Resource Requirements for Transgenic Livestock Research

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ABSTRACT: Transgenic research usually involves a large investment in carrying out a series of low-efficiency steps to produce a few transgenic founder animals, and then characterizing a transgenic line of animals derived from each founder. There is considerable variation in phenotype among transgenic lines made with the same DNA construct. In the case of livestock species, to produce one founder animal that expresses a transgene typically requires injection of DNA into hundreds of embryos, which then are transferred to reproductive tracts of recipients for gestation to term. To reduce costs, embryos sometimes are screened by analysis of a biopsy using the polymerase chain reaction, and only those with the transgene are transferred. Characterizing a transgenic line often is a greater logistical undertaking than making the transgenic founder. Ideally, animals should be evaluated for the transgenic trait as well as for absence of undesirable side effects in both sexes in both the hemizygous and homozygous transgenic states. Producing homozygous transgenic animals requires mating relatives, resulting in inbreeding. Characterization of transgenic lines takes many years in species with long generation intervals; the whole process is much less costly in litter-bearing species. Because of safety and efficacy issues, characterizing transgenic lines for agricultural production purposes will be more demanding than characterizing them for research purposes. At a minimum, three broad areas of expertise are required for successful transgenic projects: molecular biology, embryo micromanipulation/reproductive physiology, and knowledge of the biology of the tissues/systems affected by the transgene. Facilities and equipment attendant to these areas of expertise are essential, as are appropriate animal facilities, including surgical suites for most projects. Although current procedures are expensive and inefficient, new approaches plus incremental improvements that accumulate with experience will make transgenic technology much more efficacious in the future.

Key Words: Transgenic, Homozygous, Hemizygous, Cost

Introduction

Transgenic technology is a very efficacious approach to answering some kinds of fundamental questions (Gordon, 1988; Jaenisch, 1988), can be used for making customized models of diseases, and may be used to produce valuable proteins in body tissue and fluids such as blood, urine, milk, and egg albumin (Ebert et al., 1991; Wright et al., 1991). In the longer term, this technology can be applied to production agriculture to increase genetic variance (Smith et al., 1987), to produce animals resistant to disease (Salter et al., 1987), and to make animals that produce fundamentally new products (Bremel et al., 1989) or more conventional animal products more efficiently. As one moves from mice to pigs, the costs to produce one expressing transgenic founder animal increase more than 10-fold, and there likely is another 10-fold increase when moving from pigs to cattle. I have heard $500,000 quoted as the cost of producing one suitable transgenic founder calf. This figure likely is too low for past efforts (Hill et al., 1992) and too high for future ones; estimates of costs and prognostications of success must be viewed suspiciously because so far (mid-1992) not a single case clearly documenting expression of a transgene in cattle has appeared in the literature. On the other hand, there have been numerous cases of transgenic expression in swine, sheep, goats, and chickens (see papers in these Proceedings). Remarkably, the majority of these transgenic founders have been sterile, which greatly decreases their utility for research. Note, however, that many of these were transgenic for very high expression of growth hormone, and that most future transgenic livestock likely will be fertile because of improved regulation of transgene expression. To summarize, transgenic procedures are clumsy and expensive, but improving rapidly.

1Our transgenic research was supported by the Experiment Station at Colorado State University through USDA Regional Project W-171. Numerous colleagues and students have contributed to the ideas in this paper.
More than 90% of transgenic research with mammals has been done with mice. To understand the resources required for transgenic research with farm animals, pertinent differences between livestock and mice will be analyzed in three areas: 1) intrinsic species differences, 2) generation interval, and 3) inbred lines. It should be noted that producing transgenic animals for research is much simpler than producing them for production agriculture; in the latter case, lack of deleterious side effects and safety must be addressed, which requires huge resources. The special case of using transgenic animals to produce pharmaceuticals can be relatively straightforward biologically but especially complicated from a regulatory perspective.

