

TECHNICAL BULLETIN



TRANSGENIC ANIMAL SCIENCE: PRINCIPLES AND METHODS

Transgenic Animals and Genetic Research

Prior to the current revolution in applied molecular genetics, the only practical method to study the regulation and function of mammalian genes was to utilize spontaneous mutants. To prove the genetic basis of the mutation, the animals had to transmit an observable trait to offspring. Since the 1970s, it has been possible to introduce DNA fragments into prokaryotic and eukaryotic cells *in vitro* and to induce the expression of the foreign DNA in these cells.

DNA may be introduced into cells using shock and precipitation, lipofection, electroporation of the membranes, viral vectors and direct microinjection. Approximately one out of several thousand treated cells usually takes up and expresses the foreign DNA; the DNA may be expressed as extrachromosomal satellite DNA or it may be integrated into the cellular chromosomes.

Characterized gene sequences may be introduced into cultured cells, and the protein product of the transferred gene, if secreted, may be collected from the culture medium. Although the evaluation of gene expression is relatively straightforward, the activity of a specific gene at the cellular level does not yield satisfactory information about the regulation of the gene among the complex physiological interactions of the whole animal. Current state-of-the-art cell cultures do not adequately simulate tissues and organ systems to predict responses to sophisticated environmental stimuli.

Successful attempts were made to isolate genetic defects on a different genomic background by performing breeding experiments to generate congenic mice (mice which differ from a chosen strain by a single chromosomal region, containing the gene under study). The major problem with this method of genetic evaluation is that a large amount of DNA flanking the desired genetic locus is invariably transferred from animal to animal during meiotic recombination, along with the gene(s) in question. A second disadvantage has

been the long time frames (usually about three years) to generate the congenic strain via breeding alone.

With extensive studies of the mouse genome, it is now possible to employ a technique called “speed congenics” to dramatically shorten the time required. In the speed congenics process, DNA samples from the males of each litter (with a few exceptions, discussed later) are analyzed using microsatellite based assays. The one or two males with the highest percentage of recipient genome are identified. These mice are then mated with females of the recipient strain to produce the next generation.

The Value of Transgenic Animals

The term “transgenic animal” is used here to include both animals carrying foreign DNA randomly integrated into their genomes, and animals generated by homologous recombination, allowing the researcher to control the location of the inserted DNA. These consist of both knockout animals, where an endogenous gene has been specifically inactivated, and knockins, where a gene of interest has been added to the genome or a native gene has been enhanced.

Transgenic animal systems combine the virtues of cell culture and congenic breeding strategies while avoiding the negative aspects of each system. Using transgenic techniques, a characterized genetic sequence may be evaluated within the specific genomic background of the whole animal. Therefore, transgenic animals may be utilized to study the regulation of a specific genetic sequence in a realistic fashion. Many uses have been developed and many more are forecast, particularly in these areas:

Models of human disease processes

More than one thousand transgenic rodent lines have been produced by introducing into the genome genetic sequences such as viral transactivating genes and activated oncogenes implicated in specific pathologies. The phenotype and regulatory parameters of the gene may then be evaluated in an animal model with a relatively short generation time. Also, normal rodent genetics and physiology are highly characterized. The predictability of many transgenic phenotypes permits the innovative testing of diagnostic and therapeutic

agents while using a reduced population of experimental animals.

The generation of novel cell lines from transgenic organs also promises to reduce the number of research animals required to evaluate a therapeutic compound. In addition, transgenic genomes may be created in which more than one transgene may interact, or in which a transgene may interact with an endogenous normal or mutated gene. The use of transgenic disease models in biomedical research promises to dramatically accelerate the development of new human diagnostic and therapeutic treatments. Increasingly, transgenic animals are being used for drug discovery, toxicology, and pharmacokinetic studies (Eastin, 1998; Rosenberg and Bortner, 1998-99).

