

In vivo transfection of testicular germ cells and transgenesis by using the mitochondrially localized jellyfish fluorescent protein gene

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Received 21 September 2000; revised 10 November 2000; accepted 13 November 2000

First published online 27 November 2000

Edited by Matti Saraste

Abstract We aimed to introduce foreign DNA into spermatogenic cells in the testis by injection of the DNA encoding jellyfish fluorescent proteins, green fluorescent protein (GFP) and yellow fluorescent protein (YFP) into the seminiferous tubules and in vivo electroporation. We obtained fluorescent spermatozoa only when using the gene of the YFP protein fused to a mitochondrial localization signal peptide. Intracytoplasmic injection into oocytes of these spermatozoa gave fluorescent fetuses and pups. Almost all of the individuals produced from fluorescent spermatozoa were transgenic. We confirmed integration of the gene into chromosomes and its transmission into offspring. This is the first report of gene transfer into germ cells and subsequent production of transgenic offspring. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Transgenesis; Testis; Spermatozoon; Green fluorescent protein/yellow fluorescent protein; Electroporation; Intracytoplasmic injection

1. Introduction

Experimental modification of mammalian genomes has mostly been accomplished by pronuclear microinjection of the one-cell stage embryo, or gene transfection into embryonic stem cells followed by chimera production and germ line transmission. Introduction of foreign genes into zygotes by use of spermatozoa that have been pre-treated with DNA has been an attractive but so far unreliable method [1,2], and its molecular mechanism is still unknown. The recent finding that the mixing of DNA vectors with membrane-disrupted sperm, followed by intracytoplasmic sperm injection (ICSI), can lead to a high rate of production of transgenic mice [3] has opened a new exciting possibility. A related method is injection of a mixture of DNA and lipofection reagent into testes and subsequent mating of these male mice. In spite of recent reports of the production of transgenic mice and rats using this approach [4,5], the reliability and efficiency of this method are still unclear. In all such sperm-mediated gene

transfer, the foreign DNA seems to be transported to fertilized eggs, and then integrated into chromosomes in zygotes.

Gene transfection into germ cells has only rarely been attempted because of difficulties in manipulating germ cells, although there is a very recent report of the retrovirus-mediated gene delivery into mouse male germ cells [6]. For example, there is still no culture method to permit long-term proliferation of germ cells to allow gene integration and selection in culture [7]. However, transfection into germ cells would have various important advantages. Firstly, such direct modification of the germ line may well turn out to be the most efficient method of transgenesis in some species. Secondly, it would enable the study of gene function in germ cell development and gametogenesis immediately after transfection, even when the same transgene causes embryonic lethality and thus prohibits production of transgenic animals. However, interpretation of the phenotype might be complicated by presence of the intercellular bridges and transfer of the transgene products between spermatogenic cells. Finally, it may be the only method to study the integration of foreign vectors into chromosomes at around the initiation of meiosis, which is the only period when endogenous homologous recombination occurs frequently along the chromosomes.

Electroporation is a method usually used for transfection into cell suspensions. Forceps electrodes are used for in vivo electroporation aimed at various organs [8]. Its application to the mammalian testis has so far produced only limited success. Previous studies [9,10] detected expression of marker genes 2 months after transfection in clusters of spermatogenic cells, but they were rare in number and there was no evidence that this gave rise to spermatozoa expressing marker genes. So far, there have been no reports of production of transgenic mice from such testicular electroporation.

2. Materials and methods

2.1. Plasmid DNA

We used vectors carrying the CAG promoter [11] upstream of the gene encoding either EGFP (Clontech, USA), a modified green fluorescent protein (GFP) with a high degradation rate (Clontech, USA), or a modified EYFP containing a mitochondrial localization signal peptide (EYFP-Mito) (Clontech, USA). In the last case, the pCAG-EYFP-Mito expression plasmid was constructed by inserting the *NheI-NotI* fragment encoding the EYFP-Mito gene of pEYFP-Mito (Clontech, USA) into the *EcoRI* site of the pCAGGS [11], which contains a cytomegalovirus immediate early promoter/enhancer, chicken β -actin promoter, β -actin intron and rabbit β -globin polyadenylation signal. For testicular injection, pCAG-EYFP-Mito was di-

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Abbreviations: ICSI, intracytoplasmic sperm injection; GFP, green fluorescent protein; YFP, yellow fluorescent protein

gested with *SaI*I and *Hind*III, and the 3.1 kb DNA fragment was purified by using a gel extraction kit (Qiagen, Germany).

