

Mini-Review

Misexpression of genes in brain vesicles by *in ovo* electroporation

Harukazu Nakamura,* Yuji Watanabe and Jun-ichi Funahashi

Department of Molecular Neurobiology, Institute of Development, Aging and Cancer, Tohoku University, Seiryomachi 4-1, Aoba-ku, Sendai 980-8575, Japan.

Transfection to living chick embryos *in ovo* by electroporation has been recently developed. In this mini-review, misexpression in brain vesicles is introduced. To transfect, expression plasmid is inserted in the brain vesicle, and the square pulse of 25 V, 50 ms was charged five times. The translation product of the transfected gene is detected 2 h after electroporation, and reaches the peak at 24 h after electroporation. Transfection is so effective that this method is contributing greatly to the study of the molecular mechanisms of morphogenesis.

Key words: chick embryos, electroporation, *in ovo*, transfection.

Introduction

In the field of experimental embryology, chick embryos are good materials because it is easy to access the embryo and manipulate it. But these days, the importance of chick embryos as experimental material for embryology has decreased because: (i) it is very difficult to grow inbred strains; and (ii) the embryos stay in the uterus for the first 24 h of the embryonic period, which makes it difficult to produce transgenic chickens or to knock out certain genes. Recently, we improved the electroporation method to transfer certain genes to chick embryos *in ovo* (Muramatsu *et al.* 1997; Funahashi *et al.* 1999). By this method, very rapid and effective expression of the introduced gene can be obtained. In this minireview, we briefly describe the method used for gene transfer into brain vesicles.

Procedure for *in ovo* electroporation

Plasmids Usually, cDNA was inserted into the expression vector, pMiwSV, which had RSV (Rous Sarcoma virus) enhancer and β -actin promoter (Suemori *et al.* 1990; Wakamatsu *et al.* 1997). pRc/CMV (Invitrogen Co., Carlsbad, CA, USA), which had CMV (cytomegalovirus) enhancer, can also be used as an expression

vector. Both work well as expression vectors in chick embryos.

Injection of DNA solution Embryos were incubated until they reached the desired stage. For transfection to the mesencephalon, stage 10 (Hamburger & Hamilton 1951; 36 h incubation) embryos are usually used. A window of about 2 cm in diameter should be opened on the top of the egg after removing 4 mL of albumen. Injection of Indian ink underneath the embryo facilitated visualization of it (Fig. 1C). The head region of an embryo was exposed by cutting the vitelline membrane with a microscalpel. To ensure that the injected DNA stays in the lumen of the neural tube, it is recommended to cut the most anterior part of the neural tube with a microscalpel. Then, DNA solution was injected with a micropipette into the lumen of the neural tube (Fig. 1D). If the anterior tip of the central canal is closed, injected DNA solution comes out when one pulls the pipette out of the neural tube. To transfect the mesencephalic region, a micropipette was inserted from the metencephalon anteriorly (Fig. 1D), and the DNA solution was injected. For this process, the micropipette was put in the hematocrit tube, and injection of the solution was controlled by mouth. Micropipettes for the injection of DNA into the embryo were made of glass capillary tubes 1 mm in diameter (GD-1; Narishige, Tokyo, Japan). About 0.1–0.2 μ L of 1 μ g/ μ L DNA solution in TE (Tris-EDTA) buffer or in phosphate-buffered saline (PBS) was injected.

Electroporation Electrodes (0.5 mm in diameter with an exposed length of 1 mm) set on a micro-manipulator (MN-151; Narishige (Fig. 1A,B)) were put

*Author to whom all correspondence should be addressed.

E-mail: nakamura@idac.tohoku.ac.jp

Received 27 September 1999; revised 24 December 1999; accepted 24 December 1999.

