Preface

The past decade has witnessed a spectacular explosion in both the development and use of transgenic technologies. Not only have these been used to aid our fundamental understanding of biologic mechanisms, but they have also facilitated the development of a range of disease models that are now truly beginning to impact upon our approach to human disease. Some of the most exciting model systems relate to neurodegenerative disease and cancer, where the availability of appropriate models is at last allowing radically new therapies to be developed and tested. This latter point is of particular significance given the current concerns of the wider public over both the use of animal models and the merits of using genetically modified organisms.

Arguably, advances of the greatest significance have been made using mammalian systems—driven by the advent of embryonic stem-cell–based strategies and, more recently, by cloning through nuclear transfer. For this reason, this new edition of *Transgenesis Techniques* focuses much more heavily on manipulation of the mammalian genome, both in the general discussions and in the provision of specific protocols.

Of all mammalian experimental systems, the laboratory mouse is probably the most widely used, a situation that almost certainly derives from the fact that it is genetically the most tractable. This second edition, therefore, devotes much space to methodologies required for the creation and maintenance of genetically modified murine strains. In addition to protocols for conventional pronuclear injection, chapters have been included covering alternative routes to the germline, by either retroviral or adenoviral infection. Extensive coverage is also given to the generation, maintenance, and manipulation of embryonic stem cell lineages, since this is now widely recognized as an indispensable approach to genotype–phenotype analysis. Part V contains protocols to facilitate gene targeting and so permit both constitutive and conditional gene targeting. The latter approach, reliant on either the Cre-lox or the Flp-frt system, is rapidly gaining favor as a method of choice for the analysis of null mutations because it solves the twin difficulties of embryonic lethality and developmental compensation—two problems that have hampered the analysis of simple "knock-out strains."

The proliferation of newly engineered murine strains has given rise to one problem within the field, namely, that of the long-term storage of lines for which

there might be no immediate requirement. Within many laboratories, this is now far from a trivial problem, and, therefore, methodologies are included that detail the cryopreservation of both male and female germlines.

Although the mouse is currently the most genetically tractable system, it is not without its limitations and clearly cannot deliver all appropriate experimental or commercial systems. Transgenic manipulation of the rat germline is now delivering valuable models across a range of fields, perhaps most notably in neurobiology and in the study of vascular diseases. This edition, therefore, also focuses on the generation, maintenance, and cryopreservation of rat transgenic lines.

The mouse and the rat remain essentially laboratory models. However, perhaps the most radical change to occur within the field relates to our emerging ability to genetically engineer livestock. In particular, the advent of cloning as a viable technology has wide ramifications for the scientific and industrial communities as well as for the wider public. Protocols are given for the generation of transgenic sheep by nuclear transfer, and, furthermore, the potential implications and future directions of large animal transgenesis are discussed in some detail.

Finally, this second edition carries a very detailed part relating to the basic analysis of transgenic organisms. Although many of the techniques included are widely used throughout molecular biology, those pertinent to transgenic analysis have been brought together to facilitate the rapid analysis of phenotype. Used in conjunction with the plethora of techniques relating to the generation and maintenance of transgenic strains, the contributors and I anticipate that this new edition of *Transgenic Techniques* will prove an invaluable asset to any laboratory either already engaged in transgenic manipulation or setting out along this route.

Alan R. Clarke

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Gene Transfer in *Drosophila*

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1. Introduction

The generation of germline transformants in *Drosophila melanogaster* has relied on the utilization of transposable elements to effect the chromosomal integration of injected DNA ***(1,2)***. The success of this approach has depended largely on our understanding of the biology of P elements and the syncytial nature of the early Drosophila embryo. The first 13 embryonic divisions following fertilization are nuclear, resulting in the formation of a syncytium. Consequently, if microinjection into the posterior end of the embryo is carried out prior to cellularization, a proportion of the microinjected DNA will be present in the cytoplasm of the pole cells, the progenitor cells of the germline.

In practice, the DNA to be injected comprises two components. The first consists of a helper plasmid containing a defective P element that, although capable of producing the P transposase, which can act in *trans* to mobilize P transposons, is itself immobile (*see* **Note 1**). The second component consists of a transposon construct in which the sequence to be integrated as a transgene is situated between the 31-bp P element inverted terminal repeats along with a suitable marker (*see* **Note 2**). The transposase produced by the helper plasmid will act on the inverted repeats of the transposon construct and facilitate the integration of the transposon into essentially random chromosomal sites of the recipient's germline. Both P element biology and the characteristics of P element–mediated transformation have been reviewed extensively (e.g., *see* **ref. *3***). In this chapter, we deal primarily with the technical details necessary for obtaining germline transformants.

