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MOLECULAR FARMING: TRANSGENIC ANIMALS AS BIOREACTORS

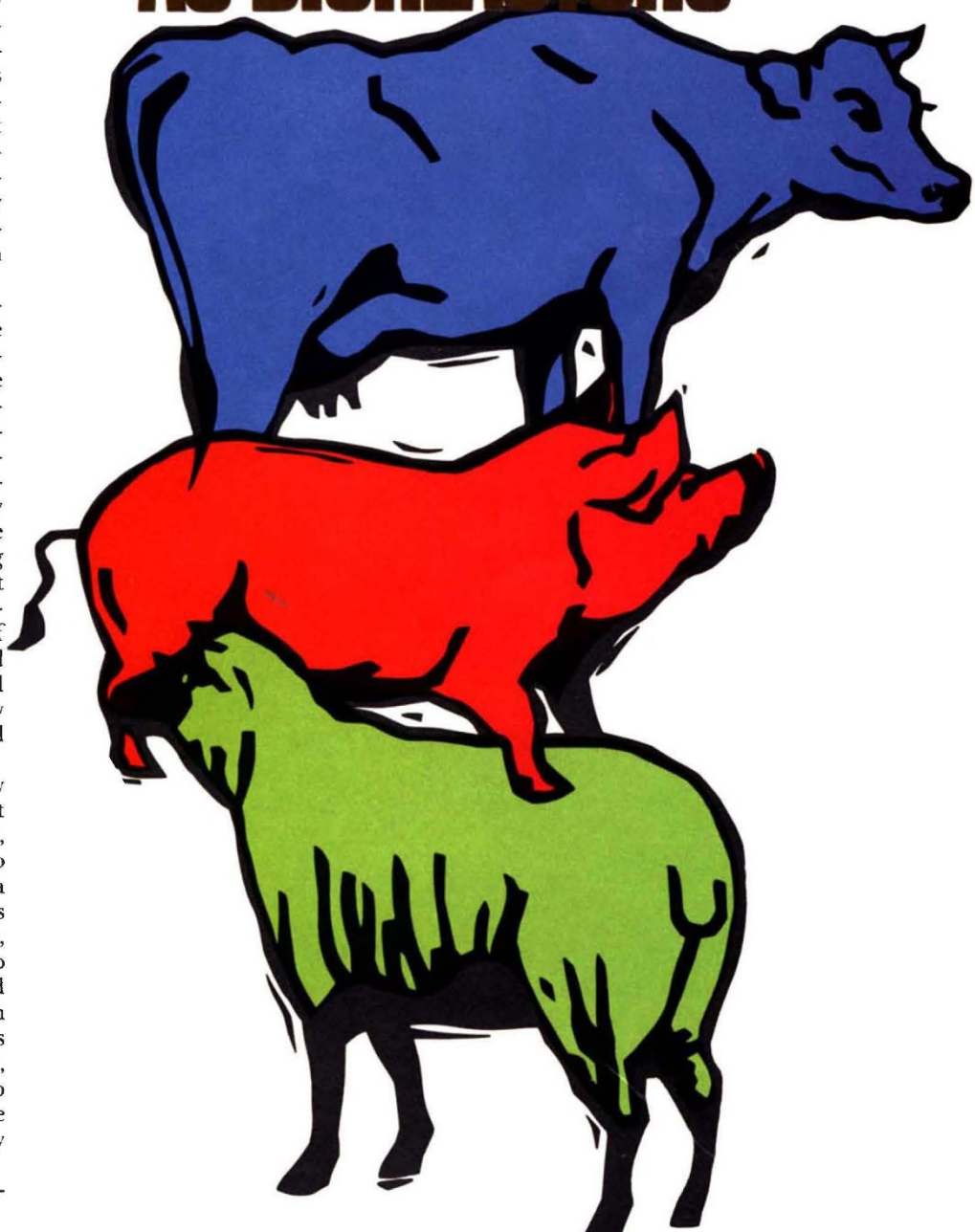
by Jennifer Van Brunt

Nobody who picked up the December 16, 1982 issue of *Nature* could doubt that there was some fundamentally significant difference between the two mice that graced the cover—beyond the fact that one was twice as large as the other. "Supermouse"—perhaps the only living rodent in history to achieve star status—was not just big, it was transgenic. Its remarkable growth had been governed by the structural gene for rat growth hormone, fused to the promoter for the mouse metallothionein-1 gene. Supermouse became a very visible manifestation of genetic engineering's ability to profoundly alter an animal's phenotype.