Transgenic rodent models have been characterized for numerous human diseases including cardiovascular disease (Walsh et al., 1990), cancer (Sinn et al., 1987), autoimmune disease (Hammer et al., 1990; Boyton and Altmann, 2001), AIDS (Vogel et al., 1988), sickle cell anemia (Ryan et al., 1990), Parkinson's disease (Betarbet et al. 2002), as well as other neurological diseases (Small et al., 1986).

Targeted production of pharmaceutical proteins

Another use for transgenic animals involves the biological production of valuable human enzymes, hormones, antibodies, and growth factors. These products may be recombinant or mutated. Collection of the functional protein from the animal employs tissue-specific regulatory DNA sequences, a strategy described below, and in more detail in a companion report. Current techniques in the biotechnology industry use large-scale cell cultures to generate products in biological systems. Eukaryotic cells or bacteria which have taken up genetic expression sequences (or constructs) are cultured in nutrient medium which is continually replaced, and from which the bioengineered product is refined. This medium must be correctly buffered, temperature-regulated, and maintained pathogen-free.

The use of transgenic animals, particularly larger mammals, as bioreactors (“pharmaceutical pharming”) is a cost-effective alternative to cell culture methods.

Animals automatically supplement their bodily fluids with fresh nutrients, remove waste products, reliably regulate their internal temperature and pH, and resist pathogens. By directing (or targeting) the expression of the transgene product so that it is produced by the secretory cells of the liver, lactating mammary gland, or kidney, “pharmers” may collect and process bodily fluids with minimal effort. The mammary gland probably is the most promising target tissue because it produces large amounts of protein in a temperature-regulated fluid that may be collected daily in a non-invasive fashion. This approach has been successful in generating sheep that produce human Factor IX, used to treat hemophilia B (Colman, 1999), and pigs that produce human protein C, an anticoagulant (Velandar et al., 1992). Transgenic animals are not only cost-effective bioreactors but, with the complex secretory cell types and organs of the mammalian organism, they can also perform much more complicated protein modifications than simple cultured cells. For a review of transgenic animal bioreactors, see Lubon, 1998.

Analysis of developmental pathways

Many transgenic animals are found to be inviable, either prenatally or perinatally. This has yielded important insights into genes required during development. Correlation of the time of embryonic lethality to known mouse embryonic gene expression patterns provides insight into the pathways and interactions of the gene that was mutated.

As transgenic technology matured, two new approaches provided significant power to this research. One advance was the use of reporter genes, which combined the regulatory regions of the gene under study with an easily-detected marker protein. More recently, the Cre-lox system has allowed both temporal and tissue-specific control of expression of knockout gene constructs. Briefly, the gene of interest is flanked by the locus of crossover (loxP) of bacteriophage P1 Cre recombinase. The loxP site is a 34 base-pair sequence consisting of two palindromes of 13 base pairs each surrounding an eight base-pair core. A gene flanked by loxP sites is said to be “floxed”. A mouse carrying the floxed gene is crossed to a mouse expressing the Cre recombinase with a promoter that regulates its expression in the desired fashion. In cells in which both Cre recombinase and the floxed gene are present, the recombinase acts to excise the floxed region, leaving behind a single 34 base-pair loxP site. There are numerous lines of

differentially-regulated Cre-expressing mice, so it is possible to examine the effect of the floxed gene at several different developmental stages or in different tissues by crossing the mouse carrying the floxed gene to several different Cre-expressing mouse lines. While other methods exist to control gene expression, the Cre-lox method is the most common (Utomo et al., 1999).

Modification of animal anatomy and physiology

The most controversial aspect of transgenic animal usage involves the “selective improvement” of species by the modification of the genome. Most often, foreign genes are added to the host genome, but selective deletion of specific genes or regions has been attempted. It has become apparent that merely adding genes for growth factors or hormones to the genome is a simplistic approach to altering the complex multigenic physiology of the mammal. The goals of this type of experiment may include decreased body fat, increased speed, novel disease resistance, or higher yields of meat or milk. At present, these types of phenotypic alterations are more realistically achieved in plants and bacteria than in animals (Larrick and Thomas, 2001).