1.1. Outline of Events Involved in Generation of Germline Transformants

1. Construct the desired plasmid containing the transgene, marker, and necessary P element sequences for transposition.

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Fig. 1. Typical arrangement of the apparatus used for injection of *Drosophila* embryos.

2. Coinject the transposon along with a defective helper plasmid supplying the P element transposase.
3. Mate the survivors (Go) to an appropriate strain that will allow for the scoring of the marker carried on the transposon construct.
4. Select for transformed progeny that have acquired the marker carried on the transposon and balance the transformants.
5. Test the structure and copy number of the transgene(s) in the transformant lines.
6. Choose unrearranged single insert lines for phenotypic analysis.

2. Materials

2.1. Microinjection System

Figure 1 shows the injection apparatus we use. This system consists of the following:

1. Leitz micromanipulator.
2. Nikon inverted phase-contrast microscope.
3. Vibration-free table, on which the microscope is mounted.
4. Loaded needle, containing the DNA to be injected.
5. Collar (Narishige, Tokyo) into which the needle is placed, which, in turn, is attached to the micromanipulator.

Although the micromanipulator is used to position the needle, injection is carried out by moving the microscope stage with the embryos on it. We use an air-filled system to deliver the DNA into the embryos. This consists of a 60-mL glass syringe attached to the collar by a piece of rubber tubing (Narishige Teflon™ tubing also may be used).

This system may appear very basic, but we find that the syringe imparts adequate control of DNA delivery without producing the problems often encountered when using a fluid-filled transmission system, and the system has the advantage of being much cheaper. Injection needles are prepared from borosilicate capillaries (e.g., Clark Electromedical [Reading, UK] GC100TF-15 capillaries, which contain an internal filament) using a pipet puller. A relatively inexpensive two-stage vertical needle puller can be used, such as the PB-7 model from Narishige.

2.2. Fly Requirements

In general, a large number of embryos (in the region of 500–1000) need to be injected for each construct in order to produce several independent transformants. In our hands, between 25 and 75% of injected embryos will hatch as larvae. Approximately 50% of the larvae will survive as adults, and between 50 and 80% of the surviving adults will be fertile. Each surviving adult will be individually mated, and approx 200 progeny from each mating will be scored for the marker present on the transposon construct. Although the frequency with which germline transformants are produced varies depending on the construct injected ***(4)***, in general, on the order of 10% of the surviving adults will produce at least one germline transformant among its progeny. Therefore, it is reasonable to aim at obtaining about 100 adult survivors for any given construct injected. We usually collect only one transformant from the progeny derived from each surviving adult with which to establish stocks. This ensures that different transformants originated from independent events.

Since the injections must be performed prior to pole cell formation, 1-h embryo collections are used (*see* **Subheading 3.3.**). Therefore, the fly strain used for embryo collections must be robust enough to provide sufficient eggs (at least 100) during a 1-h interval. One further consideration is that the presence of defective P elements in the injected host strain can affect the frequency of

transformation. Consequently, care should be taken to ensure that such elements are not present in the chosen host strain.

2.3. Miscellaneous

2.3.1. Preparation of DNA

1. Qiagen anion-exchange columns.
2. Injection buffer: 5 m*M* KCl, 0.1 m*M* Na phosphate, pH 7.8.
3. Millipore filters (0.45-µm).

2.3.2. Egg Collection and Egg Processing

1. Egg collection chamber. This can be made from open-ended plastic cylinders of any sort large enough to contain a few hundred flies. The chambers should have fine gauze placed over one end for ventilation, and once the flies have been placed into the chambers, small Petri dishes containing yeast-glucose food and smeared with moist, live yeast are taped to the other end.
2. Glass or plastic tube with a nitex gauze over one end.
3. Freshly diluted 50% household bleach.
4. 0.02% (v/v) Triton X-100.
5. Black nitrocellulose filters.
6. Fine paint brush.
7. Cover slips (22 × 40 mm).
8. Solution of Sellotape in *n*-heptane.
9. Voltalef oil.

