In Vivo Electroporation : A Convenient Method for Gene Transfer to Testicular Cells in Mice

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Abstract In the present study, transfection efficiency of the chloramphenicol acetyltransferase reporter gene was compared in mouse testicular cells by utilizing lipofection, electroporation (EP) and microparticle bombardment (MPB) techniques in both in vivo and in vitro environments. In addition, in vivo EP was conducted to attain spatial expression of the bacterial β-galactosidase reporter gene in the testis of living mice. Altogether three experiments were conducted with male ICR strain mice at 4 weeks of age. The results showed that among the three different transfection methods employed, in vivo EP and in vivo MPB were the most efficient. When the in vivo EP technique was used to transfect the bacterial β-galactosidase reporter gene, spatial gene expression was clearly demonstrated by X-gal staining in the mouse testis where among X-gal stained cells, some spermatocyte- or spermatid-like cells were also found. By taking account of maximum amounts of transfactable DNA, the extent of tissue damage, and transfection efficiency, it was concluded that in vivo EP would provide a powerful and useful means to transfer foreign genes to testicular cells of living mice.


Key words : In vivo electroporation, Gene transfer, Mouse testis, CAT, LacZ

In the past, most studies on gene transfer were conducted in cultures of somatic or germ cells simply because no in vivo gene transfection method was available. Undoubtedly, normal cell functions are not perfectly maintained in cell cultures in vitro for a long period of time since optimization of numerous factors that affect cell growth and differentiation is hardly attained as inherently achieved in vivo.

Recent progress in gene therapy technology offers, however, several means of in vivo gene transfer including virus mediated transfection, lipofection, microparticle bombardment and direct injection\(^3\). Of these, the most successful method has been the biological means, i.e. the use of viral vectors which may give rise to high risk of biohazard. In contrast, physical and chemical methods that are considered to be relatively safe are suffered from low efficiency of gene transfection.

A solution to this problem may lie in the use of either in vivo electroporation (EP) or in vivo microparticle bombardment (MPB). By in vivo EP, DNA as well as antitumor drugs and antibodies was found to be delivered to cells of interest in animals in vivo\(^1,7,9,11,13\). Similarly, in vivo MPB was also utilized to successfully transfer foreign genes to several tissues of living animals\(^6,10,14,23\). Although the both methods are simple and applicable to virtually any tissues in vivo, the former may be more useful because there is no limitation of trans-
fectable DNA dose, and of the transfectable location within the tissue such as the depth from the surface.

In the present study, gene transfection efficiency of EP was compared with that of lipofection and MPB in both in vivo and in vitro environments in mouse testicular cells. In addition, in vivo EP, which was deemed the most efficient and convenient method, was used to attain spatial expression of the bacterial β-galactosidase reporter gene in the testis of living mice.

Materials and Methods

Animals and Cells

As experimental animals, ICR strain male mice at 4 weeks of age were obtained from a local supplier (Chubu Kagaku Co. Ltd., Hamamatsu, Japan), and used in all experiments. They were cared for under Guideline of Animal Experimentation, laid down by the Committee of Experimental Animal Care, Nagoya University, Nagoya, Japan. Altogether three experiments were conducted. Firstly in experiment 1, the effect of heating treatment on the sensitivity of chloramphenicol acetyltransferase (CAT) determination was investigated in the testis to which a reporter plasmid DNA was transfected by in vivo EP. In experiment 2, gross comparison of transfection efficiency was done by three different methods in vivo and in vitro. They were lipofection11), EP1,7,9,11,13) and MPB2). For this purpose, the dose of plasmid DNA to be transfected was adjusted to 5μg per one testis for both in vivo and in vitro environments. Finally in experiment 3, the bacterial lacZ gene was transfected by the in vivo EP, and the spatial expression of the gene in the testis of living mice was investigated.

In vivo DNA Transfection

For in vivo EP, skin surrounding the testis was excised under the light anaesthesia, and the testis was exposed. Plasmid DNA at 1μg per testis in experiment 1, 5μg per testis in experiment 2 and 30μg per testis in experiment 3 was dissolved in 50μl TE buffer (10mM Tris, and 1mM EDTA, pH adjusted to 7.5), and injected into the left testis with a 1ml syringe and a 27G needle. Immediately after the injection, square electric pulses were applied eight times with an electro–square porator T 820 in combination with an optimizor 500 (BTX, San Diego, USA) at 25 V with a time constant of 50 msec. After the in vivo EP, the skin was stitched, and the mice were maintained for the following 48 hrs.