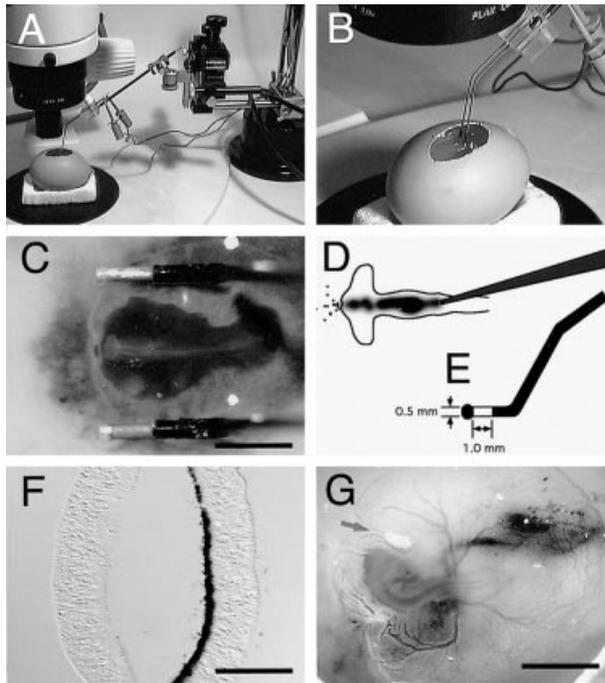


Fig. 1. *In ovo* electroporation. A pair of electrodes held by a manipulator (A) was inserted from a window opened on the shell (B). The electrode was put on the vitelline membrane overlying the embryo (C), and a 25 V, 50 ms pulse was charged five times. Plasmid solution was injected into embryonic stage (E2; HH stage 10) chick neural tube (D) prior to pulse charge. Dimensions of the electrode are shown schematically in (E). Most of the electrode was insulated (black in figure) so that only the tip was exposed (white area). The cells in the neural tube look normal 1 h after electroporation (F). Deposits on the right-hand side of the neural tube are a complex of plasmids and the color substrate that was not removed by washing in dimethylformamide. Twenty-four hours after electroporation, the development of yolk sac plexus, vitelline veins and vitelline arteries was retarded in the area in contact with the electrodes (arrows in (G)). Bar, 2 mm (C), 50 μ m (F), 4 mm (G).

on the vitelline membrane at a distance of 4 mm apart (Fig. 1C), and a small volume of Hanks' solution was placed between the electrodes. Then the square pulse (25 V, 50 ms) was charged five times. Pulses were generated every 1 s so that a pulse of 50 ms was followed by a 950 ms rest phase. Pulses were generated by Electroporator T820 and Optimizer™ (BTX, San Diego, CA, USA), or by CYU 21 (Tokiwa Science, Tsukushino-city, Fukuoka, Japan).

Resistance between the electrodes was dependent on the volume of Hanks' solution, but the current through the embryo may be constant if the distance between the electrodes is fixed. After electroporation, the window was sealed with Scotch tape, and the embryos reincubated at 38°C.

As DNA is negatively charged, DNA moved toward the anode in the electric field so that the anode side of the tissue was transfected. In this sense, the neural tube is a very convenient tissue to use for applying *in ovo* electroporation. The DNA solution was easily injected into the canal of the neural tube, and stayed there. The cathode side served as a control so that we could compare the effects of transfection in the same embryo. The electric charge in our experimental conditions did not injure the cells of the neural tube (Figs 1F, 2E). Only places where the electrodes were directly attached were damaged so that vascularization was affected to some extent, which may be the main cause of embryonic death (Fig. 1G). We found the above conditions optimum for transfection to the mesencephalon at around stage 10 for chick embryos. If the distance between the electrodes was shorter, a lower voltage can produce similar transfection efficiency. To do so, however, we had to put the electrodes near the embryo, which interfered with

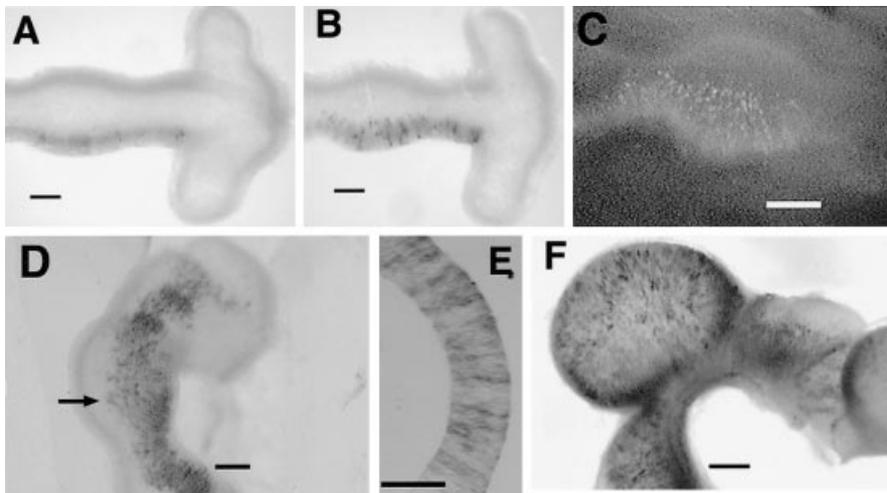


Fig. 2. Efficiency of electroporation. Efficiency of *in ovo* electroporation was assessed by injecting *lacZ* expression vector (pMiwZ), or by green-fluorescent protein (GFP) expression vector (pEGFP-N1) at stage 10. The *lacZ* signal was already recognizable 2 h after electroporation (A), and became strong 3 h after electroporation (B). Efficiency of the transfection of the introduced gene can be assessed *in ovo* by coelectroporation with GFP vector (C); 9 h after electroporation). Twenty-four hours after electroporation (D, E), more than half of the cells expressed *lacZ* at the

transfection zone. The expression was transient, but the *lacZ* signal was still strong 72 h after electroporation (F). The *lacZ* transfection exerted no morphological effects. Arrow in (D) indicates the section in (E). Bars: 200 μ m (A–D, F), 50 μ m (E).

subsequent vascular development, and caused more embryonic death. Widening of the distance between the electrodes reduced transfection efficiency.

