

## Introducing foreign genes into fish eggs with electroporated sperm as a carrier

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### Abstract

A new method has been developed for introduction of foreign genes into fish eggs. The procedure is based on the incubation of fish sperm cells suspended in dilute citrate solution with plasmid DNA, followed by application of high-field-strength electrical pulses (electroporation) to increase DNA binding, uptake, or both. Tissue homogenates and genomic DNA extracts of free swimming fry developed from eggs fertilized with treated sperm was tested to evaluate the efficiency of gene transfer. Dot blot hybridization and gene expression assay demonstrated the presence and expression of the reporter genes introduced in 2.6 to 4.2% of several hundreds of tested larvae of common carp (*Cyprinus carpio* L.), African catfish (*Clarias gariepinus*), and tilapia (*Oreochromis niloticus*). No transgene have been found in the fry resulting from parallel experiments without sperm electroporation. This is the first report on

successful application of electroporated sperm cells for production of transgenic fish.

### Introduction

Transgenic fish are traditionally produced using microinjection, which is a technique developed in mammalian systems. In mammals, the foreign gene is introduced into the male pronucleus of fertilized eggs by microinjection (Palmiter and Brinster, 1986), whereas cytoplasmic microinjection (Fletcher and Davies, 1991) is the commonly used method with fish eggs because of the lack of pronuclei visibility. Additional difficulties implicit in this technique (e.g., slowness, thickness of chorion of several species, mechanical damage to eggs) prevent researchers from capitalizing on the advantage of large numbers of eggs; the method therefore creates a "bottleneck" in the procedure of transgenesis applied to fish (Guise et al., 1991). Our aim has been to find a mass gene transfer method to replace the microinjection technique.

It has been previously reported by several groups that sperm cells of different species, including carp, are able to bind exogenous DNA (Arezzo, 1989; Castro et al., 1991; Atkinson et al., 1991). Successful gene transfer to eggs with this procedure has only been reported by one group working on mouse (Lavitrano et al., 1989); however, other laboratories have been unable to repeat their results (Brinster et al., 1989).

During mammalian fertilization, the acrosome reaction is followed by penetrance of sperm nucleus only, whereas the whole sperm head and sometimes the tail enters the ova in most finfish species (Ginzburg, 1972). The latter mechanism allows entrapment and transfer of foreign genes bound only to the surface of sperm cells as well; therefore, the chance for transformation is expected to be better with fish.

In our experiments, incubation with plasmid solution was followed by exposure of sperm cells to electrical pulses (electroporation) to enhance the incidence of binding and possible uptake of exogenous DNA. Cells can be reversibly permeabilized to

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macromolecules (e.g., DNA molecules) by application of electroporation (Neumann et al., 1982), a widely used technique for transforming many kinds of animal cells, plant protoplasts, and bacteria (Potter, 1988). The first use of electroporation for producing transgenic animals was achieved in a fish species by introducing genes into fertilized eggs of medaka (Inoue et al., 1990).

Applicability of sperm binding followed by sperm electroporation as a method for transferring genes has been tested on three freshwater fish species: common carp (*Cyprinus carpio* L.), African catfish (*Clarias gariepinus*), and tilapia (*Oreochromis niloticus*).

## Results

Spawning of broodstock was induced by hypophysis injection in carp and catfish and by exposure to a special light program in tilapia. Sperm and eggs were obtained using conventional propagation methods (Horváth et al., 1984).

### Incubation media for sperm

In the natural fertilization process, sperm cells of freshwater fish species become activated by dilution in water and remain viable usually for 1 minute or less. In our experiments, 1.8% (w/v) sodium citrate solution was used as incubation medium, in which a sufficient proportion (30–80%) of sperm cells of all 3 species are active and constantly motile for several

hours (Magyari et al., 1991). Because sodium citrate was found to disturb the normal mechanism of egg activation and fertilization in carp, 2.8% potassium citrate was used for this species.

### Vectors

The incubation solution contained 10 µg of one of the following three plasmids: pRSV/lacZ, pHSVtk/neo or pGM3H4/CAT (approximately  $5 \times 10^4$ – $10^5$  copies/sperm cell) in circular or linearized form dissolved in the citrate solution.

