'Shocking' developments in chick embryology: electroporation and *in ovo* gene expression

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Efficient gene transfer by electroporation of chick embryos *in ovo* has allowed the development of new approaches to the analysis of gene regulation, function and expression, creating an exciting opportunity to build upon the classical manipulative advantages of the chick embryonic system. This method is applicable to other vertebrate embryos and is an important tool with which to address cell and developmental biology questions. Here we describe the technical aspects of *in ovo* electroporation, its different applications and future perspectives.

The avian embryo is a classical system used to investigate questions of vertebrate development. Accessibility, ease of tissue manipulation and *in ovo* culture, and its planar topology make this a widely used model. Chick–quail grafting, as well as the tracing of fluorescently labelled cell lineages, has generated a wealth of information on cell fates and lineage relationships during chick embryogenesis[1–5]. Furthermore, the development of time-lapse analysis, in combination with explant and *in ovo* culture methods, has provided a means by which to follow the dynamic movements and fate of cells over time[6,7].

To understand how developmentally important genes are integrated with developmental processes, it would be extremely valuable to couple these techniques with methods for manipulating and analysing gene expression. However, a limitation of the chick system is the absence of established methods for genetic manipulation, as compared with the mouse and fish. Although there are some spontaneous chick mutants, targeted or chemical mutagenesis and transgenic analysis are not routinely available in the chick. Ectopic expression has relied primarily on gene-transfer systems, using retroviral vectors and host strain restrictions[8,9]. While useful, this approach is subject to DNA size constraints and a number of other problems. So there has been a real need for alternative methods of gene transfer that allow a wider range of approaches to study gene function, regulation and expression.

In ovo electroporation

Progress in this area has been made with the development of efficient methods for gene transfer into living embryos by electroporation. Electroporation is an effective method for introducing DNA into cultured bacterial or eukaryotic cells[10,11], but this involves exposure to high voltages, which result in 50–75% cell death. Although this may be acceptable for transfecting cultured cells, such as bacteria or embryonic stem cells, massive cell death destroys tissue architecture and kills embryos, making it impractical for efficient application to embryological approaches. A modified type of electroporation, using a low fixed voltage, made it possible to enhance cell viability[12]. Improvements in voltage control have enabled its application to tissues, living embryos and animals[13–15]. Figure 1a shows the technique for generating expression in the chick neural tube. This consists of cutting a window in the eggshell and injecting DNA vectors into the neural tube of chick embryos. At Hamburger and Hamilton stage (HH) 10 of development (when the embryo contains ten somites) the neural folds have fused, creating a closed system with a lumen into which it is easy to inject DNA constructs *in ovo* (Fig. 1a). The neural epithelium at this stage consists of a single layer of cells, all exposed to the DNA confined to the lumen. Electrodes are applied in parallel on the surface of the embryo, along the anteroposterior axis. A difference in voltage is created that allows the directional entry of DNA into cells along one side of the neural tube, while the other side constitutes an untransfected control. The embryos are cul-

Table 1 Parameters for electroporation of mouse and chick embryos

Species	Stage/tissue	Voltage (V)	Number of pulses	Pulse length (ms)	Electrode dimensions: length/diameter (mm)	References
Chick	HH 9/neural	15	5	50	4/0.4–0.5	This paper
	HH 11–12/trunk	10–25	3	50–99	12/?	Ref. 13
	HH 10-15/neural	20–25	5	50	4/0.4–0.5	Refs 17, 18 and this paper
	HH 9–10/ectoderm	25	5	50	?	Ref. 20
	HH 7–9/limb	12–17	1–3	90	Needle type	Refs 15, 21
	HH 10–11/placode	10	3	50	Needle type	Ref. 15
Mouse	8.5 d.p.c.	25–35	5	50	Plates 5×3	This paper
	9.5 d.p.c.	25–45	5	50	Plates 5×3	This paper
	9.5 d.p.c.	90	5	50	?	Ref. 19

HH is Hamburger and Hamilton stage; d.p.c., days post-coitum; needle type refers to the point-source type of electrode described in ref. 15; references shown in the last column are the references in which the respective conditions were described; ?, refers to the fact that this parameter was not specified in the relevant reference.