The remarkable results of this experiment and others conducted in the early 1980s crowned years of frustrated attempts to refine and perfect the techniques necessary to create transgenic animals. Constructing the foreign fusion gene was just the beginning: microinjecting it into the pronuclei of single-cell ova; successfully implanting those into surrogate mothers; bringing the developing embryos to term; demonstrating that the foreign gene has been stably, heritably incorporated into the DNA of at least some of those newborns; and proving that this gene is regulated well enough to function in its new environment—all presented, and still present, formidable challenges.

Many of these challenges are now being tackled in model systems that are large to begin with—cattle, sheep, pigs, even chickens. The goal is not to produce cows as big as elephants (a popular misconception that persists even today), but to introduce specific, economically significant traits into livestock. Conventional breeders and molecular geneticists alike envision that tomorrow's transgenic animals will be more efficient at utilizing feed, will have leaner meat, will grow to marketable size sooner, and will be immune to those diseases that today decimate populations and profits.

And many biotechnologists see an-



other way to profit from transgenic animals: as living bioreactors that secrete valuable recombinant proteins and pharmaceuticals into their milk or blood stream. The concept of "molecular farming" has also caught the fancy of venture capitalists; the last year or so they have financed a host of transgenic startup companies—many of which are still headquartered in executive desk drawers.

Experimental results in large animals have been slow in coming, but seem to be worth the wait. It *is* possible to achieve the expression of incorporated, heritable, foreign genes in livestock. Successful "takes" are still very low and expression levels are nothing to brag about, but the research scientists who have devoted the last three or four years to such experiments are fairly encouraged by their progress. While technical challenges still remain, the main barrier to successful molecular farming—how to regulate a foreign gene's expression in its new host—remains in the cloner's domain. To him falls the task of manipulating regulatory signals so that the gene's spatial and temporal expression can be controlled at will.

The Growth-Hormone Lesson

While there is no doubt that transgenic mice are still the most reasonable model organisms for studying the integration, expression, and regulation of a foreign gene construct, they don't always prove to be reliable predictors of the same construct's behavior in large animals.

The early experiments using the zinc-regulatable metallothionein (MT) promoter and growth hormone gene (GH) constructs did, indeed, demonstrate that the integrated construct could pump out enough GH to grow giant mice. In most cases, it was even possible to control the gene's expression at will. What those experiments did not reveal, however, was the adverse physiological effect of chronic elevated hormone levels, or the fact that the zinc switch doesn't always work. As important, large animals transgenic for GH don't necessarily grow any bigger.

Experiments by Robert E. Hammer and colleagues (*Nature* **315**:680, June '85), in which they injected the MT-human GH fusion gene into more than 5,000 ova (collectively) of rabbits, pigs, and sheep, were moderately successful in producing transgenics in all three species. And some of the pigs and rabbits even expressed low levels of human GH (none were exposed to high levels of zinc, however, which raises the gene's expression in mice by 10-fold). In no case, however, were the frequencies of transfer or expression anywhere close to what was possible in mice. Interestingly, month-old transgenic pigs, with plasma levels of hGH greater than 300 ng/ml, showed no dramatic increase in body weight. (In mice, 20-80 ng/ml is enough to set off a growth spurt.)

Mouse models can be equally misleading for experiments in sheep. James M. Murray, principal research scientist at CSIRO's division of animal production (Sydney, Australia), and his collaborators have found that their GH construct, which worked beautifully in mice, failed miserably in sheep. Murray opted for constructs consisting entirely of sheep DNA—both the MT promoter and the GH gene. In mice, he could regulate this construct at will with zinc; in sheep, he couldn't get it to turn off. (Murray is now working with a modified MT promoter that may be down-regulated.) In mice, elevated GH levels doubled growth rates and had no adverse physiological consequences. In sheep, the chronic elevated levels of GH killed three out of four—one in 10 weeks, the others at 11 months. Murray's conclusion: "For this gene, anyway, the mouse is not a good model."

In fact, creating livestock transgenic for growth hormone might not be the best way to enhance their commercial appeal. According to Murray, to date, all such large animals suffer from common ailments—they have a reduced ability to fight infection, they tend to die young, the females are infertile—and there is no increase in growth rate. On the positive side, GH does cause the animals to grow leaner and can increase feed efficiency.

