Transgenic Animal Technology

Minireview

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The definition of transgenic animals is evolving. The original definition described a transgenic animal as one carrying recombinant DNA molecules that were introduced by intentional human intervention (Gordon and Ruddle, 1981). That definition nicely characterizes animals in which transgenes are introduced at the preimplantation embryonic stage of development and are transmitted through normal Mendelian inheritance. However, the original definition has been broadened to include animals in which genes are introduced postnatally, as exemplified by gene therapy. The goal of postnatal gene transfer, also known as somatic cell engineering (SCE), is to use genes as a "drug" delivery system for the treatment of an individual (Brenner, 1995). A subset of SCE is the rapidly emerging field of nucleic acid vaccines (NAV) (see "The DNA Vaccine Web" hosted by Robert Whalen, www.genweb.com/Dnavax/dnavax.html). One wonders if once NAVs become part of the medical communities' therapeutic repertoire whether patients will be referred to as transgenic. The primary focus of this minireview is on production of transgenic mammals (with an emphasis on large animals) that are intended to contain transgenes in their germ cells.

Why Produce Transgenic Animals?

Transgenic animals are first and foremost an exquisite tool for discovery. The vast majority of transgenic animals (mice) have been produced to answer basic research questions. Molecular biologists have used this technology to characterize genetic regulatory elements. In some systems such as the mammary gland that lack good cell culture models, transgenic animals are one of the few approaches available to researchers to identify which genetic se-

quences confer tissue specificity, developmental gene regulation, and feedback control of gene expression. Physiologists have used transgenic technology to perturb homeostasis of various systems to study immunology, neurology, development, thyroid function, circulatory and cardiac function, intermediary metabolism, muscle development, bone growth, hemoglobin switching, and reproduction. The biomedical community has used transgenic technology to generate a wide array of disease models including those for sickle cell disease, prostatic hyperplasia, atherosclerosis, retinoblastoma, diabetes, learning impairment, and cystic fibrosis to name just a few (Wagner et al, 1995). In all cases mentioned, the mouse served as the animal model. For many of these studies a larger animal model would be desirable.

Agriculturists also have espoused the potential value of transgenic animals in livestock production systems. To date, most transgenic livestock projects have focused on enhancing growth in swine by overexpression of growth hormone, IGF-I, or estrogen receptor (Pursel and Rexroad, 1993). A smaller number of projects have been designed to enhance disease resistance in pigs and sheep, and recently, transgenic sheep with enhanced wool production have been produced. In general, however, projects designed to improve animal agriculture through genetic engineering have proceeded slowly, in part because of the low efficiency of producing transgenic livestock, because of the long generation interval of such animals, and because ideal genetic strategies for the improvement of production traits have yet to be identified. The rate of progress in applying biotechnology to large animals may improve as animal scientists begin to explore alternative approaches such as SCE for vaccine development and a way of down-regulating various hormones such as testosterone in boars to eliminate boar taint and follicle-stimulating hormone (FSH) in feedlot heifers to decrease behavioral expression of estrus (Wall, 1996).

While the agricultural applications of transgenic livestock have been slow in achieving success, transgenic livestock projects designed for use by the biomedical community are making significant strides. In the last 5 years a new industry, the transgenic animal bioreactor industry, has formed. The goal of that industry is to produce pharmaceuticals and nutraceuticals (food with therapeutic value) primarily in the milk of farm animals (Clark, 1992). One company has also envisioned manufacturing human hemoglobin in pigs to serve as a principal component of a human blood substitute. There are

now about a half dozen transgenic animal bioreactor companies world-wide producing transgenic livestock, and at least three of them have products isolated from the milk of either pigs, sheep, or goats in the early stages of human clinical trials. In another arena, transgenic pigs are being produced to serve as organ donors for humans. The xenograft companies intend the transgenic organs for temporary use (Fodor et al, 1994). However, as the technology develops, it is hoped that organs can be produced for extended use.

What Is a Transgene?

A transgene is a recombinant DNA molecule that includes, at a minimum, two parts: a regulatory element and a structural element. The regulatory element confers tissue specificity, controls when the gene will be expressed during development, and modulates the amount of gene expression. It does this by controlling transcription of the structural element. If the transgene includes elements that are responsive to feedback control they usually reside in the regulatory element. The structural element is composed of DNA sequences that encode the genetic information needed to synthesize the gene product. There are three basic forms of structural sequences. Genomic structural sequences contain both exons (the actual coding sequence) and introns (the function of which is not fully understood but can be thought of as spacer sequences that intervene between the exons). Introns are spliced out immediately after transcription; thus the mRNA molecule generated is complementary to the genomic sequence minus its introns. Structural sequences without introns can be created by reverse-transcribing mRNA. These sequences are termed cDNA sequences because they are complimentary to the mRNA. The term minigene is used to describe structural sequences that contain some, but not all, of the introns native to the genomic version of the structural gene.