The 6.7 kb long pRSV/lacZ plasmid (provided by Campos-Ortega, Cologne, Germany) was constructed by combining the long-terminal repeat (LTR) sequence of Rous sarcoma virus (RSV) with the β-galactosidase (lacZ) gene of *Escherichia coli* and SV40 termination and polyadenylation sequences. The 4.7 kb long pHSVtk/neo plasmid contained the 5' promoter region of herpes simplex virus type 1 thymidine kinase gene regulated by the RSV enhancer region, and the Tn5 kanamycin phosphotransferase gene (constructed by J. Szelei, Szeged, Hungary). The pGM3H4/(chloramphenicol acetyltransferase) (CAT) construct contains the trout histone-4 gene promoter linked to the CAT gene of Tn9 transposon of *E. coli* (provided by G. Dixon and S. Valla for N.M.).

### Incubation of sperm cells with DNA

Freshly stripped semen of carp and catfish (5–10 µl) (representing the range of  $5 \times 10^7$ – $10^8$  sperm cells,

**Table 1.** Gene transfer into fish eggs by sperm electroporated or incubated in the presence of plasmid DNA<sup>a</sup>

Species	Treatment	Construct	No.		
			Analyzed	Positives <sup>b</sup>	%
DNA hybridization analysis					
Common carp	I + EP	pRSV/lacZ	192	3+(2)	2.6
	I + EP	pHSVtk/neo	44	0	—
	I	pHSVtk/neo	94	0	—
Tilapia	I + EP	pGMH4/CAT	126	2+(2)	3.2
African catfish	I + EP	pRSV/lacZ	86	2+(1)	3.5
	I	pRSVLacZ	126	0	—
Expression assay					
African catfish	I + EP <sup>c</sup>	pHSVtk/neo	72	3	4.2

<sup>a</sup> Details of experiments and description of vectors used are found in the Results Section.

<sup>b</sup> Numbers in parentheses represent fish showing faint signals, suggesting the presence of less than one copy per cell plasmid.

<sup>c</sup> Incubation and electroporation were performed in the presence of 10% DMSO in the incubation medium.

I = incubated with DNA; EP = electroporated after incubation in the presence of DNA.

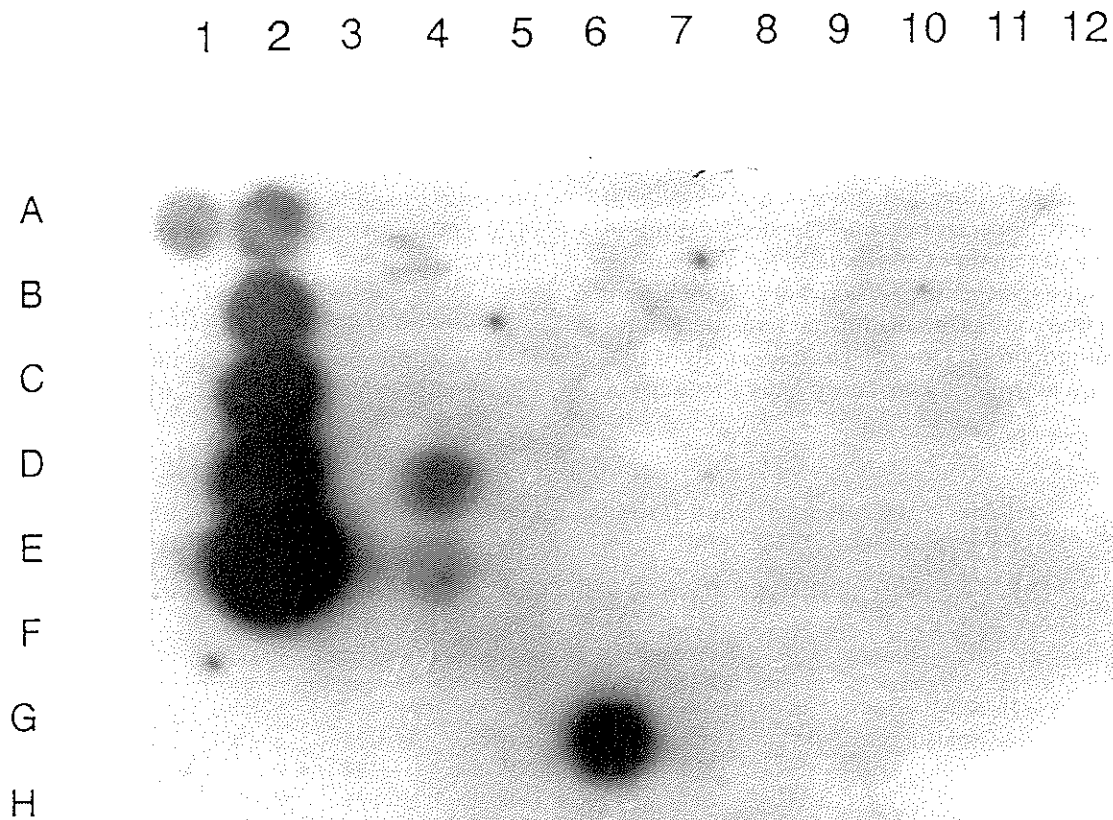
depending on the density and quality of sperm) was incubated with plasmid DNA in 100 to 500  $\mu$ l of the solution described. In tilapia, 20  $\mu$ l sperm was used to provide a similar number of cells. Incubation was performed at room temperature and lasted for 40 to 60 minutes depending on the viability of sperm.