3. Methods

3.1. Preparation of DNA

Plasmid DNA for microinjection may be prepared either by the cesium chloride–ethidium bromide centrifugation method, or by the more convenient Qiagen anion-exchange columns produced by Qiagen (Chatsworth, CA). The latter method produces clean DNA and is not only quicker but also avoids the use of ethidium bromide and organic solvents, such as phenol and chloroform, which could potentially reduce embryonic survival rates.

The concentration of DNA for microinjection needs to be quite high (between 400 and 600 µg/mL) with "helper" plasmid, if used, at a concentration of 200 µg/mL. The DNA to be injected should be ethanol precipitated and given an 80% ethanol wash before being redissolved in injection buffer. Aliquots of 20 µL can then be stored at –20°C.

Prior to loading the DNA into injection needles, the aliquots should be heated to 65°C for 10 min to ensure that the DNA is fully dissolved and then spun through 0.45-µm Millipore filters for a couple of minutes to remove any dust or particles, which could potentially block the needle.

3.2. Preparation of Needle

To obtain a needle that possesses the appropriate shape, the first-stage pull should generate a stretch with a length of about 8 mm and a diameter of approx 200 μm. The heating filament should then be moved to the center of this stretch so that the second pull produces a very fine tip of approx 2 mm in length with an end of between 1 and 5 μm in diameter. The heater settings for the first and second pull will need to be determined empirically in order to produce a good-quality needle.

Once a needle has been prepared, the simplest way to load it with the DNA solution is to add 1 to 2 μL of the injection DNA at the back of the capillary with a micropipet. The internal filament that runs along the length of the capillary draws the DNA solution to the front of the needle, which can then be placed into the collar of the microinjection system.

The survival of injected embryos is affected to a large extent by the sharpness of the needle. To obtain a sharp point, the needle can be broken at an angle against a cover slip mounted onto a glass slide. This process is visualized using the inverted-phase microscope and is made easier by placing a drop of Voltalef halocarbon oil on the junction between the slide and the cover slip where the needle is to be broken. When the needle breaks, a small amount of the oil can usually be seen to enter the tip. The flow of DNA can then be tested by applying a little pressure to the syringe. The needle is now ready to use for microinjection.

In between injecting embryos, the needle can be lowered into a small (5-cm) Petri dish lid containing Voltalef halocarbon oil. This helps prevent evaporation of the DNA solution and the concomitant clogging of the needle that can otherwise occur.

3.3. Egg Collection

Synchronous and abundant batches of eggs are required for injections. In general, 300–600 adults will produce enough eggs for a few days of microinjections. The flies should be transferred into collection chambers. To optimize egg laying, the flies should be kept at 25°C for a further 2 d in the chambers before starting egg collections for injection, and the Petri dishes containing the food should be changed every day. At the end of the second day, and every subsequent day, the flies should be transferred to 18°C overnight and then returned to 25°C on the morning of collection. The first hour's collection should be discarded because female flies tend to retain eggs until fresh food is supplied. Thereafter, at 60-min intervals, the collection plates can be removed and replaced with new ones.

The eggs to be injected are washed off the collection plates with distilled water and passed down a glass or plastic tube containing a nitex gauze over one end to retain the embryos. The eggs are then ready for dechorionation.

3.4. Preparation of Embryos for Microinjection

1. The first step in preparing the eggs for microinjection requires the removal of the tough outer chorion (*see* **Note 3**). To achieve chemical dechorionation, place the tube with nitex gauze and embryos into a beaker containing 10 mL of a 50% solution of household bleach. Gently shake the beaker and tube and, after 2–2.5 min of dechorionation, dilute the bleach by adding an equal volume of a 0.02% Triton X-100 solution. Then remove the tube from the beaker and wash the eggs thoroughly with distilled water.
2. Transfer the embryos onto a black nitrocellulose filter with a fine paint brush and line up along one of the ruled lines on the filter in such a way that the micropile is nearest to you. It is important to keep the filter damp to prevent the eggs from drying out.
3. When 50–60 embryos have been lined up, transfer them to a 22 × 40 mm cover slip; the cover slip can be made adhesive by the prior application of a solution of Sellotape in *n*-heptane. Stick the cover slip with attached embryos onto a microscope slide using a small drop of Voltalef oil and a little pressure. Place the whole slide inside an airtight box containing silica gel in order to desiccate the embryos (*see* **Note 4**).
4. At the end of the desiccation period, take the eggs out of the box containing the silica gel and cover with a layer of Voltalef oil. This oil, although being oxygen permeable, is water impermeable and therefore prevents any further desiccation of the embryos. The embryos are now ready to be injected.