Results and Discussion

The Typical Transgenic Mouse Model

Ideally, a transgene is constructed by combining a regulatory sequence of DNA with a structural sequence of RNA to produce the desired mRNA and resultant protein in the desired tissues at the desired time of the life cycle (e.g., in mammary tissue during lactation or in hemopoietic tissue at all times). In some cases, it is appropriate to test constructs in cell lines in vitro before proceeding with in vivo studies.

Typically, 30 or 40 one-cell embryos are recovered per superovulated mouse, and these are automatically synchronized within a relatively narrow stage of the cell cycle (because ovulation is induced with a timed hCG injection). Hundreds of copies of the DNA construct then are injected into the easily visible pronuclei, the embryos are cultured overnight, and those developing normally are transferred the next day to the oviducts of pseudopregnant recipients (perhaps 20 embryos/recipient) for gestation to term. About 1 wk after birth, the tip of each newborn mouse’s tail is removed, and DNA is extracted and probed for the transgene with a Southern blot. Although the objective usually is to have one copy of the transgene integrate at one site in a chromosome, the more common outcome is multiple copies integrated at one site; in over 10% of transgenics, integration occurs at multiple sites.

Each transgenic founder mouse is studied carefully but used primarily for reproduction. In most cases, half the offspring produced are transgenic (but only on one chromosome; i.e., hemizygous). In the founder parent, some of the cells in the body may not be transgenic. However, in transgenic offspring, the transgene will be in every somatic cell in the body because the founder’s gamete resulting in the offspring was transgenic.

Hemizygous offspring then are mated with each other (brother-sister, or sometimes parent-offspring). These matings result in 25% homozygous transgenic, 50% hemizygous transgenic, and 25% nontransgenic offspring (Table 1). Because mice usually are inbred or at least are selected for absence of lethal genes, there is no practical consequence of brother-sister matings, and reasonably homogeneous animals are produced with ideal, nontransgenic controls. These populations then can be studied in various ways.

Not all transgenic experiments proceed in this way, but most do. The seven types of animals listed in Table 1 may be quite different from each other. For production agriculture, it would be important to characterize each type. It is especially important to characterize the homozygous transgenics, because insertional mutagenesis often results in homozygotes for the transgene that die at the embryonic stage or become very abnormal animals (Jaenisch, 1988). Although this occurs only in about 10% of transgenic lines, a huge effort is required to remove such deleterious recessives from populations. Past examples of deleterious recessive alleles in cattle include ovarian hypoplasia, dwarfism, uridine monophosphate synthase deficiency, and leukocyte adhesion deficiency (Robinson et al., 1984; Shuster et al., 1992).

Sometimes the situation is even more complex than that outlined in Table 1. For example, the transgene may integrate on the X-chromosome and therefore likely will be inactivated in half the cells of females, there may be multiple sites of insertion, or the gene may be imprinted differently when inherited via the sperm or the ovum (Swain et al., 1987). With imprinted genes, there is expression of mRNA when inherited from one sex but not the other; interestingly, it is the sex of the parent, not the sex of the offspring that governs this effect (Swain et al., 1987).

Intrinsic Species Differences

In vitro techniques such as composition of culture media for embryos have been optimized for mice but not yet for farm animals. Intensive research on production of embryos from in vitro maturation and fertilization of oocytes from ovaries of slaughtered farm animals is rapidly closing this gap. Another difference between mice and livestock species is that variation in stage of the cell cycle is higher for zygotes from livestock than for those from mice because ovulation (and thus fertilization) among females is not as well synchronized. The result is that some embryos are not at the correct stage to be injected. In vitro fertilization can be used to reduce this problem, but it simultaneously leads to other problems, such as reduced viability compared with that of embryos fertilized in vivo.

Pronuclei are difficult or impossible to visualize in embryos of cattle and swine without a centrifugation step (typically 15,000 x g for 3 min), which complicates the process. An important logistical consideration for non-litter-bearing species is that only one to three embryos can be transferred per recipient, which
greatly increases recipient costs. Ideally, embryos should be returned to the oviducts on the day after microinjection. Surgery is required for this step in all species. In the case of cattle and horses, embryos frequently are cultured in vitro or in vivo in oviducts of intermediate recipients such as rabbits or sheep for several days until they are at a suitable stage for nonsurgical embryo transfer to the uterus.