Production of organs for xenotransplantation

As there is a severe shortage of healthy donor organs for transplantation, there has been much interest in developing animals whose organs would be suitable for xenotransplantation (Cozzi and White, 1995; Lonberg and Huszar, 1995; Sandrin et al., 2001). Organ rejection is the major barrier to xenotransplantation. Transgenic models have been useful in identifying the molecular basis of rejection, which includes foreign antigens, coagulation defects, and interspecies ligand-receptor interactions. Transgenic pigs have been developed that do not express the surface glycoproteins that are so highly antigenic to the human immune system (Lai et al., 2002; Phelps et al., 2003). Although not yet successful, this remains an active and promising area of research.

Development of Transgenic Science

In the 1970s, experiments were conducted with embryonal carcinoma cells and teratocarcinoma cells to construct chimeric mice (Brinster, 1974; Mintz and Illmensee, 1975; Bradley et al., 1984). The term chimera is derived from the name of a monster in Greek mythology whose body was part eagle, part lion, and part serpent. In modern chimeric



Figure 1. Transgene DNA construct. A cDNA of the gene of interest is used. The promoter and 3' untranslated region must be included for proper gene regulation. There is some evidence that intron splicing plays a role in gene expression, so an intron (not necessarily from the gene of interest) is included. The construct is linearized before injection.

animals, cultured cells derived from one strain of mouse are introduced into the embryos of another strain of mouse by direct embryo aggregation or by injection into the blastocyst-stage embryo. The resulting mouse has tissues derived from cells of both strains.

Another type of animal genome manipulation involved the transfer of the entire nucleus from an embryo directly into the enucleated oocyte of a different recipient strain (McGrath and Solter, 1983). This is the method Ian Wilmut and his laboratory used to produce the famous transgenic sheep, Dolly (Campbell et al., 1996). These transgenic animals are produced without any recombinant DNA techniques. However, they represent important milestones in the elucidation of genetic regulatory mechanisms in mammalian systems.

Another method for the production of transgenic animals is accomplished by the infection of preimplantation mouse embryos with retroviruses (Jaenisch and Mintz, 1974; Jaenisch, 1976). The viral information was successfully transferred into the genome of the recipient animal, and the technique of utilizing retroviruses as vectors for specific foreign DNA sequences was soon developed (Stuhlmann et al., 1984). Retrovirus-mediated transgenesis produces a high degree of mosaicism; the size of the transgene sequence is limited, and the viral sequences may interfere with expression of the transgene. However, the integration of single copies of the transgene flanked by the viral DNA can be advantageous if it is desired to clone the locus of integration.

Several other techniques have also been developed to produce transgenic animals. These include embryonic stem (ES) cell-mediated techniques (Gossler et al.,

1986), transfer of entire chromosomal segments (i.e., “transomic” mice; Richa and Lo, 1988), and gamete transfection in conjunction with *in vitro* fertilization (Lavitano et al., 1989). Of these, two major techniques are widely used to produce transgenic animals. Pronuclear microinjection allows transgene sequences of variable length from viral, prokaryotic, plant, invertebrate or vertebrate sources to be introduced into the mammalian genome, where they may be expressed in both somatic cells and germ cells. ES cell techniques permit selection for rare recombination events, allowing the investigator to identify cells in which homologous recombination has occurred between the gene of interest and the transforming DNA construct. ES cells are transformed with the desired transgene in tissue culture, selection is applied for the transgene construct, and homologous recombinants from the selected cells are used to produce a chimeric embryo *in vitro*.

Pronuclear Microinjection Techniques *Transgene DNA preparation*

The transgene DNA is engineered in the molecular laboratory to achieve fairly predictable expression in the animal. Using restriction enzymes and ligase, different functional regions of genes from different species may be recombined in the test tube. All components of endogenous genes may be isolated and recombined to form a transgene expression cassette or construct (Figure 1). The ends of the completed construct may be modified by the addition of polylinker sequences containing several different restriction enzyme recognition sites. The polylinker permits the construct to be inserted into a variety of vectors for testing and cloning.