3.5. Microinjection of Drosophila Embryos

1. Once the needle is lifted safely out of the way, place the slide containing the embryos on the microscope stage so that the eggs have their posterior facing the needle. Use the micromanipulator to bring the needle into the same plane as the line of eggs.
2. Bring the tip of the needle level with the center of the first egg; this is gaged by running the very end of the needle up and down the edge of the embryo. This method ensures that the needle will not slide over the surface of the egg and will also help decrease the amount of damage to the embryo. Then move the embryo toward the needle with a purposeful motion so that the vitelline membrane is just penetrated. Draw back the needle so that the tip is only just within the cytoplasm. Most of the embryos to be injected will be in the early cleavage stage (15 min to 1 h 20 min) and will have a space between the posterior pole and the vitelline membrane. It is important that the needle be inserted through the space and that the DNA be deposited in the posterior pole of the embryo proper. It is here, at the posterior pole, that the germline will be formed. Next, inject the embryo with a quantity of DNA solution equivalent to approx 1% of the egg's total volume and

remove the needle. Repeat the procedure until all the embryos have been injected (*see* **Note 5**).

3. Kill any embryos in which pole cell formation has already taken place running them through with the needle. Do not count these among those eggs that have been successfully injected.
4. Remove the cover slip containing the injected embryos from the slide and place onto a flat yeast-glucose-charcoal plate. Apply a further thin layer of Voltalef oil to the line of embryos and place the plate into a box kept humid by damp tissues. Then place the box on a level surface in an 18°C incubator for 48 h. If the plate is not kept level, the Voltalef oil will run off, and the embryos will overdesiccate and die.
5. After this time, count the hatched larvae, transfer into vials containing fly food, and return to the 18°C incubator to develop. The percentage survival to first instar larvae can be determined by dividing the number of survivors by the number of successfully injected embryos.

4. Notes

1. There exist a number of plasmids that, when injected, can provide the P element transposase necessary to mobilize the coinjected transposon. Two of the most widely used sources are pp25.7wc (wings clipped; **ref.** ***5***) and pUChsΔ2-3 ***(6)***. The wings-clipped transposase source contains a complete 2.9-kb P element in which the last 22 bp has been deleted so that the element is no longer mobile. The pUChsΔ2-3 transposase source comprises the engineered transposase gene (Δ2-3) in which the intron separating the second and third exons (normally only spliced in the germline) has been removed ***(6,7)***. This modified transposase gene is placed under the control of the *HSP70* promoter, although the constitutive expression of this promoter is of a sufficiently high level such that heat shock is not necessary. Injecting this construct will result in the transient expression of a functional transposase in both germline and somatic tissues. An alternative approach to coinjecting a plasmid that provides a transposase source is to inject embryos that possess a chromosomal source of the Δ2-3 transposase ***(3)***.
2. Many vectors suitable for constructing transposons have been described. We consider here three of the more widely used ones. The transformation vectors based on *rosy* (*ry*) as a scorable marker were the first to be used. One of the most versatile versions of the *ry*-based vectors is pDM30 ***(8)***. The major advantage of using *ry*-based vectors is that since 1% of wild-type *ry* expression is sufficient to yield ry^+ eye color, insertions into positions that result in a low level of expression can still be recovered. However, the *ry* gene is large (usually a 7.2-kb *Hin*dIII fragment carrying *ry* is used), and this results in a less-than-optimal vector size. For example, the largeness of *ry*-based vectors can make the construction of transposons more difficult and can also contribute to a decreased transformation frequency.

   Another popular series of transformation vectors use the *white* (*w*) gene as a marker ***(9)***. In the most widely used *w* vectors, a mini-*white* gene ***(10)*** with a

subthreshold of w^+ activity is used. There are several advantages associated with these mini-*w*-based vectors. First, the gene is small, ~4 kb, compared with *ry*. Second, since mini-*w* has subthreshold activity, for most insertions, flies that are heterozygous for mini-*w* can be distinguished from flies that are homozygous on the basis of eye color. Finally, *w* is easier to score than *ry* when large numbers of flies are involved. The latest versions of these vectors (the *Casper* series) may be requested from the Thummel or Pirrotta laboratories.