Incidence of integration of the transgene into chromosomes is markedly lower in farm animal species than in mice. This may be due entirely to a lower efficacy in the series of steps in making transgenic animals (e.g., due to visibility of pronuclei), or there may be inherent biological differences such as DNA repair enzyme activity. A summary of selected studies on efficiencies of producing transgenic founders for livestock is presented in Table 2. In most of these studies it was unclear how many embryos could not be injected due to absence of visible pronuclei, or how many zygotes were lysed during microinjection (usually these are on the order of 20% and 10%, respectively). Also note that bovine embryos were screened for normal development (see Table 2 footnotes) before embryo transfer, and other species were not. Some of the embryos in the study by Hill et al. (1992) and all of them in the study by Krimpenfort et al. (1991) were derived from in vitro fertilization.

The proportion of embryos surviving microinjection (usually these are on the order of 20% and 10%, respectively). Also note that bovine embryos were screened for normal development (see Table 2 footnotes) before embryo transfer, and other species were not. Some of the embryos in the study by Hill et al. (1992) and all of them in the study by Krimpenfort et al. (1991) were derived from in vitro fertilization. The proportion of embryos surviving microinjection that developed into transgenic, expressing founders ranged from 1/200 in goats to less than 1/1,000 in cattle. Such results are a consequence of doing a large number of steps in series; the final success is the product of the probabilities of success of each step.

Little is known about regulation of gene expression in any species, and indeed transgenic procedures are increasingly valuable in obtaining such information. It remains very difficult to predict the phenotype of a specific transgenic animal, and results cannot always be extrapolated reliably among species. This is illustrated by metallothionine promoter-growth hormone structural gene constructs; these result in much more dramatic growth in transgenic mice than in sheep or swine (Pursel et al., 1990; Rexroad et al., 1991). They also induce diabetes in sheep (Rexroad et al., 1991).

### Generation Interval

The times required for producing the animals listed in Table 1 are summarized in Table 3.

It is clear that characterization of transgenic livestock is a long-term proposition. Even more time must be added to figures in Table 3 to evaluate sex-specific traits such as lactation. If the founder animal is a bull, one will not even have an inkling of lactation-specific transcription until his daughters begin lactation, about 4.5 yr after the DNA injection step under ideal conditions. This also brings up the desirability of starting with the appropriate genetic background for the founder (e.g., beef vs dairy breed).

There are special cases with much shorter time frames. For example, if expression of the transgene were desired in d-14 bovine embryos, it is possible to recover these nonsurgically, biopsy them to measure expression, and nonsurgically retransfer only the expressing embryos for gestation to term. One thus can determine transgenic status, including expression, 9 mo before birth!

### Lack of Completely Inbred Lines of Livestock

Lack of completely inbred livestock results in two experimental disadvantages. First, there is much more variation from animal to animal than that which

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### Table 1. Types of animals produced from transgenic experiments

| Species | Δ or Φ hemizygous founder (possibly mosaic)
|---------|---------------------------------|
| Cattle | p and Δ nontransgenic
| Goats | Φ and Δ hemizygous
| Sheep | Δ and Φ homozygous (always inbred)

a Non-mosaic founders will be like Group 3.
b Types 2, 3, and 4 are produced in a 1:2:1 ratio when mating hemizygous animals with each other.

d Ranges from 1/200 in goats to less than 1/1,000 in cattle. Such results are a consequence of doing a large number of steps in series; the final success is the product of the probabilities of success of each step.