2.2. DNA injection and electroporation

We used ICR strain and [C57BL/6×DBA/2] F1 mice purchased from SLC, Japan. Postnatal day 14 ICR strain mice were anesthetized with Nembutal solution, and testes were exposed under a dissecting microscope. A micropipet was inserted into the rete testis for injection into seminiferous tubules. Approximately 6–10 µl of the DNA/HBS solution (100–120 µg/ml) was injected into each testis. Electric pulses were delivered with an electric pulse generator (Electrosquare Porator T820, BTX, USA). Testes were held between a pair of tweezers-type electrodes, and square electric pulses were applied four times and again four times in the reverse direction. Each pulse was at 30–50 V and 50 ms in duration.

2.3. Collection of fluorescent spermatozoa

Transfected testes were examined using a stereomicroscope equipped with an excitation light source and appropriate filter sets for GFP and YFP (yellow fluorescent protein) (LEICA MI APO, Leica, Germany). Seminiferous tubules containing clusters of fluorescent spermatozoa were dissected out from the testis and placed in Dulbecco's phosphate-buffered saline (PBS) supplemented with 5.6 mM glucose, 5.4 mM sodium lactate, and 1% polyvinylpyrrolidone (PVP). Samples were cut into small pieces and treated with 0.5 mg/ml trypsin in PBS to obtain dispersed spermatogenic cells and spermatozoa.

2.4. ICSI and embryo transfer

The isolated spermatozoa expressing YFP were transferred to a microdrop of 12% PVP in HEPES-buffered CZB solution [12]. ICSI was carried out as described by Kimura and Yanagimachi [13]. A YFP-positive spermatozoon was aspirated into an injection pipet attached to a piezo electric pipet-driving unit (Prima, Japan). After the sperm tail was separated from the head by applying piezo pulses to the neck region of the sperm, the sperm head was injected into an oocyte, which was obtained from a [C57BL/6×DBA/2] F1 female. Sperm-injected oocytes were incubated in CZB at 37.5°C with 5% CO₂ for 4–6 h. Morphologically normal zygotes were transferred into the oviducts of pseudo-pregnant ICR females.

2.5. Southern blotting analysis

Genomic DNA was isolated from mouse tails by using the Qiagen DNeasy DNA isolation kit (Qiagen, Germany). 5 µg of genomic DNA was digested with restriction enzymes, separated in a 1.0% agarose gel, transferred to Hybond-N+ nylon membrane filters, and hybridized according to the manufacturer's protocol (Amersham, UK).

3. Results

3.1. Expression of GFP or YFP genes in the testis after electroporation

Transient expression of transfected genes was examined 2 days after the injection of DNA solution and electroporation using 14-day-old ICR strain mice. Our histological study confirmed that at this age there are spermatogonia and primary spermatocytes up to the pachytene stage. When we used the conditions described in Section 2, we found widespread expression of GFP fluorescence in many round germ cells (Fig. 1A–D), as well as in Sertoli cells, throughout the whole testis.

The number of fluorescent germ cells was maintained until 7 days after electroporation but decreased drastically during the following week. Sertoli cells maintained stronger fluorescence longer than the germ cells. The remaining fluorescent germ cells were present mostly as clusters of 20–50 cells. Examination of dispersed cells after enzymatic dissociation of seminiferous tubules indicated the presence of fluorescent primary spermatocytes and round spermatids in testes 14 days after electroporation. The occurrence of such fluorescent germ cells was rare, suggesting that the GFP protein may be toxic to spermatogenic cells. In fact, we noticed abnormal or degenerating fluorescent germ cells in testes 14 days after transfection of the GFP gene, and we found no fluorescent spermatozoa when using the original GFP vector.

To reduce the presumed toxicity, we tested a modified GFP gene, of which protein has a faster degradation rate, and a YFP vector (pCAG-EYFP-Mito) in which a mitochondrial localization signal was linked to the YFP protein so that the protein is sequestered inside the mitochondria (Fig. 2A,B). There was no improvement using the modified GFP gene. However, in the testis 20 days after electroporation of pCAG-EYFP-Mito, we found clusters of fluorescent spermatozoa in seminiferous tubules (Fig. 3A). They were present as a group of several clusters, each of which contained a few spermatozoa.

