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Gene Transfer into Cultured Mammalian Embryos by Electroporation

Noriko Osumi*,†,[1] and Takayoshi Inoue*

*Division of Biochemistry and Cell Biology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1, Ogawahigashi, Kodaira, Tokyo 187-8502, Japan; and
†Department of Neural Development, Tohoku University Graduate School of Medicine, 2-1, Seiryo-cho, Aoba-ku, Sendai, Miyagi 980-8575, Japan

To gain a better understanding of mammalian development at the molecular level, technology is needed that allows the transfer of exogenous genes into desired embryonic regions at defined stages of development. Our strategy has been to use electroporation (EP) of plasmid DNA following whole-embryo culture (WEC). In our gene transfer system, postimplantation rodent embryos are taken out of the uterus and a purified DNA solution of mammalian expression plasmid constructs is injected into the neural tube. A square-pulse current is delivered using an electroporator with an optimizer. Electroporated embryos are allowed to develop in the WEC system for 24–48 h. Within the targeted area, the proportion of transfected cells varied from 10% to approximately 100% depending on the test conditions (e.g., DNA concentration, voltage, duration of EP, and pulse number). The EP–WEC system has several advantages including rapid gene expression, minimal laboratory work, precisely targeted regions, and no risk for human beings. Application of the method is useful in improving our understanding of early neural development (E7–E12 in mice), e.g., alteration of gene function via ectopic expression, interference with dominant negative proteins, and fate mapping with marker genes. In addition, EP can complement genetic approaches such as the generation of knockout and transgenic mice.

Key Words: whole-embryo culture; electroporation; mammalian development.

Among the various methods available for manipulating mammalian embryos, the whole-embryo culture (WEC) system has been used for more than 100 years (see Ref. 1 for review), although it seems less popular than modern techniques such as transgenic mice and gene targeting. This is because WEC techniques were used primarily for teratologic studies, and developmental biologists preferred more accessible embryos of other species such as amphibians and avians. WEC of mammalian embryos at postimplantation stages, however, provides a vast amount of knowledge that cannot readily be obtained from explant cultures or genetic manipulation. Recent advances in molecular technology now allow us to manipulate mammalian embryos under WEC at molecular levels.

Figure 1 is an outline of the method used for transferring genes into cultured mammalian embryos by electroporation (EP). Rodent embryos are taken from the uterus and transferred to a chamber filled with saline. The chamber contains a pair of electrodes to provide appropriate unidirectional current. DNA solution of the desired genes is injected at a targeted area and square electrical pulses are applied immediately. In this method, the electric pulse results in a brief opening of plasma membrane channels, allowing the entry of small molecules. The electroporated embryo is returned to WEC and allowed to develop. Following completion of WEC, the harvested embryos are examined for ectopic gene expression and morphological changes. This type of direct gene targeting of the mammalian embryo can lead to changes in downstream gene expression that affect subsequent development. The possibility of direct

[1] To whom all correspondence should be addressed at Dept. of Developmental Neurobiology, Tohoku Univ. Graduate School of Medicine, 2-1, Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. Fax: -81-22-717-8205. E-mail: osumi@mail.cc.tohoku.ac.jp.

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targeting can be of advantage in a variety of applications, such as alteration of gene function via ectopic expression and interference with dominant negative proteins. In addition, this method may complement other genetic approaches such as the generation of knockout and transgenic mice, which cannot be performed using *Xenopus* or chick embryos.

CULTURE OF MAMMALIAN EMBRYOS

1. Culture Apparatus

We use the system of rotating bottles (Model RKI10-0310, Ikemoto Co., Tokyo, Japan), which is a refinement of the original system designed by New and Cockroft (2). This apparatus can be supplied with a continuous flow of oxygen (see below) over the entire culture period. This system provides a better support during development of cultured embryos compared with the "roller-bottle system" (1) because embryos are allowed to grow to more advanced developmental stages, i.e., until Embryonic Day 14 (E14) in rat embryos. The simplicity of this rotating culture system allows easy experimental manipulation of the embryo at any desired stage. Embryos are housed in see-through glass bottles containing the culture medium, each of which has a center-holed silicon plug connecting it to the hollow rotator drum. This makes it easy to observe embryos without their removal from the incubator. Up to 12 bottles can be connected to one rotator drum and with one embryo per bottle, this provides easy manipulation of individual embryos (see Fig. 2).