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### Table 2. Efficiency of producing transgenic founders in farm animals

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of embryos surviving injection</th>
<th>No. of offspring (%)</th>
<th>No. of transgenic (%)</th>
<th>No. expressing construct (%)</th>
<th>% expressing/embryo surviving injection</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swine</td>
<td>7,936</td>
<td>661 (8.3)</td>
<td>45 (6.9)</td>
<td>25 (55.6)</td>
<td>.32</td>
<td>Pursel et al., 1990</td>
</tr>
<tr>
<td>Sheep</td>
<td>4,225</td>
<td>347 (8.2)</td>
<td>34 (9.8)</td>
<td>11 (37.5)</td>
<td>.26</td>
<td>Rexroad et al., 1990</td>
</tr>
<tr>
<td>Goats</td>
<td>203</td>
<td>29 (14.3)</td>
<td>2 (6.9)</td>
<td>1 (50.0)</td>
<td>.49</td>
<td>Ebert et al., 1991</td>
</tr>
<tr>
<td>Cattle</td>
<td>11,206&lt;sup&gt;a&lt;/sup&gt;</td>
<td>193 (1.7)</td>
<td>7&lt;sup&gt;b&lt;/sup&gt; (3.6)</td>
<td>&lt;? .03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;.03</td>
<td>Hill et al., 1992</td>
</tr>
<tr>
<td>Cattle</td>
<td>981&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21 (2.1)</td>
<td>1&lt;sup&gt;d&lt;/sup&gt; (4.8)</td>
<td>?</td>
<td>&lt;.10</td>
<td>Krimpenfort et al., 1991</td>
</tr>
</tbody>
</table>

<sup>a</sup>1,058 of these developed normally enough to transfer.

<sup>b</sup>Three of the seven calves were stillborn or died neonatally.

<sup>c</sup>129 of these developed normally enough to transfer.

<sup>d</sup>One additional calf was a mosaic for a rearranged version of the gene.
Table 3. Effects of generation interval on time points in transgenic experiments

<table>
<thead>
<tr>
<th>Event</th>
<th>Time from Microinjection of DNAa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Founder's birth</td>
<td>Mice  Sheep Cattle</td>
</tr>
<tr>
<td>Founder's offspring's birth</td>
<td>3 wk  5 mo  9 mo</td>
</tr>
<tr>
<td>Birth of homozygote</td>
<td>13 wk 20 mo 32 mo</td>
</tr>
<tr>
<td>Homozygote reproduces</td>
<td>23 wk 35 mo 55 mo</td>
</tr>
<tr>
<td></td>
<td>33 wk 50 mo 78 mo</td>
</tr>
</tbody>
</table>

aAssumes puberty occurs at 7 wk, 10 mo, and 14 mo for mice, sheep, and cattle, respectively.

one finds within inbred mouse strains. This makes it more difficult to characterize the true phenotype of the transgenic line. A related problem is interaction between the transgene and the genetic background. A special example might be imagined for genes affecting lactation in beef vs dairy cattle (Hart et al., 1978).

The second disadvantage to lack of inbred livestock is especially insidious. The breeding scheme in Table 3 assumes brother-sister or parent-offspring matings to make homozygous individuals; this results in 25% inbreeding with full-sibs and 12.5% with half-sibs. Animals with these levels of inbreeding usually have compromised phenotypes for survival traits independent of transgenes. To sort out decreased performance due to the offsprings' being homozygous transgenic from the effects of their being inbred will require huge numbers of animals. Note that because each founder has a unique site of insertion of the transgene, inbreeding is necessary to produce homozygous animals to test.

There are several solutions to this problem. The first is to add one more generation to Table 3 and mate cousins instead of siblings (Smith et al., 1987). This of course requires more animals and more time. A second solution is to not test homozygotes. This is the appropriate course for many research applications and for special cases such as producing pharmaceuticals in milk. However, if in the future both mates have the transgene, in about 10% of transgenic lines such matings will result in very deleterious consequences, such as dwarfism, embryonic death, and so on, due to insertional mutagenesis (Palmiter and Brinster, 1986). A third solution, using embryonic stem cells and homologous recombination to obtain the same site of insertion in unrelated animals (Mansour et al., 1989) many embryos must be transferred, preferably one per recipient in cattle, to avoid freemartins. From the summary by Hill et al. (1992), approximately 150 embryos were transferred (even more were injected) to produce each founder. To be sure to obtain an expressing founder perhaps these numbers should be doubled. Sometimes more than one construct must be used to test a hypothesis, necessitating another doubling or tripling of the number of animals needed. The number of donor animals is small in comparison, although costs per donor can be high if embryos are recovered surgically. Alternatives to conventional procedures that reduce the number of animals needed will be considered further on.