3.2. ICSI and fluorescent pups

We dissected out fragments of seminiferous tubules that contained clusters of fluorescent spermatozoa and dissociated them. Using micromanipulators, we picked up fluorescent spermatozoa (Fig. 3B) and used them for ICSI. In the first such experiment (Table 1), three injected ova produced eight-cell stage embryos, all of which showed fluorescent protein expression. In the second experiment (Table 1), 11 injected ova were transferred into oviducts of two foster mother mice which resulted in pregnancies. Two out of three fetuses isolated from one foster mother showed ubiquitous expression of YFP throughout the whole body (Fig. 4A,B). Two live pups were born from six ova transferred to the other foster mother, and both exhibited fluorescence of the YFP protein. In the third experiment, all three pups born from the transfer of six injected ova showed fluorescence (Table 1).

3.3. Examination of gene integration and transmission into offspring

We prepared genomic DNA from the fluorescent fetuses and pups produced by ICSI and carried out Southern blotting analysis. The results indicated that the introduced gene was integrated into chromosomes and represented by distinct bands in all such samples (Fig. 5). The gene was inserted as single or multiple copies. Moreover, we found that a pair of the fetuses obtained in the second experiment seemed to carry

Table 1
Production of fluorescent embryos and offspring by intracytoplasmic injection of fluorescent spermatozoa

Sperm-injected oocytes	Oocytes with sperm pronuclei	One-cell stage embryos transferred to foster mother	Fluorescent early embryos/total embryos	Fluorescent fetuses/implanted fetuses	Fluorescent pups (F0) /pups born	Fluorescent pups (F1) /pups born
3	3	–	3/3	–	–	–
18	11	5	–	2/3	–	–
		6	–	–	2/2	5/8, 4/7
11	6	6	–	–	3/3	7/12, 3/7

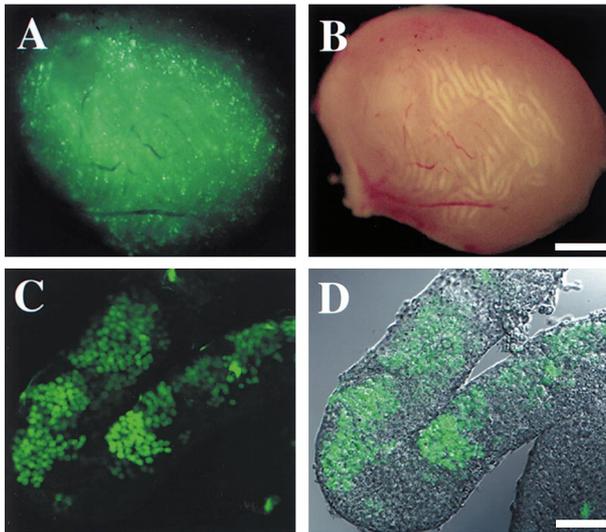


Fig. 1. EGFP expression 2 days after electroporation. Low magnification view of a transfected testis observed by fluorescence microscopy (A) or bright field (B). Examination at a higher magnification indicates EGFP expression in the spermatogenic round cells and Sertoli cells in the seminiferous tubules (C). D: Superimposition of the fluorescence and bright field images. Scale bars indicate 600 μ m (A, B) or 120 μ m (C, D).

the gene at the same integration site, because experiments using four different restriction enzymes showed the same pattern (results for three enzymes are shown in Fig. 5). In addition, a pair of fluorescent pups obtained in the third experiment also exhibited the same pattern (data not shown).

We also confirmed transmission of the transgene into the F1 generation from male and female transgenic F0 mice. The ratio of fluorescent to non-fluorescent F1 pups was approximately 1:1 as expected (Table 1), and thus there was no apparent indication of toxicity or disadvantage of the transgenic offspring compared to non-transgenic siblings.

4. Discussion

We tried to develop a novel method of mammalian transgenesis by gene transfection into spermatogenic cells immediately before or during meiosis. Therefore, we injected DNA into young testes, which contained spermatogenic cells only up to primary spermatocytes at the pachytene stage. We obtained widespread expression of the marker protein in germ cells until 7 days after transfection, but it decreased drastically

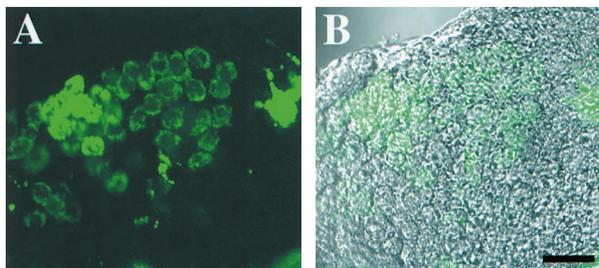


Fig. 2. EYFP-Mito expression 2 days after electroporation. Gene expression was detected in spermatogenic cells as fluorescent dots representing mitochondrial localization (A). B: Superimposition of the fluorescence and bright field images. A scale bar indicates 20 μ m.