2. Preparation of Culture Medium

Various cell or tissue culture media have been used for culture of embryos (3), although the results are so far insufficient or poor. Homogeneous serum offers the best support for embryonic growth in culture. At present, however, rat serum is generally used for the culture of both rat and mouse embryos. A key step in the preparation of the culture medium is the need for immediate preparation of the serum. This is achieved by immediate centrifugation of blood samples withdrawn from adult male rats starved for more than 24 h, followed by heat inactivation at 56°C for 30 min (4). Previous studies have shown that the use of delayed-centrifuged serum for culture is associated with the development of embryos with a double heart due to failure of fusion of the heart primordia (5). The rat serum can be stored at −20°C for several months.

For preparation of the culture medium, the rat serum is thawed and then incubated at 37°C for 10–15 min to avoid ether vapor, followed by the addition of glucose at 2 mg/ml. We do not routinely use antibiotics because the risk of contamination is very low in our WEC system. If desired, penicillin/streptomycin solution (GIbco) can be added at 1:200 to 1:400. Prepared medium is filtrated using a 0.45-μm filter

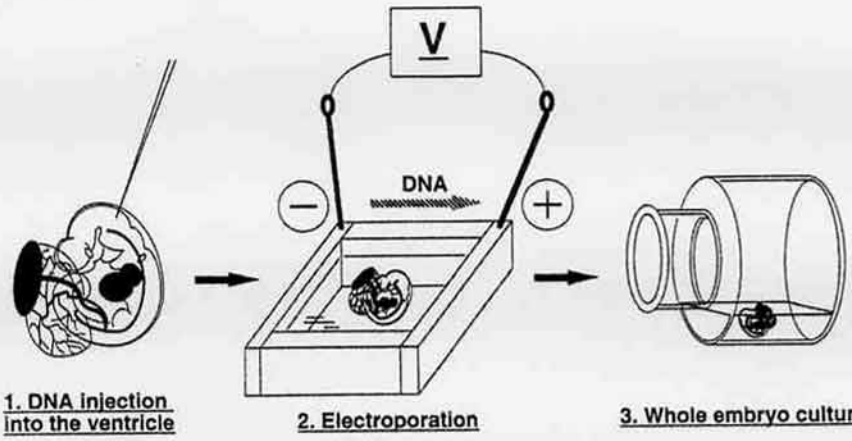

FIG. 1. Strategy of electroporation during whole-embryo culture. First, remove mouse/rat embryos at a defined developmental stage, and microinject DNA solution of expression vector(s) into the ventricle at the desired position of the neural tube (step 1). Then, position the embryo with the to-be-targeted wall of the ventricle facing the anode in the chamber (see Fig. 3), and apply square pulses immediately (step 2). Gene expression commences almost immediately (step 3). This strategy allows the expression of any gene at any time and

(Millipore, Bedford, MA) and added into each glass bottle (1.5–3 ml/bottle, Ikemoto Co.).

3. Explantation of the Embryo

Explantation of postimplantation mammalian embryos requires a certain level of skill, especially in the dissection process. The methods used for this purpose have been described previously in detail (1, 3, 6, 7). Embryos are dissected out under a surgical microscope using low-power magnification and freed from the uterine wall, decidua, and outside membrane layer including Reichert's membrane, which are attached to the trophoblast and parietal endoderm. The yolk sac, amnion, and chorioallantoic placenta are left intact and cultured with the embryo. The yolk sac and amnion are opened after E12 in the rat (roughly corresponding to E10 mouse embryo) to expose the embryo to higher concentrations of oxygen (8). The yolk sac can be opened at E11 if direct manipulation of these embryos for EP is desired at this stage. Opening the sac at an earlier stage (e.g., E10), however, is not advisable as it leads to deterioration of further development of the embryos. There are two key points that should be remembered when dissecting embryos and their membranes. First, the layer of giant trophoblast cells covering the allantoic placenta should be completely dissected away after E10 in the rat embryo. The giant cells proliferate rapidly, resulting in necrosis of the remaining portion of the placenta if they are not removed, as well as allowing the formation of a large blood vessel that bypasses umbilical vessels in the embryo (9). Second, blood vessels of the yolk sac membrane should be as intact as possible to avoid blood loss. It should be emphasized, however, that even using the present techniques, development of the placenta *in vitro* is not as efficient as that *in vivo* (6).