General Considerations of Animal Resources Needed for Transgenic Livestock

With a few exceptions, to date transgenic procedures have been used to obtain basic information, not to produce agriculturally or commercially salable animal products. Considerable information can be obtained without all the steps in Tables 1 and 3 (although following those steps usually is desirable, even for research). Furthermore, results from experiments to obtain basic information frequently are so dramatic that only a few animals are needed to form robust conclusions that might be quite useful even outside the context of additional transgenic research. In contrast, for production agriculture, in most cases traits are not likely to be changed so dramatically that two or three animals will constitute convincing data. Furthermore, one generally will be obligated to examine effects in both sexes, preferably in both the homo- and hemizygous states, before animals are released for production agriculture (Hoeschele, 1990).

Animal Resources Needed to Generate the Founder Animal

At a minimum, to make a transgenic animal one needs a source of semen, oocytes (e.g., donors to superovulate), and recipients for embryo transfer. In some cases, intermediate recipients also will be used. By far the biggest expense is the recipients to gestate the embryos to term. Without screening to eliminate nontransgenic embryos (King and Wall, 1988; Ninomiya et al., 1989) many embryos must be transferred, preferably one per recipient in cattle, to avoid freemartins. From the summary by Hill et al. (1992), approximately 150 embryos were transferred (even more were injected) to produce each founder. To be sure to obtain an expressing founder perhaps these numbers should be doubled. Sometimes more than one construct must be used to test a hypothesis, necessitating another doubling or tripling of the number of animals needed. The number of donor animals is small in comparison, although costs per donor can be high if embryos are recovered surgically. Alternatives to conventional procedures that reduce the number of animals needed will be considered further on.

Numbers of Animals Needed to Characterize Transgenic Lines

To characterize transgenic lines, large numbers of animals must be kept for years (Table 4). Smith et al. (1987) and Hoeschele (1990) provide a more detailed treatment of this subject. The scheme outlined in Table 4 is a minimal one to test presence of desirable transgenic traits and absence of deleterious ones. The assumptions of exactly 50% transmission of the transgenic allele from hemizygous parents and a 50:50 sex ratio will rarely be met exactly, so there will be
subclasses in which three progeny are expected and none or one actually is produced. These expectations can be improved by biopsying each embryo to determine its sex and transgenic status. With this approach, some unneeded animals in steps 3 through 5 could be eliminated.

With the assumptions in Table 3, step 5 in Table 4 begins 78 mo after the beginning of the experiment for cattle and ends at 101 mo when reproduction/lactation can be evaluated. The homozygotes from mating cousins will be 3.1% inbred, a level that seems reasonable for the purposes of testing absence of severe deleterious effects of the homozygous transgenic state (Smith et al., 1987). The last steps in Tables 3 and 4 can be shortened in some cases by in vitro fertilization of fetal or prepuberal oocytes (Betteridge et al., 1989).

The situation for swine, and to some extent for sheep and goats, is much simpler than that for cattle, buffalo, or horses because swine are litter-bearing and have a shorter generation interval. Of course, this does not eliminate the steps in Table 4. Even with rodents, the huge proliferation of animals to test hypotheses using transgenic approaches results in considerable space requirements and high animal care costs. These costs are exacerbated if special feeding or housing is required because of the nature of the transgene or because of regulations that may apply even if the transgene is expected to be innocuous. Note that most transgenic experimental animals cannot be salvaged for meat in the absence of convincing safety studies, and there are some hurdles in using meat from nontransgenic animals from such studies as well (Federal Register 56:67054, Dec. 27, 1991).