For in vivo MPB5,6,23,24), similar surgical procedures were carried out to expose the testis. Subsequently, a small area of the testicular capsule about 2×2 mm was excised. In contrast to in vivo EP, this removal procedure was necessary for in vivo MPB as the DNA-coated particle could not reach beyond 2–3 mm deep from the surface of the tissue23). In in vivo MPB, 3 mg tungsten with 5μg DNA was shot with Machimpacter (Kansai Paint, Osaka, Japan) at the capsule-removed target area by the pressure of nitrogen gas at 80 kgf/cm² at a distance of 3 cm from the nozzle. After the in vivo MPB, the skin was stitched, and the mice were then maintained for the subsequent 48 hrs.

For in vivo lipofection12), 5μg DNA was mixed with 15μl of Lipofectamine (Gibco-BRL, USA), and diluted in total of 50μl with phosphate buffered saline (pH 7.4). The solution was directly injected into the left testis without any surgical treatment. The mice were then maintained for the following 48 hrs.

In vitro DNA Transfection and Primary Cultures of Testicular Cells

For in vitro gene transfer experiments, primary cultures of mouse testicular cells were used. Testes were excised quickly after the mice were sacrificed by decapitation, and placed onto a plastic dish containing approximately 2 ml F12 solution prewarmed at 33°C. Testicular membrane and extraneous connective tissue were trimmed off, and remaining
blood in the testis was washed. The testes were then transferred to a new plastic dish with approximately 2 ml fresh F12 solution, and minced into small pieces with scissors. Ten to fifteen minced testis samples were pooled, and transferred to a 125 ml flask to which 20 ml dissociation medium was added. The dissociation medium contained 0.17 mg/ml collagenase, 20 mg/ml trypsin, 25 mg/ml DNase and 0.19 U/ml protease in F12. Dissociation was conducted in an orbital shaking incubator at 33°C at 200rpm for 30 min with mechanical dissociation by pipetting at 10-min intervals. After dissociation, the cell suspension was centrifuged at 80×g at room temperature for 10 min, the supernatant was removed, and the cell pellet was resuspended in transfection medium which contained 5% charcoal-stripped horse serum, 5 U/ml streptomycin, and 250 ng/ml fungizone in F12.

In vitro DNA transfection was attempted by the same methods as in in vivo transfection: lipofection\textsuperscript{12) was done with Lipofectamine (Gibco-BRL, Grand Island, NY, USA), EP\textsuperscript{21) with Gene Pulsér Apparatus (Bio-Rad, Hercules, CA, USA), and MPB\textsuperscript{22) with Machimpacter. Then, the transfected cells (1×10^6 cells) were transferred into 35 mm culture dishes containing 3 ml of F12 and Dulbecco’s modified Eagle’s medium at the ratio of 1:1 with 0.1% bovine serum albumin, 5 U/ml penicillin, 5 mg/ml streptomycin, 250 ng/ml fungizone and 50 ng/ml insulin, and cultured under a gas phase of 5% CO₂ and 95% air at 33°C for 48 hrs.

Plasmid DNAs

The plasmid DNAs used were pCAT control (Promega, Madison, WI, USA) for expressing CAT activities in experiments 1 and 2, and pmiwZ obtained from Japanese Cancer Research Resources Bank (No. VE052) for histochemical staining of testicular cells\textsuperscript{19).}

Assays

For analyzing the in vivo transfected samples, the mice were sacrificed by decapitation at 48 hrs after the transfection, and the testis samples were excised quickly, and weighed. Whole testis samples were then homogenized with three volumes of buffer A (15 mM Tris, 60 mM KCl, 15 mM NaCl, 12 mM EDTA, 1 mM dithiothreitol, 0.15 mM spermine and 0.4 mM phenylmethylsulfonyl fluoride, pH adjusted to 8.0) followed by heating treatment at 60°C for up to 120 min (experiment 1) or for 60 min (experiment 2) in order to increase the sensitivity as suggested\textsuperscript{16). The possible reason for the increased sensitivity would be that the heat treatment inactivates a number of enzymes which would compete for the acetyl-CoA as substrate for enzymatic reactions. The homogenate was centrifuged at 6000×g for 11 min, and the supernatant was used for measurements of protein content by using a Bradford assay kit (Bio-Rad, Hercules, CA, USA) and CAT activity as described previously\textsuperscript{17,22).}

For detection of the bacterial β-galactosidase activity in the transfected testes in experiment 3, histochemical staining was used. The testes were fixed with 4% glutaraldehyde solution in phosphate buffered saline (PBS) at room temperature for 1 hr, and rinsed with PBS twice. Soaking in the X-gal reaction mixture (pH adjusted to 7.6, 0.1% X-gal, i.e. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5% N,N-dimethylformamide and 0.1% Triton-X dissolved in a buffer composed of 2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 3 H₂O and K₄Fe(CN)₆ in PBS) was conducted for less than 1 hr to minimize endogenous greenish-blue color development in the entire testis samples. Light microscopic examination was done by embedding the testis in paraffin for cross sectioning. In some sectioned preparations, co-staining with hematoxylin was also performed.