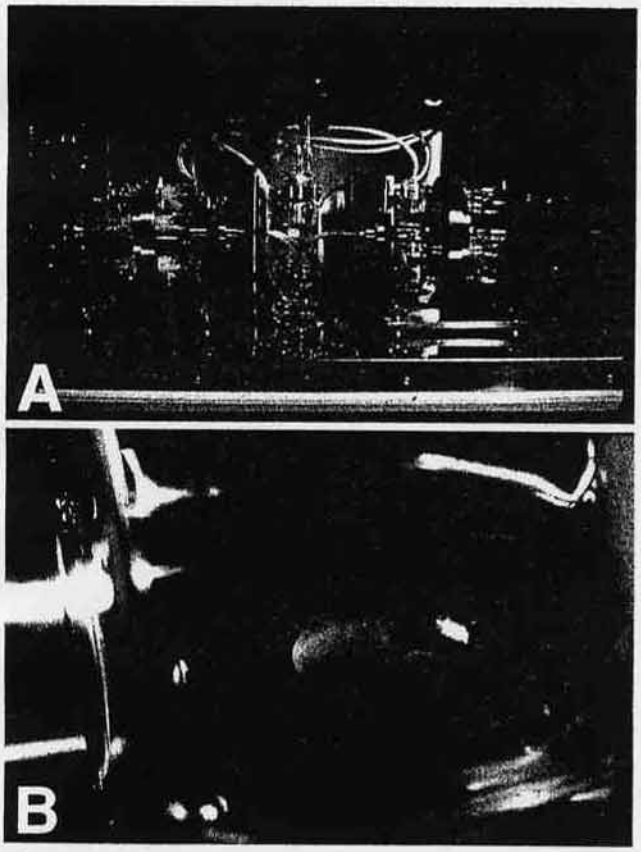

FIG. 2. Whole-embryo culture apparatus. (A) WEC equipment of Ikemoto Company (RKI10-0310) with two rotating drums each with 12 bottles. Gas mixture can be continuously supplied in this system, which allows better embryonic development. (B) Cultured mouse embryo in a glass bottle. A mouse embryo at E10.5 is cultured for 48 h.

Note: Animal Species for Whole-Embryo Culture

Both mouse and rat embryos can be cultured, although development of the latter in culture is more advanced due to the reasons described above. One should be careful about the embryonic stage of rodent embryos because it differs among strains (e.g., ICR mouse embryos develop faster than C57BL/6 and C3H mouse embryos), litters, and sometimes within the same litter. Genetically mutant embryos can also be cultured using the same method.

4. Oxygen Supply

Oxygen supply is very important for the success of WEC. Based on our experience in successful experiments, i.e., those associated with good embryonic development in culture, we have determined the optimal gas exchange schedules for rat and mouse embryos (see Table 1). At a rotation rate of 20 rpm, oxygen concentration should be increased from 5% at the primitive-streak and early-somite stages, to 20% at 10- to 15-somite stages, 60% at 15- to 25-somite stages, and 95% for embryos with more than 25 somites. For culture of embryos explanted at more advanced stages, the yolk sac is opened to directly expose the embryo to high concentrations of oxygen in the medium. In the uterus, blood circulation of the yolk sac and that of the allantoic placenta begin at E10.5 and E11 in rat embryos, respectively. Both organs play important roles in respiratory exchange

throughout the gestation period. *In vitro*, however, only the yolk sac, but not the allantoic placenta, seems to be functional (6). Opening the yolk sac after the 30-somite stage enhances diffusion of oxygen directly into the embryo and/or via the yolk sac blood circulation, thereby compensating for the loss of function of the allantoic placenta *in vitro* (8).

5. Culture Period

Our culture system can support growth and tissue differentiation of rodent embryos during the entire period of organogenesis, from before the egg cylinder stage to after the formation of the allantoic placenta. The total duration of the culture period, however, depends on the embryonic stage at explantation (also reviewed in Ref. 1). Rat embryos explanted at the egg-cylinder stage (E8) can develop *in vitro* for a period equivalent to 3 to 4 days *in vivo* (6), while those explanted at later stages can be cultured for a comparatively shorter period. As for growth in the culture medium, the commencement of circulation in the allantoic placenta (at E11 in rat embryos) is a crucial developmental event. Rat embryos explanted before E11 show almost the same rate of protein synthesis *in vitro* as *in vivo*, whereas the protein content *in vitro* gradually decreases in embryos explanted after E11 in comparison with *in vivo* embryos.[2]

TRANSFERRING GENES BY ELECTROPORATION

1. Preparation of DNA Solution

Plasmid DNA constructs generally used for gene expression in mammalian cells can be applied using WEC-EP. In our experience, both CA (CMV enhancer + β-actin promoter) and EF-1 promoters work well. The LacZ or GFP construct is sometimes cotransferred for visualization of targeted areas. The constructs, however, should be tagged with FLAG, *myc*,

[2] For assessment of condition of cultured embryos, see Ref. (10).