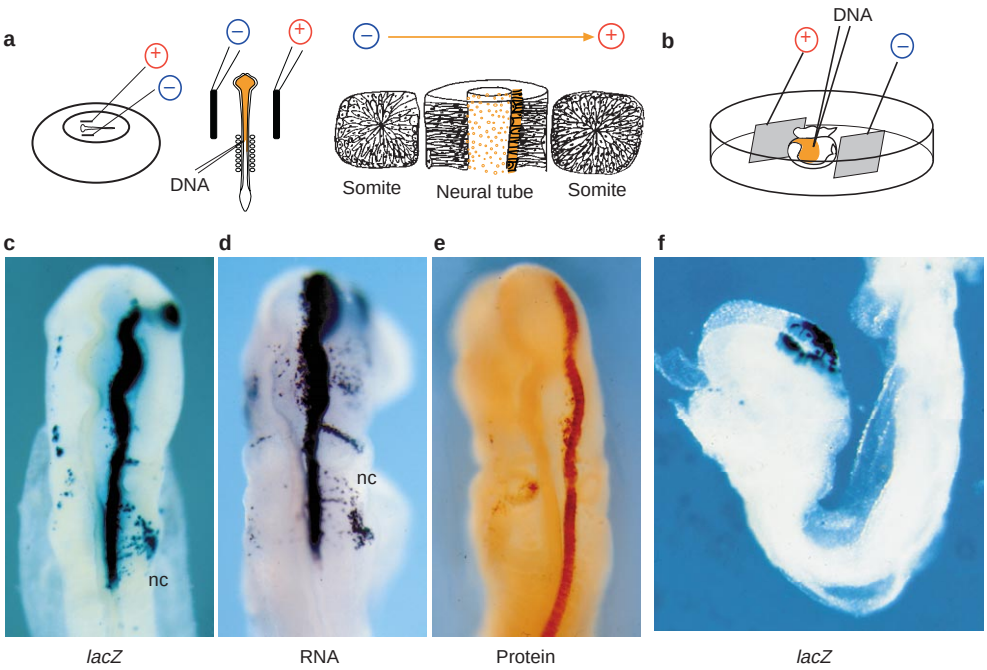

Figure 1 **Electroporation-mediated gene expression in cultured chick and mouse embryos. a**, **b**, Basic methods. **a**, For chick *in ovo* electroporation, a window is opened in the eggshell and a DNA solution (orange) is injected into the neural tube of an 8–10-somite embryo. Two wire electrodes (separation 4 mm) are then placed, touching the blastoderm, in parallel along the anteroposterior axis of the embryo. The DNA is transferred unilaterally to the side of the positive pole by application of electric pulses. Embryos are cultured *in ovo* and assayed for gene expression at later stages. **b**, For the mouse, embryos at 8.0–8.25 d.p.c. are dissected, keeping membranes intact for *in vitro* culture, and are placed in a small chamber. DNA is injected into the area overlying the open neural folds; wire or parallel plate electrodes are placed anterior and posterior to the embryo to deliver the electric pulses; and the embryos are then cultured *in vitro*. **c–e**, Gene expression in chick embryos following 18–24 h of *in ovo* culture can be detected by: **c**, staining for a reporter gene (*lacZ*); **d**, whole-mount *in situ* analysis (RNA); or **e**, immunohistochemistry of an epitope-tagged cDNA (protein). More than 90% of electroporated embryos efficiently express the transgene. In **c–e**, the positive electrode was placed to the right. **f**, Bilateral reporter staining in the mid/hindbrain region 12 h after electroporation of an 8.0–8.25-d.p.c. mouse embryo. In **c–f**, gene expression is driven by an RSV enhancer, which should allow expression in all cells taking up the DNA. In these experiments, either a BTX T820 Electroporator with enhancer 400 (BTX, San Diego) or a Cuy21 electroporator (Tokiwa Science, Japan) was used to deliver electric pulses; DNA concentrations were 0.1–1.0 $\mu g\,\mu l^{-1}$ in H_2O. nc, neural crest cells.

tured *in ovo* for varying lengths of time to allow expression of the injected DNA.