Embryo collection

For the pronuclear microinjection technique, the step following transgene construction is collection of embryos. With ES cell methods, there is an additional step. Transformed cells are grown in tissue culture under selective conditions before being introduced into the embryo. This procedure is described in more detail in a subsequent section.

The choice of the donor parental strains for production of embryos is a point of extreme proprietary concern to most laboratories. Many factors are cited including the response to superovulation, frequencies of embryo survival following microinjection, size of pronuclei, and incidence of specific pathologies inherent to various strains. The relative merit of inbred versus outbred backgrounds may be important for the evaluation of a specific transgene expression. Other factors may involve coat color, the availability of a certain strain, or simply anecdotal rationales. Certain hybrids (e.g., C57BL/6 x SJL/N) and outbreds (e.g., CD1) are reported to yield large numbers of viable pronuclear embryos following superovulation. The FVB/N inbred strain is reputed to survive microinjection procedures better than many other strains and has been shown to possess pronuclei of relatively larger volumes.

Whichever strain is chosen to provide embryos, fewer animals will be needed and less variability encountered if exogenous gonadotropins are used to superovulate the donor females. Successful superovulation protocols must consider the strain, age, and weight of the animals. Breeding should be monogamous, and the light cycle in the breeding room must be strictly regulated. The superovulation and synchronization of rats, rabbits, and larger mammals present additional technical challenges.

At the appropriate time post-mating (vaginally plugged females are selected the morning following introduction of a male), oviducts are removed from euthanized donors, and embryo clumps are collected from the oviducts by flushing or by dissection into a microdrop of sterilized buffered medium. The embryos are clumped together with sticky follicular cumulus cells that must be removed by brief treatment in a series of microdrops. The first drop, a solution of the enzyme hyaluronidase, is followed by two or more wash drops. With heat-pulled tapered micropipets controlled by suction (a new pipet for each drop), the embryos are transferred from drop to drop until they are free of cumulus cells, debris, and enzyme. Finally, the embryos are transferred into a pool of medium in a Petri dish that will be placed under the microscope.

The embryo-containing pool is covered by a layer of sterile-filtered, autoclaved mineral oil to prevent contamination by microorganisms and debris and to prohibit evaporation and the resultant pH changes that would kill the embryos. All collection and manipulation media contain a buffering system (i.e., bicarbonate or HEPES) and protein source (e.g., bovine serum albumin) to prevent embryos from adhering to the dishes and pipets. In addition, media may contain antibiotics (e.g., penicillin and/or streptomycin) and a heavy-metal chelating agent (e.g., EDTA).

DNA microinjection

The first successful production of transgenic mice using pronuclear microinjection was reported in 1980 (Gordon et al., 1980). Although the recombinant viral construct was proven to have integrated into the mouse genome, it was rearranged and did not express. Subsequent reports (Brinster et al., 1981; Costantini and Lacy, 1981) proved that integrated transgenes were capable of functional expression

TIMELINE FOR DEVELOPMENT OF A TRANSGENIC MOUSE LINE



Figure 2. Timeline for development of a classical transgenic mouse line

following pronuclear microinjection. The first visible phenotypic change in transgenic mice was described in 1982 for animals expressing the rat growth hormone sequence (Palmiter et al., 1982). The publication rate for these types of papers has grown rapidly ever since.

The pronuclear microinjection method of producing a transgenic animal results in the introduction of a purified double-stranded DNA sequence into the chromosomes of the fertilized mammalian egg. If this transferred genetic material (transgene) is integrated into one of the embryonic chromosomes, the animal will be born with a copy of this new information in every cell. The foreign DNA must integrate into the host genome prior to the doubling of genetic material that precedes the first cleavage or a mosaic animal may be produced in which many cells do not possess the new gene. For this reason, the transgene DNA is introduced into the zygote at the earliest possible stage (the pronuclear period) immediately following fertilization.