*Electroporation of sperm cells*

Incubation with DNA was followed by application of high-field-strength electrical pulses. The sperm cells were pulsed once using a homemade electroporator apparatus (designed by A. Nagy, ELTE, Hungary, and C. Hawkins, University of Southampton, UK) that discharged 175 or 270 microfarads capacitance on catfish or carp and tilapia, respectively. The electroporator was charged to the described capaci-

tance by using a conventional electrophoresis power supply (Hoefer PS500TX). The pulse amplitude (field strength) varied between 750 to 2,250 V/cm by using two different electroporation cuvettes (165–2086, 165–2088, Bio-Rad) with either 0.4 or 0.2 cm electrode distances.

Oscilloscopic measurements of the pulse showed an exponential decay curve-type featured by a pulse width of 5 to 15 msec. These measurements also revealed that the actual voltage was not significantly different from the set voltage. After electroporation, the treated cells were incubated for 5 minutes; the motility of the sperm was then tested and fertilization was performed. Reduced motility of electroporated sperm cells resulted in a decreased fertilization rate (70–80% of nonelectroporated controls).



**Figure 1.** Autoradiogram of DNA dot blot of African catfish larvae produced by fertilization with electroporated sperm cells. Samples containing 2  $\mu$ g denatured DNA (prepared by overnight proteinase K digestion followed by phenolchloroform extraction and precipitation in ethanol from individual 10-day-old free-swimming fry) of African catfish were blotted on positively charged nylon membranes (Hybond N+; Amersham). Prehybridization was followed by hybridization to probes labeled with alpha<sup>32</sup>P]dCTP by nick translation. A 773-bp-long fragment of the plasmid containing the SV40 polyadenylation sequence and termination region was used as probe. Order of samples were as follows: 1A, 2A–2E (10–500 pg, circular); 1B–1H (DNA of nontreated negative control larvae); 2F–12H (DNA of African catfish larvae developed from electroporated sperm).

Fertilization, incubation of eggs to hatching, and culture of early fry were achieved according to standard warmwater fish breeding procedures (Horváth et al., 1984).

#### Analysis of DNA and detection of gene expression

Free-swimming fry were used for both DNA analysis and gene expression assay. Several plasmid-containing specimens were found to demonstrate the uptake and efficient transfer of the exogenous DNA fragments by electroporated sperm to the larvae of all three species investigated. Summarized results of this study are shown in Table 1.

Evidence of successful gene transfer in 3 of 86 ten-day-old catfish larvae was provided by dot-blot DNA hybridization (Figure 1). Five carp individuals showed presence of plasmid introduced among the 236 specimen tested by the same method. DNA slot-blot analysis of 1-month-old tilapia larvae also yielded 4 samples containing the plasmid of 126 larvae developed from eggs fertilized with treated sperm.

