

Such bioreactors are enormously costly to construct, and they prove in operation to be extremely sensitive to small changes in the temperature and composition of the broth in which the cells are grown. In contrast, transgenic livestock bioreactors can be created merely by breeding more animals. Transgenic livestock need only routine attention to control their living conditions and nutrient supply, and yet they can easily produce the desired proteins at much higher concentrations than their metallic counterparts.

Although some risk exists that pathogens could be transmitted from livestock to humans, formal procedures are available to establish pedigreed animals that are free of known diseases. Indeed, such specific-pathogen-free herds are a well-established part of the agriculture industry. In addition, decades of the clinical use of pigs to produce insulin for diabetics give us confidence that swine can readily serve as bioreactors for therapeutic human proteins without presenting undue hazard.

Still, like all new medicines, the human proteins produced in this way need to be carefully tested for safety and effectiveness before the government approves them for widespread use. The

What's Good for Genie…

The advent of transgenic techniques for manipulating livestock also raised legitimate concerns about the health and welfare of the animals altered in this rather unorthodox way. After all, engineered "transgenes" of the kind we implanted in pig embryos can ultimately become part of each and every cell of the mature animals. What if an introduced gene turns on inappropriately and produces the foreign protein in a way that damages the surrounding tissue?

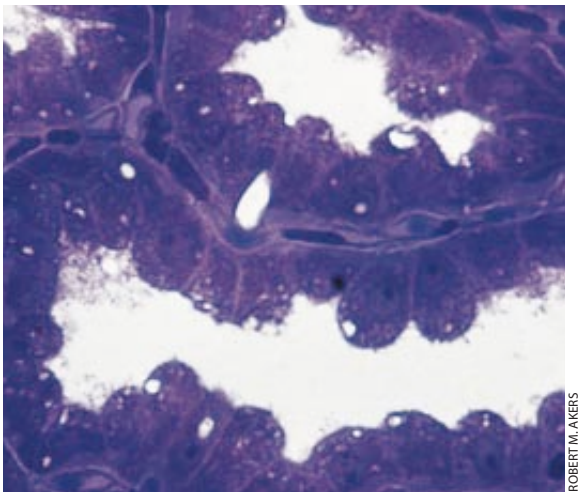
Such worries made it critically important that we design our genetic manipulations so that the foreign gene would be driven into action only in the mammary gland—that is, within tissues that have a natural ability to produce and export protein without harming themselves or their host. We could expect to achieve such targeted control of protein production in our transgenic pigs because we used a promoter from a milk gene—a genetic switch of a type that is present in all mammals.

Yet we recognized that even such well-behaved genes can show some promiscuous activity. The genes we introduced into pigs, for example, also produce small amounts of their foreign proteins in the animals' salivary glands. These tissues are, in fact, quite similar in composition to mammary tissue. So we fully expected this incidental production, and we are quite sure that this minor side effect does not harm the pigs in any way.

The lack of detrimental side effects is crucial—for the animals involved and also for the success of this pioneering method. One of the primary reasons for developing transgenic livestock to supply human proteins is to limit the possibility of transmitting diseases to the recipients of these drugs. Using anything but the healthiest livestock to produce these substances could increase the animals' susceptibility to disease as well as the possibility that they might accidentally pass on some unknown pathogen. Genetically engineering weakened livestock would thus, in the end, only prove self-defeating in the quest to produce safe and plentiful medicines. *—W.H.V.*

first example to be so examined (an anticlotting protein called antithrombin III, manufactured by Genzyme Transgenics Corporation using transgenic goats) began clinical trials just a few months ago.

It is possible that the subtle differences between human and animal cells in the way post-translational modifications are carried out may affect how such proteins function in people. For example, certain modifications cause proteins to be cleared from the blood quickly by the liver, and so we suspect that some of the differences between the animal and human forms of these proteins could actually constitute improvements in the way these substances function as long-lived therapeutic drugs.



ROBERT M. AKERS

MAMMARY TISSUE from a genetically engineered pig contains a dense array of cells (*purple*) that produce a therapeutic human protein. The structure of the mammary gland allows the human protein produced in this way to flow through the secretory channels (*white*), along with other components in the animal's milk.

It is tempting to view the development of transgenic livestock bioreactors purely as a triumph of technology. But the history of this science also highlights the limits of what people can do with sophisticated machines. The mammary gland is optimized to maintain a high density of cells, to deliver to them an ample supply of nutrients and to channel the valuable proteins produced into an easily harvested form. Mammary tissue proves far superior to any cell-culture apparatus ever engineered for these tasks. Despite all their efforts to improve industrial cell-culture facilities, it turns out that a generation of biochemical engineers were unable to match the abilities of a tool for making proteins that nature had already honed. SA

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Further Reading

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