For several hours following the entry of the sperm into the oocyte, the male and female pronuclei are microscopically visible as individual structures. The transgene may be microinjected into either of these pronuclei with equivalent results. However, X-chromosome or Y-chromosome integration events do occur and obviously may be influenced by the choice of pronucleus. Usually, the male pronucleus may be distinguished because it is larger than the female pronucleus and also because it is closer to the oocyte surface.

The animals that develop after receiving the transgene DNA are referred to as the founder (F0) animals of a new transgenic lineage. If the germ cells of the founder (mosaic or not) transmit the transgene stably, then all descendants of this animal are members of a unique transgenic lineage. Integration of foreign DNA into the embryonic genome generally is a random event with respect to the chromosomal locus. Therefore the probability of identical integration events in two embryos receiving the same transgene is overwhelmingly unlikely. Thus each pup born from a single pronuclear injection procedure has the potential to be a genetically unique founder animal. Mating a pair of animals with identical transgenes but from different founder lineages cannot result in a true homozygote in which independent segregation of the loci is predictable. Because the new transgenic locus is present in only one member of a particular paired chromosome, the genotype of the founder is described as hemizygous for the transgene rather than heterozygous. A homozygous genotype, in which a pair of transgene alleles is present, may be produced by the mating of a pair of hemizygous F1 siblings.

In addition, it is impossible to regulate exactly how many copies of the transgene will be introduced into the embryo and how many will join together to integrate (usually at a single site) as a single linear array called a concatamer (Brinster et al., 1981, 1985; Bishop and Smith, 1989). Many studies have found dramatic differences in the expression of a specific transgene within individual sibling embryos simply due to different integration loci. The number of copies of the transgene

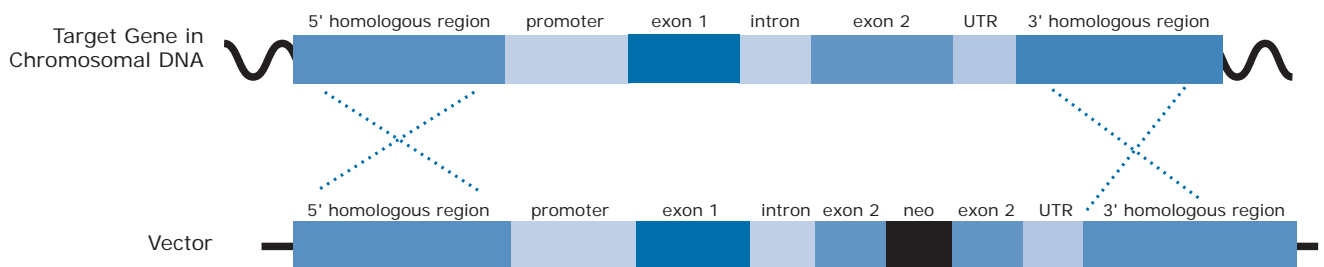


Figure 3. Targeted DNA construct. The gene of interest is modified, usually by replacing one or more exons with a selectable marker such as neo. In order to promote homologous recombination, the construct includes several kB of flanking DNA on both the 5' and 3' ends. The construct is introduced into ES cells by transfection or electroporation.