**Animal Facilities Required**

Requirements for animal facilities vary enormously depending on the species, the approach (e.g., in vitro fertilization using slaughterhouse material vs surgical collection in vivo), the perceived danger in the transgene, and the vagaries of government regulations that still are in the process of being formulated. Very likely, a surgical suite suitable for use of general anesthesia will be needed as well as sufficient facilities for housing the numbers of animals described in Tables 3 and 4. In some cases, transgenic constructs for livestock should first be tested in transgenic mice, necessitating a mouse colony.

A reasonable level of security is essential to prevent animals from escaping and unauthorized persons from interfering with experiments. In the February 1, 1991, issue of the *Federal Register* (p 4134) the following major items relating to facilities for transgenic live-

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**Table 4. Example of a transgenic breeding program for cattle**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Founder (F₀)</td>
</tr>
<tr>
<td></td>
<td>If ♀, inseminate females artificially to obtain 20 offspring (all half-sibs).</td>
</tr>
<tr>
<td></td>
<td>If ♂, superovulate repeatedly using semen from several bulls to obtain 20 offspring by embryo transfer (full- and half-sibs).</td>
</tr>
<tr>
<td>2.</td>
<td>F₁ offspring</td>
</tr>
<tr>
<td></td>
<td>80% (16 head) reach breeding age</td>
</tr>
<tr>
<td></td>
<td>8 head (50%) not transgenic (controls)</td>
</tr>
<tr>
<td></td>
<td>8 head (50%) hemizygous transgenic (♀ and ♂)</td>
</tr>
<tr>
<td></td>
<td>Mate transgenic half-sibs in all possible combinations (embryo transfer greatly facilitates this step) to produce at least 30 offspring.</td>
</tr>
<tr>
<td></td>
<td>Also, mate F₁ to unrelated cattle to produce 20 offspring.</td>
</tr>
<tr>
<td>3.</td>
<td>F₂ offspring from half-sib × half-sib mates</td>
</tr>
<tr>
<td></td>
<td>80% (24 head) reach breeding age (Note: all these are 12.5% inbred; 25% inbred if full-sib matings)</td>
</tr>
<tr>
<td></td>
<td>3 head (12.5%) non-transgenic, control ♂</td>
</tr>
<tr>
<td></td>
<td>3 head (12.5%) non-transgenic, control ♀</td>
</tr>
<tr>
<td></td>
<td>6 head (25%) hemizygous transgenic, ♂</td>
</tr>
<tr>
<td></td>
<td>6 head (25%) hemizygous transgenic, ♀</td>
</tr>
<tr>
<td></td>
<td>3 head (12.5%) homozygous transgenic ♂</td>
</tr>
<tr>
<td></td>
<td>3 head (12.5%) homozygous transgenic ♀</td>
</tr>
<tr>
<td>4.</td>
<td>F₂ offspring from half-sib × unrelated mates</td>
</tr>
<tr>
<td></td>
<td>80% (16 head) reach breeding age</td>
</tr>
<tr>
<td></td>
<td>8 head (50%) not transgenic (controls)</td>
</tr>
<tr>
<td></td>
<td>8 head (50%) hemizygous transgenic (4 of each sex)</td>
</tr>
<tr>
<td>5.</td>
<td>Mate transgenic cousins (from 4 above) to produce less inbred homozygotes</td>
</tr>
<tr>
<td></td>
<td>80% (24 head) reach breeding age (Note: all these are 3.1% inbred)</td>
</tr>
<tr>
<td></td>
<td>3 head (12.5%) non-transgenic, control ♂</td>
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<tr>
<td></td>
<td>3 head (12.5%) non-transgenic, control ♀</td>
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</tr>
</tbody>
</table>
stock were listed: follow good agricultural research practices; control and maintain integrity of site and organisms; exclude predators, other animals, and unauthorized personnel; and keep good records including a record of all persons entering and leaving the research site. For transgenes that may be pathogenic or result in animals likely to thrive in the wild if they escape, suggested requirements are stricter and can include alarms on exits and continual surveillance. Under some circumstances, it will be prudent to house animals with a given transgene in two locations to prevent loss of the line in case of a disease outbreak or some other catastrophe.