To make it easy to monitor the efficiency of expression of electroporated DNA, we have used a *lacZ* reporter gene (which encodes β-galactosidase) under the control of either a Rous sarcoma virus (RSV) enhancer[16] or a cytomegalovirus (CMV) enhancer element (pcDNA3; Invitrogen). These regulatory elements are capable of mediating expression in all cells that take up the DNA, making it relatively easy to test a number of variables and work out the optimal conditions. In Table 1, we summarize the conditions that we and others have used for electroporation. The electrode parameters can be a significant variable. Small changes in size influence the amount of heat generated and, as a consequence, viability of embryos. Therefore, conditions that optimize efficiency and viability need to be calibrated for each set of electrodes. The age of the embryo and the type of target tissues are also a source of variability, and it is necessary to find a combination of parameters that are optimal for each case. For example, higher voltages may be used without affecting viability by increasing the separation between electrodes. In general, the current should be kept at less than 30 mA.

Figure 1c provides an example of the efficient and high-level expression of a *lacZ* reporter gene on one side of the embryonic neural tube, 18 h after electroporation. *lacZ* reporter staining is a very sensitive means of assessing the efficiency of transfection. However, robust expression of electroporated complementary DNA constructs can also be detected by RNA *in situ* analysis (Fig. 1d) and by immunostaining for expressed protein (Fig. 1e). The fact that there are so many positive cells and that the levels of expression are high indicates that this method of gene transfer is very efficient. The examples shown in Fig. 1 were analysed 18–24 h after electroporation, but we have detected reporter staining as early as 3 h, and strong expression 72 h following electroporation (data not shown).

Under these conditions, expression is largely confined to the neural tube, but some positive cells are detected in small populations lateral to the central nervous system (CNS), which appear to be migratory neural crest cells emanating from the neural tube (Fig. 1c, d). The extent of expression along the anteroposterior axis can vary depending upon the site of injection, the volume of the DNA solution used and the degree of diffusion. Large transfected regions are obtained by injecting DNA solutions at the posterior end, using volumes that are sufficient to replace the normal luminal fluid with the DNA solution.

This approach has been successfully used in experimental studies of neural patterning. In one case, expression of endogenous *Hoxb4* in chick embryos was specifically blocked by expressing a dominant-negative form of retinoic-acid receptor-α1 (dnRARα1)[17]. Overexpression of Pax5 has also been used to study the activity of the mid/hindbrain isthmic organizer[18].

Electroporation of mouse embryos

It would be useful to be able to introduce DNA into cultured mouse embryos in a similar manner by electroporation. This approach differs from normal pronuclear injection techniques, as DNA can be applied at specific stages to wild-type, transgenic or

mutant embryos. Mice, however, have several properties that complicate this approach in comparison with the chick. The mouse neural tube closes later than the chick tube, so the DNA is less confined at early stages; the embryo has a cylindrical topology, instead of being flat; and embryo culture and manipulation are more demanding. Nevertheless, many of these complications can be overcome, and it is possible to electroporate and culture mouse embryos *in vitro* for ectopic expression studies (Fig. 1b, Table 1). The same RSV/*lacZ* reporter construct used in the chick (Fig. 1c) can mediate gene expression in the mouse mid/hindbrain region when electroporated in an embryo at 8.25 days post-coitum (d.p.c.; Fig. 1f). This approach has also been used in 9.5-d.p.c. mouse embryos to study the functions of *Hu* genes in neuronal differentiation[19].

Electroporation in diverse tissues

It would be helpful to be able to vary this method within the CNS and extend it to other tissues. As the electroporation conditions do not significantly damage or perturb the embryo, we found that it is possible to perform multiple rounds of electroporation. For example, by simply reversing the polarity of electrodes, following a standard procedure for unilateral electroporation into the chick neural tube, it is possible to obtain bilateral reporter expression (Fig. 2a). To direct expression to dorsal or ventral territories in the CNS the electrodes can be placed directly above and below the embryo. To obtain expression in neural crest cells, the electroporation needs to be done before these cells migrate from the dorsal neural tube. Therefore, the timing of electroporation is critical, as neural crest cells emerge from different anteroposterior levels at different times. For example, by electroporating even earlier than 10 somites, gene expression can be increased in cranial neural crest cells that are migrating into the branchial arches (Fig. 2b).