GENERATION OF ES CELL-DERIVED MICE

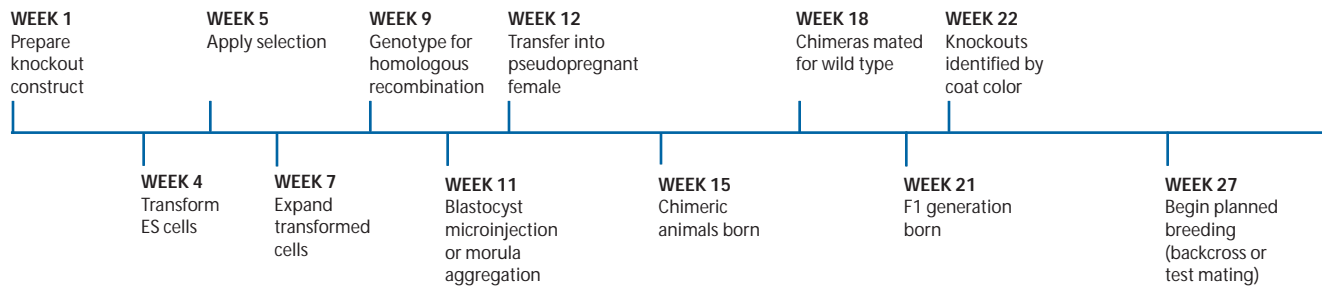


Figure 4. Timeline for development of a targeted mutant mouse line

that have joined the founder's genome is referred to as the copy number, and rarely appears to be correlated with the degree of transgene expression in the animal.

With this method, the locus of transgene integration is random. Thus the transgene may insert into functional genetic sequences. The effects of interruption of the normal expression of an endogenous gene range from inconsequential to lethal. Alternatively, observable insertional mutagenesis might be apparent when the insertion interferes with the expression of an endogenous, developmentally-active gene. These mutations are distinguished from the true transgenic phenotype because only a single lineage exhibits the defect. The mutations can involve any system including sensory, cardiovascular, neurological, and reproductive systems, and severe morphogenetic abnormalities may be observed (Woychik et al., 1985). In this case, the identification of the locus of transgene insertion is of great value because it maps the locus of an important endogenous gene.

The success of the microinjection technique relies upon the careful collection of a relatively large group of accurately timed embryos from a reproductively synchronized group of female embryo donors. In addition, the techniques of microinjection and embryo transfer to a suitable recipient female must be mastered. Of course, the combined success of all of these manipulative skills ultimately depends upon the fastidious construction and preparation of the transgene DNA fragments to be injected.

Embryonic Stem Cell Techniques

Stem cell selection

This method is critical to the development of knockout and knockin strains, where the transgene is specifically targeted

to a known location in the recipient genome (Capecchi, 1989). Growth in tissue culture allows selection for the rare homologous recombination events that result in incorporation of the transgene construct at the targeted location.

ES cells are derived from the inner cell mass of three-day post-fertilization blastocyst embryos. A number of different ES lines are available. Most are derived from mice of the 129 strain, as this strain has traditionally given a high success rate. ES cells can be maintained in culture indefinitely, although scrupulous care is necessary to prevent them from differentiating. ES cells in culture must be provided with a feeder layer of non-dividing fibroblasts, gelatinized flasks and liver-conditioned medium, or LIF (leukemia inhibitory factor, also known as cholinergic differentiation factor). These cells are pluripotent; they retain the ability to differentiate into all cell types, including germline cells.

The vector DNA used to transform these cells must be carefully constructed to support the experimental design. The construct is introduced into the cells by transfection or electroporation. Briefly, the region being mutated is flanked by sequence homologous to the desired integration site. The transgene construct may also contain selectable markers. There are two common strategies for marker selection. One uses position-dependent positive selectable markers, which rely on elements in the target site to provide necessary transcriptional signals. An incorrectly targeted cassette is not likely to integrate in a location providing the necessary element; thus random integrants will generally not be able to express the selectable marker.

Alternatively, both positive and negative drug selection markers may be employed. The positive selectable marker usually confers drug resistance upon the transformed cells. Because it is possible for the construct to insert in a random location by non-homologous recombination, the negative selectable marker is used to discriminate against cells in which this has occurred. The negative selectable marker is located on the transgene construct outside of the region of homology. In a homologous recombination event, this part of the plasmid will be lost, and the cells will not be susceptible to the treatment. In the case of a random insertion, the entire plasmid is usually retained, and the drug will be toxic to these cells.