A third series of vectors are those based on G418 antibiotic selection ***(11)***. In these vectors, the bacterial neomycin resistance gene is used as a selectable marker in place of visible markers such as *ry* and *w*. The advantage of using such vectors is that transformants can be selected on *Drosophila* food containing G418 (usually 500–1000 mg/mL), eliminating the chore of screening many flies for a visible marker. However, the major disadvantage is that the window of G418 concentration that will allow true transformants to survive, but that will reduce the leakage of nonresistant animals to an acceptable level, is narrow. Consequently, transformants owing to insertions into chromosomal sites resulting in a low level of expression will not be recovered.

Other transformation vectors, such as those based on *Adh*, which allow for selection on media containing alcohol, have also been described. In addition, a transformation vector (pCaWc) in which both the transposon and the transposase are carried on the same plasmid molecule (with the transposase located outside the P element 31-bp repeats) has been successfully employed for obtaining transformants ***(12)***. There are also "shuttle vectors" that greatly facilitate the construction of complex transposons. These vectors (e.g., pHSX, referred to in **ref. *12***) contain large polylinkers flanked by restriction enzyme sites such as *Not*I (which occurs only very rarely) and enable several DNA fragments to be assembled and then excised as one contiguous piece. The construct can then be inserted into the single *Not*I site of transformation vectors such as pDM30 or the *Casper* series. Finally, transformation vectors designed for placing genes under the control of *HSP70* and actin promoters have been described ***(13)***, as have transformation vectors designed to facilitate the insertion of desired sequences upstream of a *LacZ* reporter gene to drive its expression ***(10,13)***.

3. Two methods of dechorionation can be employed: chemical and mechanical. However, we favor the chemical method because it is far easier and less time-consuming.
4. This stage is of vital importance if the embryos are to withstand being punctured and accommodate the volume of DNA being introduced. Moreover, this step of the procedure is probably the most crucial, in terms of survival rates, because there is only a narrow margin between a sufficient reduction in egg turgor and excessive drying, which kills the embryos. If possible, embryos should be prepared in an environment with constant temperature and humidity conditions, because this will facilitate the determination of the optimum desiccation time. However, if this is not possible, the experimenter will have to determine the desiccation time empirically, since this will tend to fluctuate depending on the cli-

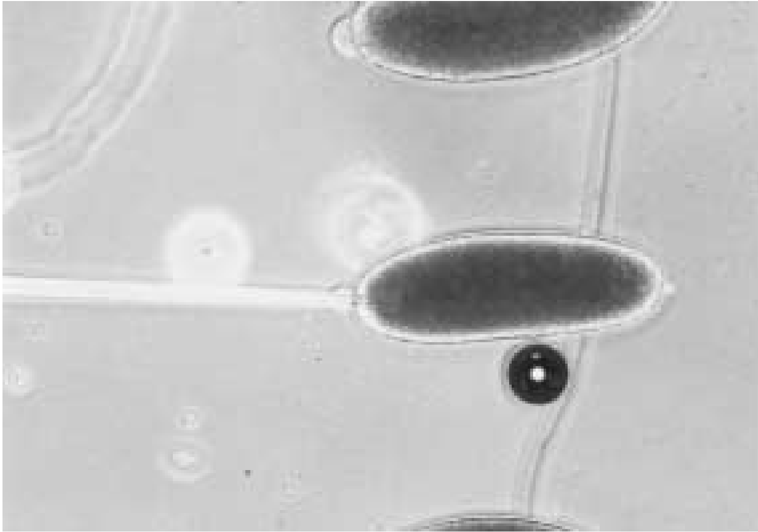

Fig. 2. Microinjection of *Drosophila* embryos illustrating the region of the embryo targeted for injection. Note also the "bubble" of cytoplasmic material leaking from the embryo, which should be removed.

matic conditions. As a starting point, we generally have used desiccation times of between 10 and 15 min.

5. If the embryo has not been desiccated enough, or if too much DNA solution has been injected, cytoplasm may leak out of the egg, reducing its chances of survival (*see* **Fig. 2**). We have found that increased survival rates can be achieved by removing the "bubbles" of cytoplasm. This is easily achieved by having a constant flow of DNA coming out of the needle, which is then brushed passed the line of embryos.

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