It is also possible to target tissues other than the CNS, such as somites, limb mesenchyme, lens or surface ectoderm. To target the surface ectoderm, the DNA solution is placed on the outer surface of the embryo, with the electrodes above and below the embryo (Fig. 2d). Electroporation of the gene *L-Maf* into embryonic surface ectodermal cells has been useful for examining lens differentiation; *L-Maf* induces these cells to form lens fibres[20]. Focal injections into the segmental plate, paraxial, lateral or intermediate mesoderm will allow gene expression in small mesenchymal populations surrounding the site of injection. By injecting the DNA next to the neural tube or within an epithelialized somite, expression can be targeted to somites and their derivatives (Fig. 2c). We have found

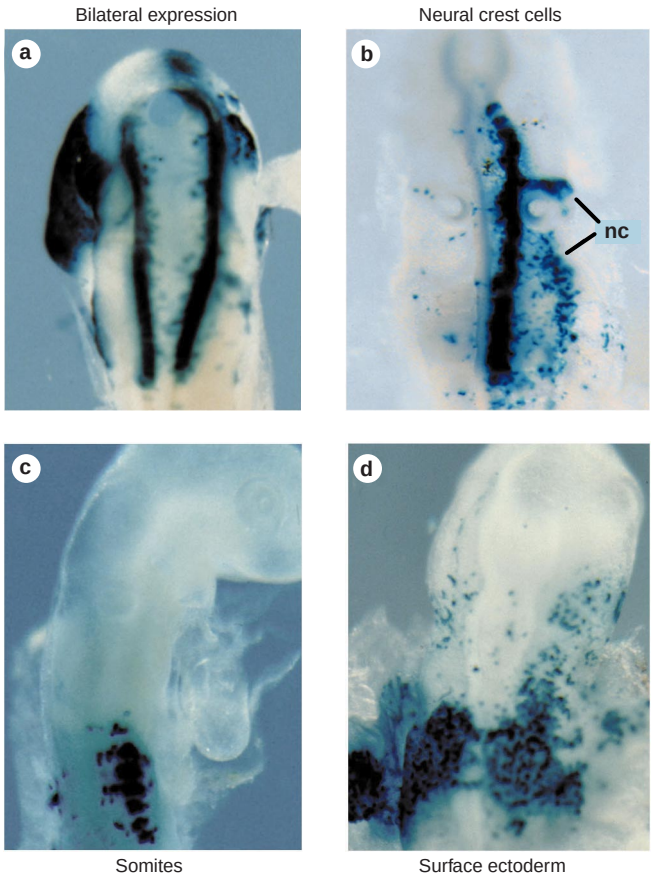

Figure 2 **Electroporation into different chick tissues. a**, Expression on both sides of the chick neural tube can be achieved by reversing the polarity of the electrodes following the initial electroporation, and applying a second round of electric pulses. **b**, Expression in neural crest cells (nc) migrating from the neural tube can be obtained by performing electroporations into the neural tube of early embryos before their migration. **c**, Injection of DNA between the neural tube and somites or directly into the somites results in reporter expression in somitic mesoderm. **d**, Application of the DNA solution to the outer surface of a chick embryo, followed by placement of electrodes above and below the embryo, allows gene transfer and expression in surface ectoderm. In **a–d**, gene expression is again driven by an RSV enhancer, which should allow expression in all cells taking up the DNA.