TABLE 1

Culture Protocol and Optimal Oxygen Conditions[a]

	0 h	6 h	12 h	18 h	24 h	36 h	48 h	60 h	72 h
ICR mouse									
E8.0 (4-somite)	5% 50 cm³	5% 75 cm³	5% 100 cm³	5% 125 cm³	20% 50 cm³	60% 50 cm³	60% 75 cm³	OYS, 60% 50 cm³	→
E8.5 (10-somite)	5% 100 cm³	5% 125 cm³	20% 50 cm³	20% 75 cm³	60% 50 cm³	60% 75 cm³	OYS, 60% 50 cm³ (by 44 h)	60% 75 cm³	→
E9.5 (22-somite)	60% 50 cm³	→	60% 75 cm³	OYS, 60% 50 cm³	60% 75 cm³	95% 50 cm³	95% 75 cm³	→	
E9.5 w/OYS	20% 50 cm³	→	20% 75 cm³	60% 50 cm³	60% 75 cm³	95% 50 cm³	95% 75 cm³	→	
E10.5 (34-somite) w/OYS	60% 50 cm³	→	95% 50 cm³	→	95% 75 cm³	95% 100 cm³	→		
E11.5 w/OYS	95% 50 cm³	95% 75 cm³	95% 100 cm³	→	95% 125 cm³	→			
SD rat									
E9.5	5% 50 cm³	→	5% 75 cm³	→	5% 100 cm³	20% 50 cm³	60% 50 cm³	60% 75 cm³	→
E10.0 (4-somite)	5% 50 cm³	5% 75 cm³	5% 100 cm³	5% 125 cm³	20% 50 cm³	60% 50 cm³	60% 75 cm³	OYS, 60% 50 cm³	→
E10.5 (10-somite)	5% 100 cm³	5% 125 cm³	20% 50 cm³	20% 75 cm³	60% 50 cm³	60% 75 cm³	OYS, 60% 50 cm³ (by 44 h)	60% 75 cm³	→
E11.5 (23-somite)	95% 50 cm³	→	95% 75 cm³	→	OYS, 95% 50 cm³ (by 20 h)	95% 75 cm³	95% 100 cm³	95% 125 cm³	→
E11.5 w/OYS	60% 50 cm³	→	60% 75 cm³	→	95% 50 cm³	95% 75 cm³	95% 100 cm³	95% 125 cm³	→
E12.5 (34-somite)	OYS, 95% 50 cm³	95% 75 cm³	95% 100 cm³	→	95% 125 cm³	95% 150 cm³	→	→	

[a] Optimal oxygen conditions under WEC for mouse and rat embryos at various embryonic stages. OYS, open yolk sac.

or HA to confirm the expression of exogenous genes. DNA is purified by routine methods (e.g., cesium chloride, Qiagen Kit, Santa Clara, CA) and finally dissolved in sterilized phosphate buffered saline (PBS) at 2–5 $\mu g/\mu l$. Linearization seems unnecessary for transient expression. We usually add 1/10,000 Fast Green (Sigma Chemical Co., St. Louis, MO) for visualization of the solution within the neural tube. Fine glass needles are prepared from microcapillary tubes using a standard micropuller equipped with a heating element. The prepared needle is connected to a syringe pump via a fine silicone tube to inject the DNA solution.