Blastocyst microinjection of ES cells

The most commonly used method for generating a chimeric mouse from ES cells is blastocyst injection. ES cells are injected into the blastocoele. There is sufficient plasticity at this stage of development that the injected cells incorporate into the developing embryo.

Morula aggregation of ES cells

Recently the technique of morula aggregation has gained popularity (Wood et al., 1993). The expense, time, and level of skill required to generate transgenic mice has been significantly reduced by the development of this method. The major advantage of morula aggregation is that it greatly reduces the need for specialized equipment, as no microinjection is required. This technique thus makes transgenic technology accessible to a wider range of laboratories. However, this method does not yet have the proven track record of blastocyst microinjection.

Freshly compacted, eight-cell embryos must be collected from superovulated mice as described earlier. The ES cell culture is removed from the culture flask with trypsin, and then the trypsin is quickly inactivated by serum-containing medium. This process will detach both the ES cells and the mitotically inactive feeder fibroblasts. The fibroblasts are removed by a brief incubation in a standard tissue culture flask. The fibroblasts will adhere to the flask within 20 minutes, leaving the supernatant enriched for the slowly-attaching ES cells. The cells are washed and concentrated in co-culture medium, placed by

drops on a Petri dish, and covered with paraffin oil.

The zona pellucida of the embryos must be removed to allow the ES cells to contact and adhere to the embryo. Once stripped of the zona pellucida, the embryos are added to the droplets of ES cells, and allowed to associate for several hours. They are then provided with fresh culture medium and allowed to incubate overnight. Although this initially results in the ES cells being on the outside of the embryo, they preferentially migrate to the inner cell mass.

Equipment

The equipment required to perform microinjection can cost between \$70,000 and \$85,000 and includes:

- CO₂ incubator to maintain manipulated embryos at 37-38° C in an atmosphere of 5-6% CO₂
- Inverted microscope with a fixed stage
- Pipet-pulling apparatus
- Microforge apparatus to heat-polish and bend pipets
- Microphotographic equipment (optional) including 35mm camera and/or video recording apparatus
- Pipet beveling apparatus (optional)
- ◆ Phase contrast, Nomarski differential interference, or Hoffman modulated contrast optical systems to visualize pronuclei. With 10x or 15x eyepieces, a 20x or 40x objective is required.
- ◆ A pair of micromanipulators to control the DNA injection pipet and the embryo-holding pipet
- ◆ A pair of micro-volume syringes and associated tubing to regulate the fluid dynamics in the injection and holding pipets (Expensive automatic microinjection systems are available in lieu of the injection syringe.)
- ◆ Vibration-free pneumatic table (optional)
- ◆ Supply of clean capillary pipets for the manufacture of holding and injection pipets
- ◆ Fluorinert solution (optional) to provide optimal fluid dynamics in the pipets

The aforementioned list includes only the supplies required for DNA microinjection. The items marked with round bullets are also necessary for the aggregation

method, whereas those marked with diamonds are only required for the pronuclear injection or blastocyst injection methods. It does not include the animal maintenance supplies (e.g., cages, food, hormones, etc.) nor the vast investment in equipment, manpower, and supplies necessary for the cloning and preparation of the transgene DNA fragments.

Microinjection Techniques

The pipets used for micromanipulation must be custom-made from thin-walled glass capillary tubing using a pipet puller and a microforge. The holding pipet should have a 15-25 μm internal diameter (embryo diameter = 85 μm). It must be perfectly flat at the tip or much difficulty will be encountered when the embryo is held in place during injection. The DNA injection pipet should have an internal diameter of 1 μm or less and must be tapered toward the end. Each pipet is held in place by an instrument holder and controlled by a separate micromanipulator that regulates movements in three dimensions. Talc-coated gloves should be avoided during all procedures because the powder will clog pipets and may be lethal to embryos.