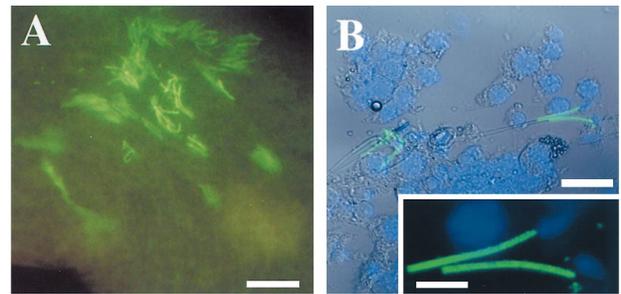


Fig. 3. EYFP-Mito expression 20 days after electroporation. Gene expression was detected in a group of spermatozoa in the seminiferous tubules (A). Higher magnification views of the dispersed spermatozoa show fluorescence localized in the middlepiece containing mitochondria (B and an inset to show a magnified view). Cell nuclei were visualized with DAPI staining. Scale bars indicate 80 (A), 40 (B) or 15 μ m (inset).

during the following week. Only when using the YFP gene linked to a mitochondrial localization signal, we found clusters of fluorescent spermatozoa 20 days after transfection. The earlier widespread expression represents the transient expression from episomal vectors, and later expression in a small number of germ cells probably represents expression after integration into chromosomes.

All of the previously reported sperm-mediated transgenesis [3–5] made use of spermatozoa as a carrier of DNA into ova, aiming at integration inside zygotes. Our results may represent the first case of the integration during spermatogenesis. In fact, there were two cases in which a pair of transgenic offspring showed the same banding pattern, thus suggesting the common integration site. The most likely explanation is that we used spermatozoa derived from a common progenitor cell, in which the transgene had already been integrated into the chromosome.

The number of fluorescent spermatozoa was very small when compared to the large number of fluorescent germ cells immediately after electroporation. It could be caused by either toxicity of the fluorescent marker protein or a very low probability of chromosomal integration around meiosis. Our observation of degenerating spermatogenic cells after transfection of the GFP gene is suggestive of toxicity. Toxicity of the GFP protein has only rarely been pointed out [14], but very recent papers [15,16] reporting toxicity of GFP in cardiac cells and neural stem cells indicate that it has become an important issue, particularly in the cell manipulation studies for medical application. Presence of the transgenic mouse strain that shows ubiquitous GFP expression [17] indicates that a certain

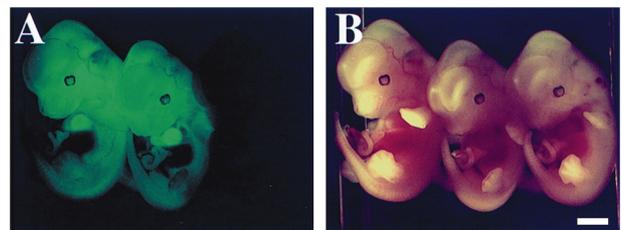


Fig. 4. EYFP-Mito expression in fetuses at 12.5 dpc, which were derived from the zygotes produced by ICSI using fluorescent spermatozoa. Two transgenic fetuses appear green under excitation light, and a non-transgenic fetus is not visible (A). B: A bright field image. A scale bar indicates 1.2 mm.

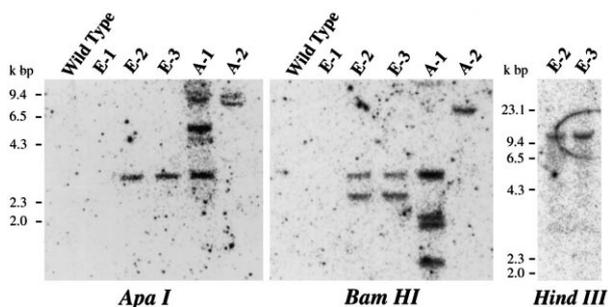


Fig. 5. Southern blot analysis of genomic DNA obtained from a non-transgenic control mouse (E1) or transgenic mice (E2, E3, A1, A2). Genomic DNA was digested with *ApaI* or *BamHI* and hybridized to the labeled 0.8 kb fragment of EYFP cDNA (*BamHI/NotI* fragment of pEYFP-Mito). DNA used for electroporation carries unique sites for *ApaI* and *BamHI*, and no site for *HindIII*. E2 and E3 transgenic mice showed the same patterns.

amount of GFP product can be tolerated. However, the transient expression level immediately after transfection may well be much higher than the expression level of integrated transgenes. We were unable to obtain fluorescent spermatozoa using GFP or a modified GFP with a high degradation rate. However, we obtained them when YFP was linked to a mitochondrial localization signal, so that the YFP protein was sequestered rapidly inside the mitochondria and thus probably decreasing its presumed toxicity. This method to reduce the toxicity may well be valuable for manipulation of stem cells and other cell populations in the medical research such as reported recently [15,16].