The power of transgenic technology is derived from our ability to combine elements from different genes within and across species.

Transgene Design and Evaluation—Unfortunately there are few rules for designing transgenes. Therefore, building transgenes is very much an empirical process. The selection of regulatory regions, coding sequence type (cDNA vs. genomic), introns, and polyadenylation signals is not difficult. However, predicting the outcome of marrying several disparate DNA sequences is still more guesswork than science. Evaluation of transgenes in appropriate cells in culture has turned out to be a poor predictor of transgene behavior in whole animals. Nonetheless, cell culture experiments do provide a method for determining if the transgene can be expressed and if the proper gene product is produced. As an initial screening tool for transgenes for which there is no adequate tissue

culture model system such as mammary tissue we are developing an *in situ* transfection technique based on jet-injection of naked DNA into lactating mammary glands. We are now using this technique to compare the potencies of various mammary-specific promoter regions (Kerr et al, 1996). We believe that this approach will speed the process of transgene evaluation. Furthermore, since the gene product (protein) can be detected after gene construct jet-injection, evaluation of post-translational capabilities of the target tissue in the target species may be possible.

How Are Transgenic Animals Produced?

Most goals of transgenic projects rely on "gain of function" strategies (introduction of a new gene) by means of pronuclear microinjection. However, eliminating or reducing the concentration of specific proteins can also be achieved, at least in theory, by adding new genetic information. This is achieved by constructing ribozyme or antisense transgenes targeted against a particular endogenous gene (Sokol and Murray, 1996). The ribozyme or antisense mRNA interferes with translation of the mRNA of the target protein, thus reducing or eliminating the production of the protein. A more widely accepted means of achieving "loss of function" (eliminating or altering gene function) is through use of embryonic stem (ES) cell-dependent gene knockout technology.

Though there are several ways of introducing genes into preimplantation embryos, pronuclear microinjection, as originally described by Jon Gordon in 1981 (Gordon and Ruddle, 1981), and as modified for livestock, is still the predominant method employed. Other methods that have been used to produce transgenic animals include use of ES cells, retroviruses, primordial germ cells (PGCs), and sperm.

Embryonic stem cell technology is based on isolating ES cells from the epiblast of a blastocyst (Bronson and Smithies, 1994). The isolated cells are cultured under conditions that maintain their undifferentiated state. During culture, a new gene or modified form of an endogenous target gene can be introduced. The transfected ES cells can then be used to produce animals or can be further characterized if a specific integration event is desired. A small proportion of the transfected ES cells will integrate the transgene by homologous recombination, thereby replacing an endogenous gene with a transgene (disrupting the endogenous gene's function or mutating its sequence). The selected population of genetically engineered ES cells are then injected into the blastocoel cavity of a recipient embryo or into the perivitelline space of a morula to form aggregate chimeras. ES cell experiments can be conducted in mice, but livestock ES cells have not been identified. Recently, lambs were produced from an established cell line derived from embryos. The mentioned

cells do not meet the classical definition of ES cells, but they may be functionally equivalent.

Genetically engineered retroviruses can be used to infect cleavage-stage embryos. This approach was the first used to produce transgenic mice in the late 1970s. It has not resulted in live-born transgenic livestock yet (Haskell and Bowen, 1995). Using viral vectors might be the method of choice for producing transgenic animals; however, there is a restriction on the size of the transgene that can be inserted into retroviruses, and the frequency at which progenitor germ cells acquire the transgene is low.

Transfected PGCs have been used to produce transgenic chickens (Sang, 1994). After harvesting PGCs from a donor fetus, transgenes are introduced using techniques similar to those employed to transfect ES cells. The PGCs are then returned to the circulation of a recipient fetus where they have a propensity to migrate to the germinal ridge. PGCs have been successfully transferred in mice, and work is underway to isolate and transfer pig PGCs.

Recently, Ralph Brinster of the University of Pennsylvania, a pioneer in transgenic animal technology, has transferred spermatagonia between males (Brinster and Avarbock, 1994). The transferred cells proliferated in the recipients seminiferous tubules and developed into functional spermatozoa that were capable of fertilizing oocytes resulting in production of offspring. Clearly, if a means is found to introduce genes into spermatogonia, yet another way would be available for producing transgenic animals.