2. Apparatus for Electroporation

Only electroporators that generate precise square pulses should be used. This cannot be obtained using a conventional electroporator. We use electroporation apparatus BTX T820 (square pulse generator) together with BTX 500 (optimizer), or Tokiwa Science CUY-21, which includes both the generator and optimizer. The exact configuration of electrodes suitable for electroporation varies according to the embryonic stage and targeting regions. For EP of the neural tube, chamber-type electrodes with a 1.5- or 2-cm gap (Fig. 3) are most commonly used. [Refer to Mr. Yasuhiko Hayakawa at TR Tech Company (Fax: +81-3-3944-6196) for various types of electrodes.] Electrodes should be disinfected by three washes with 70% ethanol just prior to EP.[3]

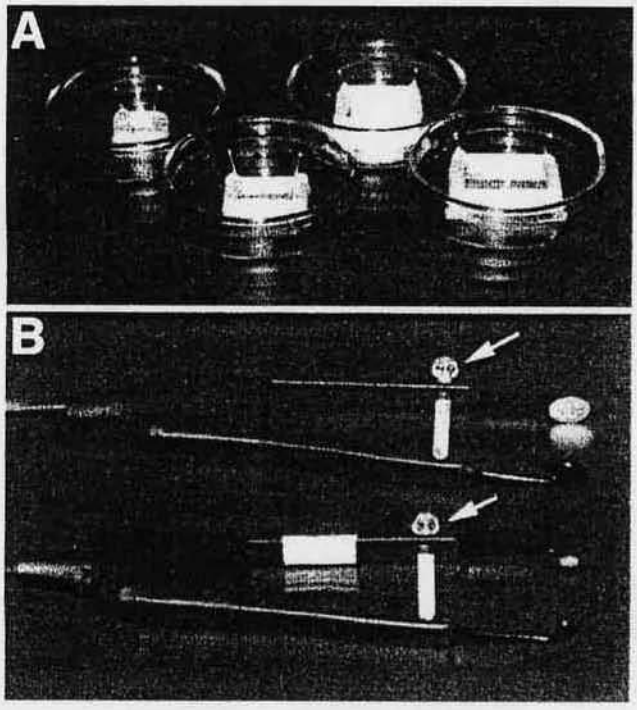

FIG. 3. Electrodes (**A**) Chamber-type electrodes. Electrodes made of platinum are composed together with silicon blocks to prepare the chamber using a glass dish. The distance between electrodes should be between 1.5 and 2.5 cm according to developmental stage (see text). (**B**) Tweezer-type electrodes. To restrict the targeted area, tweezer-type electrodes are very useful. Small electrodes (1–5 mm in tip diameter) made of platinum are arranged so as to flank the embryo at a determined distance by a fixed spacer (arrow). This type of electrode can be used for embryos at any developmental stage.

3. Electroporation

For EP in the neural tube of E11.5 rat embryos, embryos are taken from the uterus, and if possible, their yolk sac membrane is opened for easy access to the neural tube. Preincubation of the embryo for 1–2 h before EP is preferable. It has been our experience that the electric shock delivered during EP seems to cause more damage to fresh embryos than those preincubated. After transferring one embryo from the WEC apparatus to the electrode chamber, the DNA solution is injected into the desired region of the neural tube using the microcapillary tube/pump system (Fig. 4). The volume of the injectate varies according to the developmental age, but is usually less than 0.5 μl. Immediately after injection of the DNA material, the embryo is transferred to a chamber containing Tyrode's solution or PBS and placed at the desired position to target the area within the neural tube. During application of the electric shock, the DNA will migrate from the cathode to the anode. When a stable position cannot be provided, agarose gel is prepared with a hole in the middle, in which the embryo is placed to orient it in the right position.

Electrical pulses are delivered at optimal conditions, which vary according to developmental age. For example, we use BTX square pulse apparatus (see above) and deliver the following pulses for E10.5–E11.5 rat embryos: 80–100 V, 50 ms, 5 pulses at 1-s intervals. A higher voltage results in a higher efficiency of gene transfer, but the generated radicals may damage the embryo. A patchy (or clonal) transfection can be achieved by using a low voltage.

[3] For the theoretical basis of EP technique, see article by Nakamura and Funahashi and Refs. (11, 12).

The electroporated embryo is rinsed with Tyrode's solution (or PBS) and then returned to the culture apparatus. The solution in the chamber electrode should be changed after each EP.