Regulating animals' growth transgenically rather than by injections may only become feasible when it is possible to control GH expression. Hormones are tightly regulated, and interact in ways that we barely understand: creating hormonal imbalances in such a non-specific fashion is bound to cause trouble. On the other hand, adds Murray, transgenic constructs that instruct mammary glands to secrete human pharmaceuticals, or alter the protein composition of wool, for instance, should not affect an animal's physiology—thus allowing its use as a production system.

In fact, this is just the bet that many scientists—and venture capitalists—are willing to take. And the odds are looking better all the time. Transgenic mice already secrete human tissue plasminogen activator (t-PA) into their milk (Gordon et al., *Bio/Technology* **5**:1183, Nov. '87; Pittius et al., *PNAS* **85**:5874, Aug. '88). The human secretion signal appears to function normally; t-PA mRNA is confined to the target tissue; lactation induces gene expression; and the t-PA is biologically active (although the protein's concentration ranged from a control level of 20 ng/ml to over 50,000 ng/ml). Moreover, transgenic, t-PA expressing mice pass these abilities on to their progeny. Will this situation obtain in large animals? Presumably—if the promoters are correct. But it takes doing the actual experiment to know.

Sheep: the First Success

The first—and, to date, the only—large animals to achieve the status of quasi-bioreactors are sheep. These transgenics secrete human factor IX or alpha-1 antitrypsin into their milk. Although expression levels are low, factor IX does seem to be active. And the trait is heritable.

These sheep culminate years of experiments done by J. Paul Simons and his associates at the AFRC's Institute of Animal Physiology and Genetics Research station in Edinburgh, Scotland. They, too, started with mice, and demonstrated that a cloned sheep beta-lactoglobulin gene (BLG) is expressed specifically in the mammary glands of lactating mice (Simons

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Transgenic sheep (seen here being milked) are able to produce human factor IX and alpha-1 antitrypsin in their milk.

et al., *Nature* **328:**530, Aug. '87). Sheep BLG levels in milk ran as high as 23 mg/ml (five times that estimated for sheep milk). Five out of seven "generation zero" mice transmitted the gene to their progeny.

For Simons, the mouse model system *did* prove an accurate predictor: the cloned BLG gene also directs tissue-specific secretion of human proteins in sheep milk (Simons et al., *Bio/Technology* **6:**179, Feb. '88). Four transgenic sheep (out of 92 live-born lambs) carried the gene for human factor IX (FIX); one received the alpha-1 antitrypsin construct (α1AT). (Two more α1AT-containing sheep have been born since.) Three sheep have transmitted the FIX construct to their progeny, although some of the resulting females do not appear to have high expression levels, says Simons.

According to Simons, FIX expression levels in "generation zero" transgenics are very low—about 20 ng/ml—but the protein is active in standard clotting assays. By comparison, this protein's normal plasma level is 5 micrograms/ml. (Engineered mammalian cell cultures can secrete up to 100 ng/ml, he says, but the protein is only two-percent active.) And at least one of the α1AT-containing sheep expresses the protein in its milk, says Simons, but he does not yet have a good estimate of its concentration.

Obviously, the experiments have just begun. Simons' overall success rate for producing live transgenic animals from microinjected one-cell ova is still only one percent, and the expression levels are probably far from maximal. Add to this the long gestation period in sheep (five months), the 12 months it takes to reach sexual maturity, and the seasonal nature of the breeding cycle (only in the winter), and time-to-commercialization becomes very much an event of the future. Even these distant prospects, however, were appealing enough to Pharmaceutical Proteins Ltd. (Cambridge, U.K.), for it to fund the Edinburgh research—and wait for the reward.

Pigs: a New Model?

Pigs may become the second-level transgenic model system. In some ways they are the prototype of the cow, but the litters are larger and gestation periods shorter, according to Steven Holtzman, COO at start-up Embryogen (Athens, OH). The efficiencies of transgenic experiments are often better than for sheep or cattle, approaching those achieved in mice. Pigs could be engineered as production systems for human pharmaceuticals, as well—with the vehicles being blood or urine instead of milk.