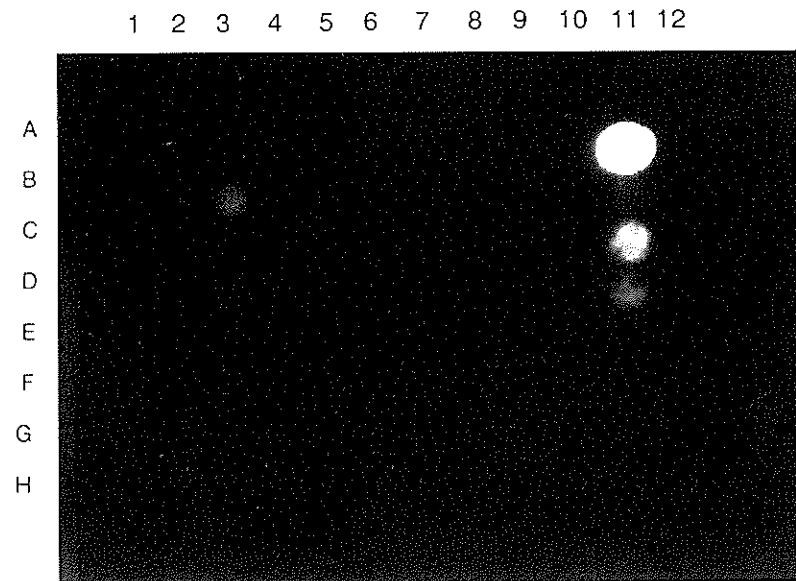
Gene expression was studied in African catfish larvae, in which DNA incubation and electropora-

tion were performed in the presence of 10% DMSO. Kanamycin phosphotransferase activity was revealed in 4.2% of homogenates from 2-week-old larvae (see Table 1, Figure 2) using the method described by Szelei and colleagues (1992).

Dot-blot DNA analysis of 1- or 2-week-old carp and catfish grown from eggs fertilized with sperm incubated with DNA (but not electroporated) failed to provide evidence for presence of the plasmid in the amount required for detection by this method (see Table 1).

#### Discussion

A new gene transfer method was applied successfully to fish. The method is based on successive application of incubation of sperm cells in DNA solution and electroporation of sperm-DNA complex. The performed assays did not demonstrate long-term persistence or integration; however, they did indicate that sperm electroporation can lead to effective transport of DNA into eggs and, consequently, to developing young fish. A similar conclusion was reported in a recently published article



**Figure 2.** Autoradiogram of neo-assay of homogenates of individual 10-day-old African catfish larvae produced by fertilization with sperm cells electroporated with pHSVtk/neo. Fish were individually disrupted by repeated freeze-thawing. Homogenates with 50  $\mu$ g protein content were assayed for kanamycin phosphotransferase activity by using gamma[<sup>32</sup>P]-labeled ATP and kanamycin as substrates. False-positive results caused by endogenous kinase activity were eliminated by TCA precipitation. Neutralized supernatants were blotted through nitrocellulose (binding all proteins) and then phosphocellulose (binding antibiotic) membranes by use of a dot-blot apparatus (Millipore). *Escherichia coli* strain pIS2-29-containing plasmids conferring kanamycin resistance were used as positive controls. Detection of radioactively labeled kanamycin was performed by autoradiography of the phosphocellulose membrane at  $-70^{\circ}\text{C}$  for 7 days. 1A–1H, nontreated negative control larvae; 2A–10H, larvae from electroporation experiment; 11A–11D, positive control.

describing successful introduction of foreign genes into bovine embryos as a result of electroporation of sperm (Gagné et al., 1991).

Because the percentage of positive results in our experiments was relatively low, further optimization of incubation conditions is required. Optimization of parameters such as duration of incubation, amount of DNA, and effect of ions, is in progress in our laboratory to increase the effectivity of DNA binding. An additional important concern is to evaluate the possible effects of different agents applied to the medium (e.g., DMSO, PEG) that may improve the results of electroporation. In our experiments, 10% DMSO in the incubation medium did not disturb motility of sperm and fertilization.

Optimal efficiency of transformation by electroporation results in a high percentage delivery in the range of field strengths, where a significant proportion of cells do not survive treatment (Förster and Neumann, 1989). To obtain more transgenics, future work should also address optimization of the electroporation parameters, such as field strength and pulse width (duration).

Motility of electroporated sperm compared to nonelectroporated controls was only slightly reduced (10–30%) because results were achieved using parameters with minimal lethal effect. We expect higher field strengths to be more effective because in small cell sizes (a diameter approximately 2–2.5  $\mu\text{m}$  for the sperm of these species), these field strengths seem to be necessary for better transformation (Förster and Neumann, 1989). Increased field strengths (2,250–2,500 V/cm, with 220  $\mu\text{F}$  capacitance and 7 msec pulse length levels) were found to be sublethal for tilapia sperm, suggesting that the optimal parameters have not been reached.