Based on preliminary studies using the pEF-1/lacZ or pCA/GFP construct, we found that expression starts 4–6 h after electroporation and seems to peak after 24 h (Fig. 5), followed by a gradual decrease

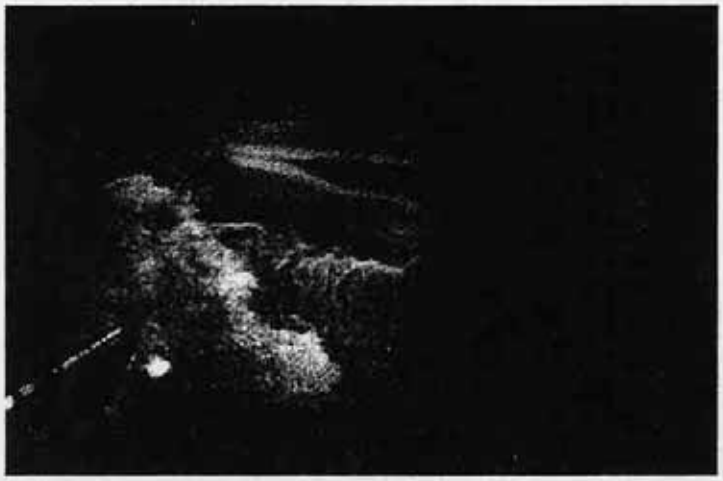

FIG. 4. DNA solution injected into the neural tube under a dissecting microscope. This E11.5 rat embryo is injected with the solution (blue colored) in the hindbrain after preincubation for 2 h.

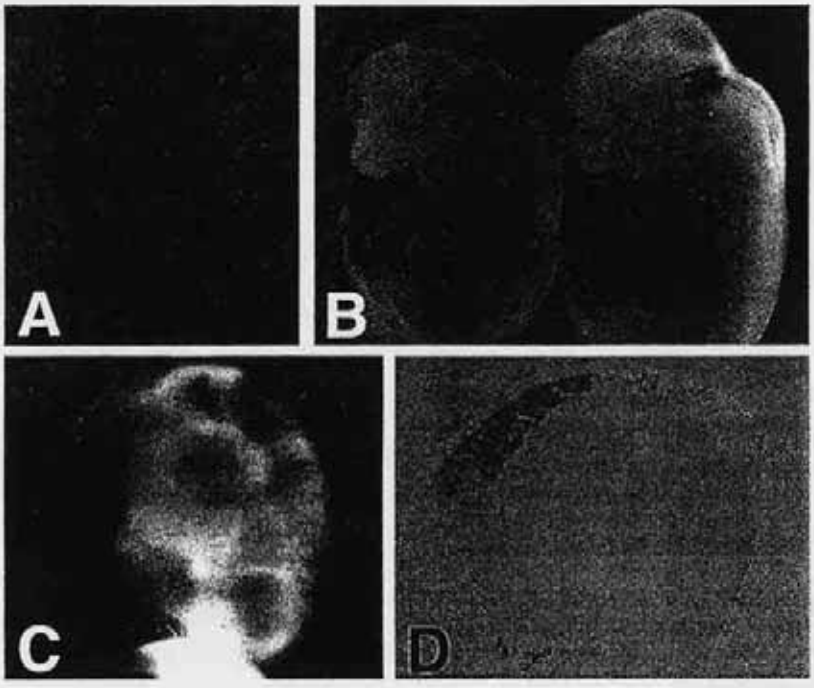

FIG. 5. Detection of transferred genes. (**A**) pCAX/GFP construct is targeted to the lateral side of the hindbrain of E11.5 rat embryo and gene expression is clearly seen after culture for 24 h. (**B**) pEF/LacZ construct is targeted to the lateral hindbrain of E12.5 rat embryos and gene expression is maintained after culture for 48 h. (**C**) pEF/LacZ construct is targeted to the dorsal midbrain of E9.5 mouse embryo; expression is maintained after 20 h of culture. (**D**) Transverse section of the embryo shown in (**C**) at the midbrain level. Note that expression of *lacZ* is close to 100% in the targeted area.

due to the high proliferation of embryonic cells. Within the targeted area, the proportion of transfected cells varied from 10% to nearly 100% (Fig. 5) at 20–24 h depending on the test conditions (e.g., DNA concentration, voltage, duration of EP, and pulse number).

CONCLUDING REMARKS

Gene transfer into embryos using EP-WEC is a powerful tool for studies of mammalian development at the molecular level. Application of the present method helps improve our understanding of embryonic development during midgestation (E7–E12 in mice), e.g., alteration of gene function via ectopic expression, interference by the use of dominant negative-proteins, and fate mapping with marker genes. In addition, this method complements genetic approaches such as the generation of knockout and transgenic mice, which cannot be executed using amphibian or avian embryos. Virus-mediated gene transfer techniques can also be applied to cultured mammalian embryos. In addition to the above uses, EP offers the following advantages: (1) less time lag for initiation of transcription, (2) less labor to prepare DNA solution, (3) most precise targeting of areas, and (4) potentially less toxicity for human beings.