**Equipment Needs**

**Molecular Biology.** Most transgenic projects require a molecular biology laboratory or close collaboration with a molecular biologist. In most departments, these laboratories already exist with their attendant electrophoresis equipment, centrifuges, laminar flow hoods, PCR equipment, and so on. Often it is more sensible to collaborate with someone with molecular biology expertise than to establish a molecular biology laboratory just to support a transgenic program. Note that separation of molecular biology and livestock facilities by thousands of kilometers presents no great logistical difficulties, although in later stages of transgenic projects there can be considerable routine work for which timeliness of results is very important. If retrovirus vectors are used to make transgenic animals, the current method of choice for poultry, then facilities and expertise relating to molecular virology are required.

**Embryology.** Easily the most common approach for making transgenic livestock is injecting the DNA construct into pronuclei at the one-cell stage or nuclei at the two-cell stage. Other approaches such as using retroviruses as vectors or an embryonic stem cell system likely will have limited use for livestock for at least the next several years. In any case, all of these systems require similar kinds of embryology equipment.

A standard in vitro fertilization/embryology laboratory is essential, including stereo and compound microscopes, CO$_2$ incubators, warming plates, a laminar flow hood, balances, pH meters, and the like. In addition, a micromanipulation system is needed, including a fixed-stage microscope with differential interference contrast or related optics and equipment to make microtools, including a pipet puller and microforge, at a minimum. Sophisticated DNA injectors are not absolutely essential.

Many of the items required for embryology and micromanipulation are present in most biological laboratories. It is easy to spend $50,000 on the embryological equipment and another $50,000 on micromanipulation equipment. For top-of-the-line equipment with some items in duplicate, twice these amounts can be invested. Another desirable feature for some species/approaches is a warm room for some of the embryo manipulations.

In the future, techniques such as absorption of DNA onto the fertilizing spermatozoon may greatly simplify the process of making transgenic animals. Accordingly, much of the micromanipulation and embryo handling equipment would no longer be needed.

**Personnel**

The simplest successful transgenic projects with livestock are likely to be complex, long-term affairs. In addition to knowledgeable administrators and dedicated technical staff, scientific expertise will be required in 1) molecular biology, 2) embryology/reproductive physiology, 3) livestock management, and 4) the biology of the tissue affected by the transgene (e.g., milk secretion, muscle biology, endocrinology, nutrition, and veterinary medicine). Animal breeding skills also will be required for long-term production agriculture applications. The above skills may reside in two or three scientists, but more scientists will be required for some projects. Of course, a cadre of graduate and postdoctoral students also will be important components of many such projects.

**Establishing Programs**

In most cases, transgenic techniques should be thought of as methodologies such as radioimmunoassay, surgery, or semen collection. Although it can be very appropriate to do research on the technique itself, for example to simplify it or to improve efficacy, in most cases techniques will be used to answer questions, make new models, or improve production agriculture. Because of the expense and complexity of currently available transgenic methods, most fundamental questions about directed modifications of DNA are done with cells in vitro by transfection procedures. Questions requiring the whole organism usually are addressed with rodent models, either via serendipitous spontaneous mutations (hundreds of these are available) or via transgenic approaches. It seems to me that most transgenic projects for livestock will concern fundamental questions in nutrition, physiology including lactation, disease resistance, and animal breeding. One approach that seems inappropriate is to establish a transgenic program and then seek applications. In some cases a transgenic program might use core facilities at other institutions for making the transgenic animals. This approach already is used routinely for much transgenic mouse research.

Use of transgenic animals in production agriculture is much more demanding than such use in research. The need to assure lack of seriously deleterious side effects and to establish true phenotypic advantage makes such projects expensive and very long-term. Note that I do not wish to imply that they should not
be undertaken. Two other concerns, of course, are safety and public acceptance. These issues are addressed by other papers in this symposium.