A controversial but promising technique for producing transgenic animals involves incubating spermatozoa (either epididymal or ejaculated) with gene constrúcts and then using those sperm to fertilize oocytes (Zani et al, 1995). This approach is considered controversial because some prominent laboratories have not been able to repeat the original work. Sperm-mediated gene transfer has been successful in introducing foreign DNA into mice and pigs. To date, all of the genes that have been transferred by this method appear to be rearranged in the transgenic founder animals produced. As a consequence those genes are not functional. If a way can be found to prevent transgenes from becoming scrambled, this would be by far the most straightforward way of making transgenic animals.

Characteristics of Transgenic Animals

Efficiency of Producing Transgenic Animals—The efficiency of producing transgenic animals is low. A compilation of data from several laboratories indicates that about one transgenic animal was produced per 40 mouse eggs injected, while the efficiency for pigs, sheep, goats, and cattle was much lower, requiring approximately 100, 110, 90, and 1,600 egg injections per transgenic animal, respectively. Furthermore, expression is not guaranteed in all transgenic lines. Low efficiency is not of particular concern to those working with mice, but it is a major impediment to those attempting to produce transgenic livestock (Wall, 1996).

Three parameters account for the low efficiency of the process: embryo survival, gene integration rate, and transgene behavior. In livestock species and laboratory animals about 15–25% of microinjected transferred embryos survive to term. However, gene integration frequency, as measured by proportion of animals born that are transgenic, is much lower for livestock species than for laboratory animals. That difference in integration rate may allude to important biological differences between the zygotes of these species. The inefficiency of the process seriously impedes widespread use of transgenic animal technology because of the attendant costs. Whereas the operational costs of making a transgenic mouse are about $100, making an expressing transgenic livestock founder can cost anywhere between $25,000 and $500,000 (Wall et al, 1992).

Transgene Integration Frequency—Genetic diversity in livestock species may play a role in the low-integration frequency observed. Laboratory animals are derived from highly inbred lines, and investigators often choose to use specific strains whose embryos "culture easily." Scientists working with livestock embryos do not have the same inbred resources.

Procedural differences between microinjection of livestock and laboratory animal zygotes are a possible cause of low transgene integration rates in livestock zygotes. Livestock eggs are more challenging to microinject than are mouse, rabbit, or rat eggs. Cow and sow eggs must be centrifuged before microinjection. Though there is little evidence that embryo survival is significantly compromised by centrifugation, the procedure may by some unknown mechanism influence integration rate.

A more compelling argument, proposed by Ken Bondioli, could be made for an association of integration failure with inappropriate timing of microinjection (Bondioli and Wall, 1997). It has been inferred that DNA replication is required for integration of foreign genes into the genome. If that is the case, then the timing of pronuclear microinjection should be synchronized with onset of the DNA synthesis phase (S-phase) of the first cell cycle to ensure the maximum likelihood of an integration event. Mouse eggs are microinjected about 8 hours postinsemination. This results in DNA being introduced into zygotes during the beginning of S-phase. Cow and pig eggs are injected toward the end of the S-phase, possibly reducing the probability of an integration event. Livestock zygotes are injected "late" because microinjection is restricted to the time pronuclei can be visualized in a nondestructive manner (differential interference contrast [DIC] microscopy). Experimental evidence to support the influence of microinjection timing on transgene integration frequency

is lacking and may be difficult to achieve because pronuclei cannot be visualized with DIC during the early stages of S-phase.

Transgene Expression—Characteristics of transgene expression in transgenic goats, mice, pigs, rabbits, rats, and sheep appear to be similar. Although data is insufficient to make the same claim for transgenic cattle, there is no reason to expect that transgenes will behave differently in that species. Transgenes appear to integrate randomly in the genome in a multicopy array, but levels of expression are rarely correlated with the number of gene copies in the array. The promoter of the transgene greatly influences the proportion of transgenic animal lines that express their transgenes for unknown reasons. In "expressing" lines, transgene expression is often inappropriate, occurring in unintended tissues (ectopic expression) or at developmentally incorrect times. These aberrant expression patterns or lack of expression have been attributed to the so-called "position effect" that suggests that neighboring genes, or heterochromatin regions, can override the control of transgenes. Adding matrix attachment region sequences or "genetic boundary" elements to transgene constructs may obviate position effects (McKnight et al, 1996).

Summary

There are numerous tools available to modify the genetic makeup of animals. They are being used to good advantage for studying basic biological phenomena. Within the decade, biomedical products derived from transgenic animals will be available, but the use of this technology for enhancing the quality and efficiency of livestock production will await further refinements in the technology.

Note Added in Proof—Recently, the cloning of sheep using nuclei from cultured fetal and adult cells has been published (Wilmut et al, Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997;385:810–813). The ability to clone animals from genetically modified, cultured cells should provide another technique for creating transgenic animals and provide an alternative to ES cells or PGCs for using gene knockout technology in livestock species.

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