The transgenesis procedures reported here can be used for introducing the desired constructs when linked to the fluorescent marker gene. For example, a useful gene for production of transgenic animals driven by its own promoter will be linked with the marker gene for sperm selection. The marker gene used in this study is relatively small with the size of less than 3 kb, and there would be no problem to construct and transfect the linked genes. Probably, such two genes should be separated by an insulator sequence to bring about independent expression patterns. The present electroporation method might be usable for other mammalian species. Although the number of fluorescent spermatozoa is still small, they could be collected from semen by using a fluorescence-activated cell sorter. Once they are obtained, the efficiency of the following steps for the production of transgenic animals can be very high, perhaps close to 100%. Such a high rate might be surprising because the intercellular bridges could produce fluorescent spermatozoa in the absence of the transgene. It is

possible that the transgene products may be rapidly incorporated into the mitochondria and not transferred to the connected spermatozoa in enough amounts for detection. This is the first report of gene transfection into germ cells and subsequent production of transgenic animals, and it may be interesting to see how frequently homologous recombination occurs when targeting vectors are introduced into spermatozoa immediately before the meiosis.

Acknowledgements: The authors would like to thank Dr. A. Ogura for help with the ICSI, Dr. M. Okabe for help with the EGFP vector. This work was supported by a grant from the Program for Promotion of Basic Research Activities for Innovative Biosciences (PRO-BRAIN).

References

- [1] Lavitrano, M., Camaioni, A., Fazio, V.M., Dolci, S., Farace, M.G. and Spadafora, C. (1989) *Cell* 57, 717–723.
- [2] Brinster, R.L., Sandgren, E.P., Behringer, R.R. and Palmiter, R.D. (1989) *Cell* 59, 239–241.
- [3] Perry, A.C.F., Wakayama, T., Kishikawa, H., Kasai, T., Okabe, M., Toyoda, Y. and Yanagimachi, R. (1999) *Science* 284, 1180–1183.
- [4] Chang, K.-T., Ikeda, A., Hayashi, K., Furuhashi, Y., Bannai, M., Nishihara, M., Ohta, A., Ogawa, S. and Takahashi, M. (1999) *J. Reprod. Dev.* 45, 37–42.
- [5] Sato, M., Yabuki, K., Watanabe, T. and Kimura, M. (1999) *Transgenics* 3, 11–22.
- [6] Nagano, M., Shinohara, T., Avarbock, M.R. and Brinster, R.L. (2000) *FEBS Lett.* 475, 7–10.
- [7] Watanabe, M., Shirayoshi, Y., Koshimizu, U., Hashimoto, S., Yonehara, S., Eguchi, Y., Tsujimoto, Y. and Nakatsuji, N. (1997) *Exp. Cell Res.* 230, 76–83.
- [8] Muramatsu, T., Nakamura, A. and Park, H.-M. (1998) *J. Mol. Med.* 1, 55–62.
- [9] Yamazaki, Y., Fujimoto, H., Ando, H., Ohyama, T., Hirota, Y. and Noce, T. (1998) *Biol. Reprod.* 59, 1439–1444.
- [10] Yamazaki, Y., Yagi, T., Ozaki, T. and Imoto, K. (2000) *J. Exp. Zool.* 286, 212–218.
- [11] Niwa, H., Yamamura, K. and Miyazaki, J. (1991) *Gene* 108, 193–199.
- [12] Chatot, C.L., Lewis, J.L., Torres, I. and Ziomek, C.A. (1990) *Biol. Reprod.* 42, 432–440.
- [13] Kimura, Y. and Yanagimachi, R. (1995) *Biol. Reprod.* 52, 709–720.
- [14] Liu, H.-S., Jan, M.-S., Chou, C.-K., Chen, P.-H. and Ke, N.-J. (1999) *Biochem. Biophys. Res. Commun.* 260, 712–717.
- [15] Huang, W.-Y., Aramburu, J., Douglas, P.S. and Izumo, S. (2000) *Nat. Med.* 6, 482–483.
- [16] Martinez-Serrano, A., Villa, A., Navarro, B., Rubio, F.J. and Bueno, C. (2000) *Nat. Med.* 6, 483.
- [17] Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. and Nishimune, Y. (1997) *FEBS Lett.* 407, 313–319.