Vernon G. Pursel, a scientist at the Agricultural Research Service's Beltsville, MD facility, has devoted much of his early work to creating pigs transgenic for growth hormone (bovine or human.) Although he found the efficiency of integration to be low, more than half the resulting transgenic pigs expressed GH—albeit at widely varying plasma concentrations. By slowly changing the composition of the feed, he was finally able to detect that the transgenic pigs grow a little faster than controls. But they still don't get larger.

Pursel has also found that continuous elevated levels of growth hormone are detrimental—even fatal. He is exploring the utility of other transgenic constructs, including ones containing growth hormone releasing factor, murine whey acidic protein gene, and sheep globulin gene. An incorporated sheep globulin gene might control foreign gene expression naturally, explains Pursel. In sheep and goats, this gene is developmentally regulated: it turns on one month after birth, and shuts down at puberty. Pursel and his associates have successfully produced pigs carrying the sheep globin gene; for reasons that are unclear, the transgenic animals are not yet expressing that gene.

Cows Take the Longest

Most scientists working on transgenic animals—be it for improving traits such as feed efficiency or for using them as factories for human pharmaceuticals—shy away from cattle. "In the cow," says Pursel, "you have a three-to-four year project." And it's a costly venture, as well. Even the very first step—microinjecting foreign DNA into ova—has its price. Generally, one can't just insert a foreign gene and transfer the egg directly back into a surrogate mother, explains Pursel. To have a decent success rate, the injected ova have to be cultured first.

Improving efficiencies of gene transfer and expression is the major goal of research scientists at Granada Genetics (Houston, TX) and their various collaborators. "What one wants for cattle," says Baylor College of Medicine's (Houston, TX) Bert O'Malley, "is very high efficiency, because it's so expensive." Joseph M. Massey, Granada Genetics' president, claims the scientists have injected 3,000–4,000 ova to date: after one week in culture, about 40 percent survive. And efficiencies are still low—overall, he says, about one percent seem to incorporate and express the transgenes.

According to Massey, the company is already field-testing cloned bovine follicle-stimulating hormone/luteinizing hormone (FSH/LH) for its ability to induce superovulation. The FSH/LH gene, supplied by collaborators at Integrated Genetics (Framingham, MA), is joined to a beta-casein promoter: Massey says they have confirmed pregnancies and fetal development, but as yet, no live births of transgenic calves. Also *in utero*, he adds, is a transgenic construct that links an actin promoter with insulin-like growth factor (IGF), as well as an actin:estrogen receptor gene construct.

Massey claims it is the choice of promoters—actin, which targets muscle, and beta-casein, which targets the mammary gland (both of which have been developed by Baylor scientists)—that makes Granada's approach unique. "The goal," explains Baylor's O'Malley, "is to develop a vector system with tissue-specific enhancer-promoter activity."

Granada's real expertise, says Massey, is its ability to clone embryos. In fact, scientists at the company's production facilities (Marquez, TX) are producing clones daily: they have al-

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Cloned triplet cattle, born April 1987. Once producing transgenic cattle embryos is routine, cloning them will result in the rapid production of herds with selected characteristics.

ready had live births of sets of two, three, up to seven cloned calves. "Now that we can do that," says Massey, "we need to identify a [candidate] embryo that has an expressing gene."

At least one transgenic cow is on the ground in Canada. Robert Church, a professor at the University of Calgary, says that his group has successfully produced an animal that expresses human beta-interferon (IFN). This animal has passed the foreign DNA on to its calf: the question is whether the calf will express that trait when challenged with live bovine diarrhea virus. The calf is still too young for this experiment, says Church.

Church has injected over 2,000 bovine embryos to date. He uses an MT-like promoter, which responds to cadmium, selenium, or zinc and turns on the IFN gene; turning it off is not so straightforward. Once the stimulus is removed, however, gene expression gradually returns to its basal level.

The majority of Church's efforts are linked to a disease resistance program. His group has also produced three calves that have incorporated a transgene consisting of a whey promoter and an antigenic epitope of coronavirus. They also have some animals that have incorporated a construct of alpha fetoprotein promoter linked to a viral coat protein epitope. There is no expression so far, says Church.

Church is trying to develop a bovine stem cell line, into which one could transfer a gene and detect its expression before introducing it into the host as a blastocyst. Actually producing transgenic cattle, he says, is enormously costly and logistically complex, a situation only complicated by our limited knowledge of bovine cellular physiology.

Chickens, Too?