Unconventional Approaches

Unconventional approaches tend to become conventional very quickly if they are efficacious. I have mentioned in vitro fertilization as a way to produce large numbers of embryos inexpensively. This also has the advantage of allowing for timing of fertilization and thus stage of the cell cycle. Krimpenfort et al. (1991) already have produced a transgenic calf with an in vitro fertilized embryo. One should not get too enthusiastic about this approach, however, because embryos derived from in vitro fertilization are not as robust as those fertilized in vivo; as one adds insults such as centrifugation, microinjection, in vitro culture, and biopsy (see below), this difference in robustness can be a serious limitation. In our own laboratory, we have used both in vivo and in vitro fertilization approaches and thus far have ended up with transgenic fetuses from only the in vivo fertilized embryos.

Another special approach alluded to earlier was biopsying embryos to determine transgenic status. We have used these procedures to produce transgenic cattle at Colorado State University (Bowen et al., 1993), but again the situation is more complex than one might expect. To explain, I will outline the procedures and results.

One-cell, and occasionally two-cell, bovine embryos were microinjected with DNA constructs and cultured in vitro overnight, and those that developed normally were transferred to ligated rabbit oviducts for 5 d. Upon recovery from the rabbit, normally developing embryos were biopsied and the biopsy was subjected to polymerase chain reaction (PCR) amplification while the embryo remained in vitro for approximately .5 d. The PCR-positive embryos were then transferred nonsurgically to bovine recipients.

Recovery rates of embryos from rabbit oviducts usually were in excess of 90%, but occasionally they were much lower, even 0%. The biopsy procedure is relatively simple, a variation of that of Williams et al. (1984), but occasionally damaged embryos resulted. Furthermore, when there were dozens of embryos, the biopsy step alone took several hours. The problems of PCR included technical errors, false positives, and rarely positives. The PCR-positive embryos were transferred nonsurgically, at the end of long working days. Pregnancy rates were on the order of half those of normal embryos; note that only normally developing embryos were selected for biopsy.

The in vitro culture steps clearly are deleterious to embryos compared to transfer to a bovine oviduct. Similarly, although some bovine embryos developed well in the rabbit oviduct, many did not develop normally, in part due to damage from microinjection.

The main advantage of this system is that in our hands about 80% of embryos were PCR-negative, and we did not waste recipients on them. Because costs of recipients are high in such projects, savings were substantial. Conversely, we invested huge amounts of time, equipment, and animals to transfer compromised embryos with lowered pregnancy rates. To determine whether this approach is appropriate for a given situation, the complexities need to be understood. A related point is that some transgenic embryos were discarded because they were unsuitable for biopsy or they were damaged. Thus, this approach may not always be cost-effective, especially considering that the majority of costs for agricultural applications will occur after the transgenic founder is made.

Other technical fixes also have their associated problems, particularly when first attempted. Even so, one particularly promising approach may be to separate blastomeres of the microinjected embryos at about the 32-cell stage, test a sample of them by PCR, and if they are PCR-positive, either reaggregate those remaining to produce an embryo to transfer or clone blastomeres by nuclear transplantation. Although this approach is attractive because of in vitro screening, there still can be problems with mosaicism (Wall and Seidel, 1992), and nuclear transplantation adds another level of technology to master.

Concluding Thoughts

Although resource requirements for transgenic studies with farm animals can be intimidating, they will in many cases be a good investment. Some hypotheses can only be tested with transgenic approaches, for example, when a mutated intracellular protein is required; other hypotheses can be tested more convincingly and efficaciously than with more conventional studies. I expect to see only two or three examples of using transgenic animals directly in production agriculture within the next decade, but many more in the next half-century. However, the information obtained in basic transgenic studies will continue to be invaluable for production agriculture and for other purposes.

Implications

Transgenic research with farm animals requires considerable resources. If resources are not available, such projects should not be started. In most cases it will be prudent to add transgenic projects when dictated by existing programs, rather than to start transgenic programs without specific applications in mind. Collaboration frequently will be an appropriate way to reduce resource requirements. Transgenic procedures are improving rapidly, which will reduce resource requirements in the future.
RESOURCES FOR TRANSGENIC RESEARCH