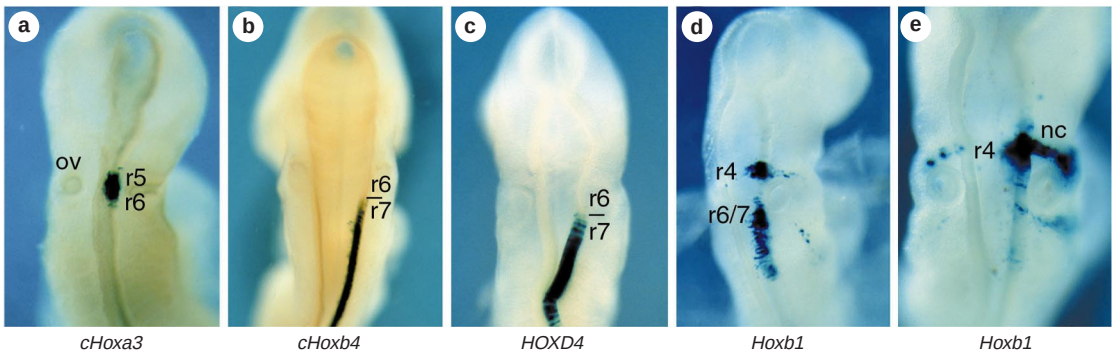

Figure 3 **Spatially restricted expression in chick embryos, using region-specific enhancers from different species. a–e**, Restricted expression from a *lacZ* reporter gene is mediated by *Hox* enhancers that have been shown previously to function in transgenic mice. **a**, A chick *Hoxa3* element[22] mediates expression in rhombomeres (r) 5 and 6. **b**, A chick *Hoxb4* enhancer[23,25,26] directs the proper expression in r7 of the neural tube. **c**, A human *HOXD4* control region[24] generates an r6–r7 boundary. **d**, **e**, A mouse *Hoxb1* element[27,28] drives expression in r4 and neural crest cells (nc) derived from r4. The difference between **d** and **e** is that the electroporation in **e** was done at an earlier stage than that in **d** to facilitate expression in neural crest. In **d**, **e**, there is also staining in r6/r7 and slightly more posterior domains that correspond to the hindbrain pattern of the endogenous chick *Hoxb1* gene[31,32]. ov, otic vesicle.

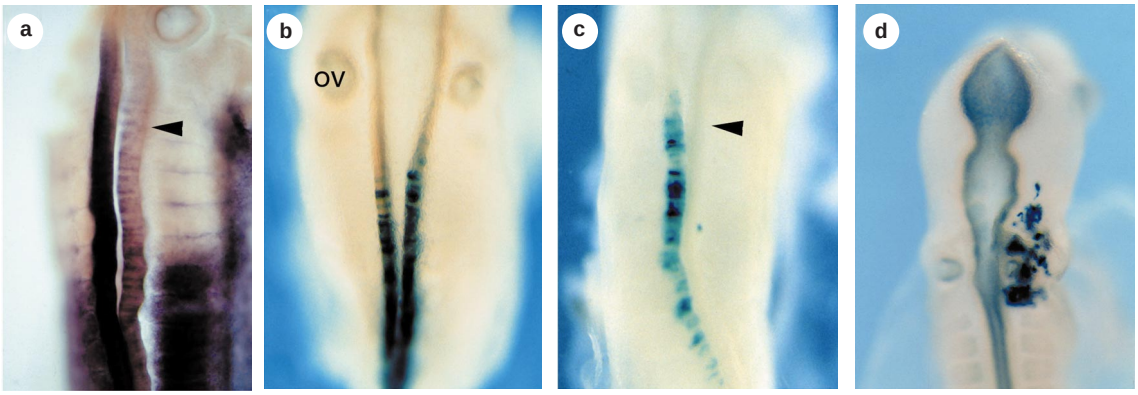

Figure 4 **Application of electroporation to analysis of gene regulation.** **a**, Blocking endogenous expression. Using a dominant-negative RAR-α1 construct (dnRAR) it is possible to inhibit the onset of *Hoxb4* expression on the electroporated side (right side; see arrowhead)[17]. **b**, **c**, Blocking reporter expression. **b**, Bilateral electroporation of the human *HOXD4* reporter construct (Fig. 3c) generates r6/7-restricted expression on both sides of a chick embryo. **c**, The same *HOXD4* construct as that used in **b** was electroporated on the left side first, and then co-electroporated on the right side with the dnRAR construct. Reporter expression is specifically blocked by the dnRAR construct (arrowhead on right side). **d**, The RSV–*lacZ* construct was electroporated into somites (as in Fig. 2c); the somites were then grafted next to rhombomeres in hosts that were not electroporated. ov, otic vesicle.

that electroporation into somites is more variable than into the neural tube, which could be a consequence of differences in the organization of these cells or their environment, such as the extracellular matrix. Successful electroporation into mesodermal derivatives has been done using replication-competent retroviral vectors, to allow a functional analysis of *Tbx4* and *Tbx5* in chick limb-bud mesenchyme[21].

Small focal electroporations into many different tissues have been achieved by modifying the shape and arrangements of electrodes used[15]. In this case, a single, large electrode is placed next to the embryo; the second electrode is needle-shaped and can be placed precisely in very specific locations[15]. This creates a voltage difference over a much smaller area and will be useful for targeting small groups of cells.