Most transgenic work with chickens is aimed at besting conventional breeders in improving the birds' muscle growth, egg production, and disease resistance. Making transgenic chickens presents its own unique dilemmas: being able to penetrate the egg shell, and then being able to inject the pronucleus, which is very small compared to the fertilized ovum (the yolk) and impossible to see. An approach developed by embryologist Margaret Perry, in the Roslin lab of the AFRC's Edinburgh branch, may prove a way out of this conundrum—at least half of it. Perry has overcome the barriers of access to the egg by developing a laboratory culture system that actually produces a chick from a zygote. "We haven't yet produced a transgenic chicken," says Perry, "but we have hatched birds that have been grown in culture from a time shortly after fertilization. The technique allows us to introduce genes into fertilized ova."

Perry grows the embryo in an airtight glass container for the first 24 hours, then transfers the contents to an egg shell, fills it with nutrient medium, seals it, and rotates the egg hourly for three days. Then, she transfers the entire contents of that shell to a larger shell, adds more medium, but leaves an air space, and waits for the chick to hatch 18 days later. "Our success rate of hatching is still pretty low, about five percent," she says.

The Edinburgh group is still in the very early stages of introducing genes into fertilized ova. At this point, the experiments involve incubating the embryos for seven days (with about 50 percent viability), and then analyzing the fate of the foreign DNA. "So far, our results indicate the genes are not integrated into the genome." Because it is impossible to see the pronuclei in the embryo, "this is very much a hit-or-miss situation," concludes Perry. Even so, this technology is the subject of a patent application, and has sparked the keen interest of investors: "Quite a number of commercial companies have shown an interest," says Perry.

Will chicken eggs ever find a role as production vehicles? According to Perry, once a foreign gene is incorporated, it should be fairly straightforward. Quite a lot is already known about the ovalbumin genes; all the albumin, which is relatively pure, is laid down in the first five hours after ovulation. Moreover, this happens every 24 hours as each ovum is produced.

In the States, several transgenic companies devote part of their research efforts to chickens. Embryogen, which was formed in 1984 as the commercial arm of the Edison Animal Biotechnology Center on the campus of Ohio University, collaborates with Merck Sharp & Dohme (Rahway, NJ) on such a venture. And Transgenic Sciences' (Worcester, MA) president F. Donald Hudson says his company maintains a program with Tufts University Veterinary School (Grafton, MA) on using chicken eggs as production vehicles for human pharmaceuticals. At this point, says Hudson, they are doing the molecular biology; once they have the constructs, they will go into mice to prove the vectors work before scaling-up to chickens. According to Hudson, most people agree the only reasonable way to introduce genes into chicken embryos is via retroviral vectors, due to the complexity of getting DNA into the pronuclei.

Embrex (Research Triangle Park, NC) is initiating a program in transgenic poultry, as well. According to president Alan G. Herosian, this is a long-term R&D goal. The company's present focus is developing high-speed technology for vaccinating eggs, a system that is just about to go into field trials. Herosian has no plans to use transgenics as production vehicles; Embrex's fledgling transgenic program is aimed at improving commercially interesting traits, by combining genetics with high-speed delivery technology.

Validating a Cow

Transgenic livestock are still a long way off. According to CSIRO's Murray, "If everything were looking well, it would probably still take five to six years for the first commercial success." Tied to that success, of course, are regulatory issues—how difficult will it be to define quality control, for instance?

Transgenic Sciences' Hudson feels that animal production systems are not that different than mammalian cell culture systems. The main issue, he says, is to be able to prove to the Food and Drug Administration (FDA) that the proteins are pure and nontoxic, and that the process doesn't affect the animal *per se*. We already have human fertility hormones isolated from the urine of post-menopausal women. And Premarin, a post-menopausal drug, comes from pregnant mare serum. We use insulin derived from pigs, and lysozyme from egg whites. Given these examples, it shouldn't be a conceptual leap for FDA, concludes Hudson. And Kevin Kinsella, acting CEO of Chimera Biotech (La Jolla, CA), agrees that there are already enough examples of animal proteins being used in humans to set a precedent. Kinsella does predict, however, that the regulatory burden will be severe on issues of purity.

Robert Church, on the other hand, forsees a major difficulty in validation issues. "When do you draw the line between an animal as a production unit and an animal as an animal? FDA is not used to looking at a situation where the animal itself is part of the test system. But if the animal tolerates [the foreign protein], and is otherwise normal, that ultimately *has* to be the test system."

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