The Petri dish containing the embryo microdrop is placed into focus at a relatively low magnification, and degenerated embryos may be culled from the healthy embryos at this time. The holding pipet is brought down into the medium, and the first embryo is gently sucked onto the end of the pipet and held in place. The tip of the injection pipet is brought into the same plane of focus as the pronucleus to be injected, and a small amount of DNA solution is ejected to ensure the patency of the pipet. The injection pipet is then thrust through the zona pellucida, cell membrane, cytoplasm, and nuclear membrane in a single smooth motion. It is difficult to ascertain visually that the pipet tip has penetrated the pronuclear membrane. Even if the membrane appears to have been pierced, the only reliable indication of success is the swelling of the pro-nucleus (volume = approximately 1 pl). The pipet is removed smoothly, and the injected embryo is moved to the far end of the pool of medium before the next is processed. Once a group of embryos has been completed, it is transferred in a single volume of medium to another dish for incubation and visual evaluation within a few hours. All apparently viable embryos are then transferred to a recipient female oviduct. Alternatively, embryos may be cultured overnight to the two-cell stage and transferred the following morning.

Embryo Transfer

The manipulated embryos must be transferred into a suitable reproductive tract in order to have an opportunity to become live-born transgenic mice. The recipient female optimally should be somewhat earlier in her reproductive cycle than the embryo donor because manipulated and cultured embryos exhibit slightly retarded development when compared to embryos that developed *in vivo*. Recipients for embryo transfer are prepared by mating with vasectomized males at the same time that the super-ovulated donor females are mated with fertile males. It is advisable to use vasectomized males and recipient females with a coat color dominant to the embryo donor so that resources are not wasted testing embryos generated by insidiously fertile vasectomized males.

Recipient females are anesthetized, the skin and peritoneum are incised, and the ovarian fat pad and bursa are exteriorized and draped over the midline. The bursa is opened, avoiding any prominent vessels, and the infundibulum is located. An embryo transfer pipet with an internal diameter of less than 150 μm is loaded in the following sequence: one small air bubble, approximately 10 μL of medium, a second air bubble, 2-15 embryos in less than 25 μL of medium, and a third air bubble. The pipet tip is inserted into the infundibulum of the oviduct, and the contents are gently transferred into the oviduct by mouth pressure until the middle airbubble is expelled. The reproductive tract is gently replaced and the incision is closed.

Pregnancy should be visible about two weeks after the embryo transfer (post-ET), and the litter should be delivered about three weeks post-ET. Offspring are analyzed for the presence of the transgene in their genomes. It should be noted that certain transgene sequences may be activated in utero and may affect embryo survival or gestation length. Also, transgenic females in subsequent generations should be observed for abnormal gestation lengths.

Characterization of Transgenic Animals

Upon birth and weaning of the pups from a transgenic experiment, genetic characterization is necessary. Not all pups will express the transgene, and pups that do may express the transgene at different levels, or in different organ systems. In addition, most targeted transgenic mice are

created on a strain 129 background, because several robust 129 ES cell lines exist. It has become apparent that there are significant differences in 129 substrains (Threadgill et al., 1997; Simpson et al., 1997). Gene targeting efficiency is increased when the ES cell line and the targeting construct are derived from the same substrain. Differences between 129 substrains can have important repercussions in terms of physiological and immunological studies. In addition, some substrains of 129 mice have a high incidence of testicular cancers and experience early hearing loss, making them unsuitable for many studies.

Thus it is usually necessary to backcross the pups onto one or more different, defined strains. The use of more than one strain is recommended in order to assure that the observed phenotype is a direct effect of the transgene and not an effect of a modifier locus in the

background strain or a particular strain/environment interaction. The recent development of speed congenics (Markl et al., 1997) can shorten this lengthy process from about three years to about 18 months.

Summary

While there are many subtleties and refinements in transgenic DNA constructs, the techniques and methods described above are general to most types of transgenic animals being produced today. These technologies have greatly expanded the limits of biomedical research, and new and exciting techniques being developed will continue to expand this important area of experimentation.

If you have any comments or questions, please call 1-877-CRIVER-1 or email askcrl@criver.com.

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