Regionally restricted expression

RSV or CMV enhancers generate global or widespread expression in electroporated cells. Although this is useful in many gain-of-function experiments designed to look at the effects of a particular gene, it would also be valuable to direct expression to specific regions or cell types. This could be achieved by using region-specific enhancers in combination with electroporation. To test this idea, we used an array of enhancers specific to particular hindbrain segments (rhombomeres, r). These enhancers were from vertebrate *Hox* genes, and were identified previously by evolutionary comparisons and transgenic analysis in the mouse[22–28]. The chick *Hoxa3* r5–r6 enhancer and the *Hoxb4* r6/r7 enhancer both direct reporter expression in spatially restricted domains in the hindbrain and spinal cord (Fig. 3a, b), in a manner identical to the expression seen in transgenic mice. We also detected appropriate rhombomeric expression when we assayed the human *HOXD4* r6/r7 and the mouse *Hoxb1* r4 neural enhancers by electroporation into chick embryos (Fig. 3c–e). Thus, region-specific enhancers from several vertebrates can function effectively in chick embryos, expanding the number of possible approaches for modulating gene expression in functional analyses. However, not all of the enhancers that we tested from other species worked in the chick, so in some cases it may be necessary to clone the relevant chick equivalent. In addition, the activity of a chick enhancer element also varied depending upon which promoter element was used, so the choice of the promoter is also critical.

Analysis of gene regulation

The fact that the region-specific enhancers generate the expected patterns of gene expression following electroporation into chick embryos opens up the possibility that this approach can be used to study gene regulation. Flanking DNA linked to reporter genes could be used to identify, map and characterize new regulatory regions from genes in chicks and other species. When applied to mouse embryos, this technique would be much quicker and easier to use than classic transgenic approaches. Another means of investigating regulatory pathways is to use electroporation to express inducers or repressors, and to monitor their effects on expression of endogenous genes. For example, we have found that dnRARα1 effectively blocks the expression of endogenous *Hoxb4* specifically on the electroporated side (Fig. 4a and ref. 17).

By performing multiple electroporations, it is possible to combine these approaches to investigate the activation or repression of reporter genes in chick embryos (Fig. 4b, c). Bilateral electroporation of the *HOXD4* r6/r7 *lacZ* construct generates reporter staining on both sides of the neural tube (Fig. 4b). However, when a dnRARα1 construct is co-electroporated on one side of the embryo, r6/r7 reporter expression is specifically blocked, in comparison with the control side (Fig. 4c).

Other applications

Together, these types of experiment illustrate the tremendous potential of using electroporation for gene transfer into chick embryos, and this opens many other investigative possibilities. Using the manipulative advantages of the chick, it is possible to electroporate embryos *in ovo* and then graft tissue or cells to host embryos for further analysis. For example, Fig. 4d shows *lacZ* reporter expression in a graft of electroporated somites. Although we have stressed *in ovo* methods, electroporation in New culture or of isolated tissues in cuvettes is also efficient and can be combined with grafting or explant culture systems. As well as *lacZ*, the gene encoding green fluorescent protein (GFP) can also be used as a reporter[15,18,20]. In combination with region-specific enhancers, this will provide a means of monitoring gene expression patterns over time in living embryos. In ectopic-expression, promoter-analysis and gene-regulation studies, plasmid and cosmid reporter constructs have been used, but analysis could be extended to larger regions through the use of bacterial and yeast artificial chromosomes.

Although we have observed reporter staining from constructs up to 3 days after electroporation, we have not investigated the maximal time that expression will persist *in ovo*. Expression of DNA can be very stable — *in vivo* electroporation of muscle in adult mice allows expression for up to 3 weeks[29]. If the DNA integrates into the host genome, this could lead to long-term expression and allow analysis in neonates or adults, following early electroporation. The earliest stage at which we have electroporated chicks is HH 5, but the technique should work in even younger embryos. It is also possible to perform electroporation in multiple stages or in adult tissues, such as rat brain[14], so this approach is not confined to the study of embryogenesis.

On the basis of initial analysis of mouse embryos, it appears that this technique will be equally beneficial to these animals and will allow us to take further advantage of transgenic lines and genetic mutants. This approach could also be applied to many other vertebrates and invertebrate embryos or tissues, and might be particularly useful for cases in which gene-transfer methods have not been previously established. Electroporation might also provide a much easier and more efficient technique for introducing DNA into fertilized mouse, fish and frog eggs or early embryos, as compared with microinjection methods. In these experiments, we have only begun to explore the full potential of gene transfer by electroporation, and many other uses are certainly possible. Furthermore, the method is not limited to transfer of DNA, and is applicable to RNA or proteins[30]. □

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