It is also possible to apply EP to the analysis of promoters for time- and space-specific expression of genes, thus complementing conventional transgenic analyses. Disadvantages of EP include dilution of the template DNA due to the high cellular proliferation, making it impossible to maintain gene expression. This point is of minor importance, however, because in WEC, embryos cannot be maintained longer than 3 days. This limitation is due primarily to moderate growth of the allantoic placenta *in vitro*.

EP itself can also be combined with other techniques. For example, fragments of embryonic tissues or dissociated cells can be isolated from embryos after EP. Such explant/cell culture systems can be used for more advanced embryos that are not suitable for WEC. Moreover, EP can be performed under *ex utero* manipulation (13), which allows fetuses to develop to full term *in utero*. Experimental studies using these manipulation systems combined with other molecular tools are expected to contribute to a better understanding of mammalian development.

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REFERENCES

1. New, D. A. T. (1990) *in* Mammalian Postimplantation Embryos: A Practical Approach (Copp, A. J., and Cockroft, D. L. Eds.), pp. 1–14, IRL Press, Oxford.
2. New, D. A. T., and Cockroft, D. L. (1979) *Experientia* **35,** 138–140.
3. New, D. A. T. (1978) *Biol. Rev.* **53,** 81–122.
4. Cockroft, D. L. (1990) *in* Mammalian Postimplantation Embryos: A Practical Approach (Copp, A. J., and Cockroft, D. L., Eds), pp. 15–40, IRL Press, Oxford.
5. Steele, C. E., and New, D. A. T. (1975) *J. Embryol. Exp. Morphol.* **31,** 707–719.
6. Eto, K., and Osumi-Yamashita N. (1995) *Dev. Growth Differ.* **37,** 123–132.
7. Morris-Kay, G. M. (1993) *in* Essential Developmental Biology, a Practical Approach (Stern, C. D., and Holland, P., Eds), pp. 55–66IRL Press, Oxford.
8. Cockroft, D. L. (1973) *J. Embryol. Exp. Morphol.* **29,** 473–483.
9. New, D. A. T., and Coppola, P. T. (1977) *J. Embryol. Exp. Morphol.* **37,** 227–235.
10. Brown, N. A., (1990) *in* Mammalian Postimplantation Embryos: A Practical Approach (Copp, A. J., and Cockroft, D. L., Eds.), pp. 93–108, IRL Press, Oxford.
11. Muramatsu, T., Mizutani, Y., Ohmori, Y., and Okumura, J. (1997) *Biochem. Biophys. Res. Commun.* **230,** 376–380.
12. Muramatsu, T., Shibata, O., Ryoki, S., Ohmori, Y., and Okumura, J. (1997) *Biochem. Biophys. Res. Commun.* **233,** 45–49.
13. Muneoka, K., Wanek, N., Trevino, C., and Bryant, S. V. (1990) *in* Mammalian Postimplantation Embryos: A Practical Approach (Copp. A. J., and Cockroft, D. L., Eds.), pp. 41–59, IRL, Press, Oxford.
14. Lee, Y. M., Osumi-Yamashita, N., Ninomiya, Y., Moon, C. K., Erickson, U., and Eto, K. (1995) *Development* **121,** 825–837.
15. Matsuo, T., Osumi-Yamashita, N., Noji, S., Ohuchi, H., Koyama, E., Myokai, F., Matsuo, N., Taniguchi, S., Doi, H., Iseki, S., Ninomiya, Y., Fujiwara, M., Watanabe, T., and Eto, K. (1993) *Nat. Genet.* **3,** 299–304.
16. Osumi-Yamashita, N., and Eto, K. (1997) *Int. J. Dev. Biol.* **41,** 187–194.

17. Osumi-Yamashita, N., Ninomiya, Y., Doi, H., and Eto, K. (1994) *Dev. Biol.* **164,** 409–419.
18. Imai, H., Osumi-Yamashita, N., Ninomiya, Y., and Eto, K. (1996) *Dev. Biol.* **176,** 151–165.
19. Imai, H., Osumi, N., and Eto, K. (1998) *Eur. J. Oral Sci.* **106** (Suppl. 1), 19–23.
20. Osumi-Yamashita, N., Asada, S., and Eto, K. (1992) *J. Craniofac. Genet. Dev. Biol.* **12,** 130–140.