Expression of introduced DNA

Expression of introduced genes was checked by pMiwZ, which encoded *lacZ* reporter. The *lacZ* translation product was detectable 2 h after electroporation (Fig. 2A). More cells express *lacZ* 3 h after electroporation (Fig. 2B). The *lacZ* signal reached peak intensity around 20 h after electroporation (Fig. 2D; Momose *et al.* 1999). More than 50% of cells were transfected (Fig. 2E) in the transfection zone. The En-2 translation product was also detected by 2 h after electroporation, and repression of *Pax6* by introduced En-2 was already detectable by 3 h after electroporation in the diencephalic region (Araki & Nakamura 1999).

The plasmid we used was not integrated into the host chromosome so that expression was transient. Expression level decreased from about 20 h after electroporation. As the *lacZ* translation product is rather stable, we detected the product 72 h after electroporation.

It was shown that coelectroporation of *lacZ* and GFP vector results in the overlapped patterns of the two genes (Momose *et al.* 1999). Co-injection of green-fluorescent protein (GFP) expression plasmids with the DNA of interest made it possible to monitor *in ovo* the transfection efficiency under a fluorescence dissection microscope (Fig. 2C). We used commercially available pEGFP-N1 (Clontech; Palo Alto, CA, USA) for this purpose.

Application for tissue-specific introduction is described by Momose *et al.* (1999). This technique is extensively applied to study the function of certain genes and the gene expression cascade in develop-

ment (Ogino & Yasuda 1998; Araki & Nakamura 1999; Funahashi *et al.* 1999; Momose *et al.* 1999; Okafuji *et al.* 1999; Takeuchi *et al.* 1999; Katahira *et al.* 2000).

References

- Araki, I. & Nakamura, H. 1999. *Engrailed* defines the position of dorsal di-mesencephalic boundary by repressing diencephalic fate. *Development* **126**, 5127–5135.
- Funahashi, J., Okafuji, T., Ohuchi, H., Noji, S., Tanaka, H. & Nakamura, H. 1999. Role of *Pax-5* in the regulation of a mid-hindbrain organizer's activity. *Develop. Growth Differ.* **41**, 59–72.
- Hamburger, V. & Hamilton, H. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92.
- Katahira, T., Sato, T., Sugiyama, S. *et al.* 2000. Interaction between *Otx2* and *Gbx2* defines the organizing center for the optic tectum. *Mech. Dev.* (in press).
- Momose, T., Tonegawa, A., Takeuchi, J., Ogawa, H., Umesono, K. & Yasuda, K. 1999. Efficient targeting of gene expression in chick embryos by microelectroporation. *Develop. Growth Differ.* **41**, 335–344.
- Muramatsu, T., Mizutani, Y., Ohmori, Y. & Okumura, J-I. 1997. Comparison of three non-viral transfection methods for foreign gene expression in early chicken embryos *in ovo*. *Biochem. Biophys. Res. Commun.* **230**, 376–380.
- Ogino, H. & Yasuda, K. 1998. Induction of lens differentiation by activation of a bZIP transcription factor, L-Maf. *Science* **280**, 115–118.
- Okafuji, T., Funahashi, J. & Nakamura, H. 1999. Roles of *Pax-2* in initiation of the chick tectal development. *Dev. Brain Res.* **116**, 41–49.
- Suemori, H., Kadokawa, Y., Goto, K., Araki, I., Kondoh, H. & Nakatsuiji, N. 1990. A mouse embryonic stem cell line showing pluripotency of differentiation in early embryos and ubiquitous beta-galactosidase expression. *Cell Differ. Dev.* **29**, 181–186.
- Takeuchi, J. K., Koshiba-Takeuchi, K., Matsumoto, K. *et al.* 1999. *Tbx5* and *Tbx4* genes determine the wing/leg identity of limb buds. *Nature* **398**, 810–814.
- Wakamatsu, Y., Watanabe, Y., Nakamura, H. & Kondoh, H. 1997. Regulation of the neural crest fate by N-myc: Promotion of ventral migration and neuronal differentiation. *Development* **124**, 1953–1962.