The measurement of CAT activities in in vitro samples in experiment 2 was done essentially in the same way as in in vivo samples. At the end of the culture period for 48 hrs, the cells were harvested, washed with phosphate buffered saline, sonicated, heat-treated and
centrifuged at 6000×g for 11 min to obtain cell extract. The extract was then used for measurements of protein content and CAT activity as in experiment 1.

**Statistical Analysis**

The data were treated statistically by analysis of variance, and significance of differences between means was tested by a Duncan's multiple range test by using General Linear Model Procedures. Where necessary the data were transformed to their natural logarithmic values to stabilize error variance.

**Results and Discussion**

Figure 1 represents the effect of heating period on CAT activity in the testis to which DNA was transfected by in vivo EP. A significant increase by approximately 30% was observed in the CAT activity due to prolonged heating treatment from 7 to 30 min (P<0.05), and no further improvement was found for the longer period up to 120 min. The extent to which sensitivity of CAT assay was improved by the combination of prolonged heating treatment with the use of buffer A was reported to range from 2 to 100 folds in a variety of cell types and tissues. In the present study, however, no such remarkable enhancement was detected, but only to a modest extent. The reason for this poor improvement was unknown, but the heating treatment alone might not predominantly contribute to the improved sensitivity. Under the conditions tested, heating period longer than 30 min was considered to be enough so that in the next experiment, 60-min heating was employed.

Gross comparison of transfection efficiency between the three different methods in vivo and in vitro is shown in Fig. 2 where the mean values were calculated only from CAT positive samples. The numbers of CAT positive to total samples were: for in vivo methods, 3/20 (15%, lipofection), 10/18 (55%, MPB) and 8/8 (100%, EP); for in vitro methods, 3/22 (14%, lipofection), 5/58 (9%, MPB) and 4/38 (11%, EP).

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**Fig. 1.** The effect of heating period on CAT activities in the mouse testis to which plasmid DNA, pCAT control, was transfected by the in vivo electroporation method at a dose of 1μg per testis. The CAT gene expression was determined at 48 hrs after the transfection. a, b Significantly different at P<0.05.

**Fig. 2.** Relative CAT activities expressed by taking the in vitro lipofection value as 1 in the mouse testicular cells. Plasmid DNA, pCAT control, was transfected by three different methods in vivo and in vitro at a dose of 5μg per testis. Vertical bars represent means±SEM of 3 to 10 replicates. a, b, c Significantly different at P<0.01. The CAT values for in vivo and in vitro lipofection, and in vitro electroporation were so low that they were virtually negligible compared with those in other groups.
The highest CAT activity was found by in vivo MPB and in vivo EP, followed by in vitro MPB in the decreasing order (P<0.01). The CAT values obtained by other three methods were considerably lower than these three methods (P<0.01), and virtually negligible.

In in vitro cell culture studies, DNA transfection to spermatogenic cells has been humpered by the lack of a suitable method. The calcium phosphate method which is most commonly used gave better transfection efficiency than lipofection or EP in cultured mouse spermatogenic cells (Nakatsuji, personal communication). The same results were obtained with the calcium phosphate method in our preliminary experiment (unpublished). However, the calcium phosphate method resulted in a drastic reduction in the cell viability: more than 80% of the cultured mouse spermatogenic cells died within 5 days. Thus, as an alternative means, lipofection, EP and MPB were tested in vitro as well as in vivo in the present study.

The poor transfection efficiency and low CAT activity by the in vitro lipofection method were not surprising since it was found that in cultured mouse spermatogenic cells, CAT gene transfection efficiency by the lipofection method was almost undetectable, only at 1.4%. The most striking contrast between the in vitro and in vivo methods was seen in the EP method, virtually no activity in vitro and the highest activity in vivo. One of the reason for explaining this difference would be that the culture condition of the mouse spermatogenic cell is far from satisfactory so that the cells are not actively transcribing genes. In fact, no good in vitro culture systems have been developed, in that complete spermatogenesis of the mouse germ cells from spermatogonia to sperms is achieved.

Although the transfected DNA dose was adjusted to 5μg per one testis, the loss of cells during cell dissociation procedures for in vitro experiments was not accounted for. In this sense, therefore, cells in in vitro culture might have exposed to higher concentration of DNA than those in the testis in vivo. Apart from the difficulty of adjusting DNA dose, the environments in which testicular cells were maintained were quite different among in vivo and in vitro methods. Especially, primary cultures in vitro might not have been optimal for the testicular cells as aforementioned. Therefore, it should be kept in mind that the comparison made here was merely rough and approximate. Nevertheless, the fact that the proportion of CAT positive samples showed a similar trend towards the intensity of CAT values among the methods tested suggests that in vivo EP and in vivo MPB may probably be the most efficient methods for transferring genes to mouse testicular cells. Besides the efficiency, both in vivo methods are much simpler than in vitro cell culture methods that require cell dissociation, and optimization of medium and culture conditions.

By taking account of practical application, in vivo EP would be more suitable than in vivo MPB for the following reasons. Firstly, according to our experience10,14) bleeding and hence possible tissue damage is less in in vivo EP than in vivo MPB since the latter utilizes high nitrogen gas pressure to launch DNA-coated microparticles. Secondly, in in vivo EP there was no limitation of DNA to be transfected. Although only 30μg was used by in vivo EP in experiment 3, in theory, several mg DNA could be transfected in one site at a time. On the other hand, in vivo MPB the maximum amount of plasmid DNA loaded onto an aluminium bullet, and thereby shot at a time in the present MPB apparatus was limited only to 5-10μg. Finally, DNA could be transferable to cells deep inside the target tissue by in vivo EP, which was not possible by in vivo MPB : the latter method allows the DNA transfer only 2 to 3 mm deep from the surface of the tissue21). Thus, in experiment 3, in vivo EP was employed to demonstrate spatial expression of the bacterial β-galactosidase gene in the testis of

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living mice.

In Fig. 3, the results of X-gal staining are presented. In macroscopic examination (A, \( \times 50 \)), some parts of seminiferous tubules were stained blue as indicated by an arrow, suggesting that the gene expression of the bacterial \( \beta \)-galactosidase was attained. Whether or not the foreign gene expression was exerted in some spermatogenic-like cells was examined in the cross sectioned preparations co-stained with or without hematoxylin (B, co-stained with hematoxylin, \( \times 200 \); C, Nomarsky optics, \( \times 200 \); D, Nomarsky optics, \( \times 400 \)). In Fig. 3B, counter-staining with hematoxylin suggested that among cells exerted blue color in cytosol, a cell located close to the basement membrane (indicated by an arrow) had a large round shape, indicating the possibility of lacZ positive spermatocyte or spermatogonium. Cross section of Nomarsky optics (Fig. 3C) implicated the existence of elongated spermatid- or sperm-like cells that were lacZ positive. In the larger magnification of a similar, but different section with Nomarsky optics (Fig. 3D), clearly demonstrated were the lacZ positive elongated cells that appeared to have flagellum. All these results suggest that the \textit{in vivo} EP might be able to transfer foreign genes to spermatogenic cells, although no further confirmation was attempted in the present study.

\textbf{Fig. 3.} X-gal staining of the mouse testis to which plasmid DNA, pmiwZ, was transfected by \textit{in vivo} electroporation method at a dose of 30\( \mu \)g per testis. The cells expressing the bacterial \( \beta \)-galactosidase were stained blue. A ; X-gal stained seminiferous tubules of the mouse testis (\( \times 50 \)). The foreign gene expression was clearly shown in several sites, one of which was indicated by an arrow. B, C, D ; Microscopic appearance of the cross section of the \( \beta \)-galactosidase expressing convoluted seminiferous tubules with (B, \( \times 200 \)) or without hematoxylin counter-staining (Nomarsky optics ; C, \( \times 200 \); D, \( \times 400 \)). In B, a lacZ-positive spermatogonium- or spermatocyte-like cell was indicated by an arrow.
For demonstrating the spermatid- or spermatocyte-specific foreign gene expression, reporter genes fused to specific gene promoters such as protamin-1\textsuperscript{13,20,25} or hsp70\textsuperscript{8} would have to be used. The related studies are currently under way.

In all experiments conducted, gene expression was deemed transient. Accordingly, transgene integration into chromosomes of spermatogenic cells was not expected because it would occur at rare frequency. Indeed, under the similar experimental conditions, lacZ gene expression was not observed by \textit{in vivo} EP in the mouse testis at 4 weeks after the transfection (unpublished). To increase the chance of integration, and to attain stable foreign gene expression, further technical and methodological progress remains to be done.

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**References**