It has also been discovered that lower voltage and higher capacitance—resulting in longer pulse duration—can be equally or more effective than high-voltage pulses (Potter, 1988). In our experiments, we compared the effect of different capacitance levels with varying field strengths using tilapia sperm without exogenous DNA. Similar mortality rates were detected using 3,500 V/cm with 50  $\mu\text{F}$ , compared with 2,500 V/cm with 150 to 220  $\mu\text{F}$ .

We could not detect the presence of the foreign gene copies in fish resulting from experiments in which electroporation did not follow DNA incubation. These results should not be considered as conclusive evidence because the number of fish samples analyzed from the incubation experiments may be too small to find the putatively scarce event of transfer by this method. Because dot-blot and

slot-blot hybridization do not provide information on the fate of the introduced genes (i.e., whether they are integrated into the genome or persist extrachromosomally), Southern blot analysis is required to show possible integration.

An appropriately optimized method with better transformation rates would provide two major advantages. (1) Because the sperm nucleus actually fuses with the female pronucleus during fertilization, exogenous DNA carried by sperm is likely to be optimally delivered in terms of location, as opposed to the generally used cytoplasmic microinjection of fish eggs. (2) The protocol of electroporation of sperm cells is rapid and simple and results in a large number of embryos in one treatment, thus it complies with the demand for a mass gene transfer method (Fletcher and Davies, 1991). Such a procedure—applied in combination with effective screening for separation of the putative transgenic specimen—would allow us to benefit from the advantages offered by a fish system: availability of large number of eggs and sperm, external fertilization, and an embryonic development that lends itself to easy visualization.

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#### References

- Arezzo, F. (1989). Sea urchin sperm as a vector of foreign genetic information. *Cell Biol Int Rep* 13:391–404.
- Atkinson, P.W., Hines, E.R., Beaton, S., Matthaei, K.I., Reed, K.C., and Bradley, M.P. (1991). Association of exogenous DNA with cattle and insect spermatozoa in vitro. *Mol Reprod Dev* 29:1–5.
- Brinster, R.L., Sandgren, E.P., Behringer, R.R., and Palmiter, R.D. (1989). No simple solution for making transgenic mice. *Cell* 59:239–241.
- Castro, F.O., Hernandez, O., Uliver, C., Solano, R., Milanés, C., Aguilar, A., Perez, A., de Armas, R., Herrea, L., and de la Fuente, J. (1991). Introduction of foreign DNA into the spermatozoa of farm animals. *Theriogenology* 36:1099–1110.
- Fletcher, L.G., and Davies, P.L. (1991). Transgenic fish for aquaculture. In: *Transgenic Fish for Aquaculture in Genetic Engineering*, vol. 13. Setlow, J.K. (éd.). New York: Plenum Press, pp. 331–370.
- Förster, W., and Neumann, E. (1989). Gene transfer by electroporation. A practical guide. In: *Electroporation and Electrofusion in Cell Biology*. Neumann, E., Sowers, A.E., and Jordan, C.A., (eds.). New York: Plenum Press, pp. 299–318.
- Gagné, M.B., Pothier, F., and Sirard, M.A. (1991). Electropora-

- tion of bovine spermatozoa to carry foreign DNA in oocytes. *Mol Reprod Dev* 29:6-15.
- Ginzburg, A.S. (1972). Fertilization in fishes and the problem of polyspermy. (Translated from Russian by Blake, Z.; Golek, B. (ed.). Jerusalem: Israel Program for Scientific Translations.
- Guise, K.S., Kapuscinski, A.R., Hackett, P.B., and Faras, A.J. (1991). Gene Transfer in Fish In: *Transgenic Animals*. First, N.L., and Haseltine, F.P. (eds.). Boston: Butterworth-Heinemann, pp. 295-306.
- Horváth, L., Tamás, G., and Tölg, I. (1984). Propagation of common carp. In: *Special Methods in Fish Husbandry*. Halver, J.E. (ed.). Budapest, Hungary: Akadémia Kiadó.
- Inoue, K., Yamashita, S., Hata, J., Kabeno, S., Asada, S., Nagahisa, E., and Fujita, T. (1990). Electroporation as a new technique for producing transgenic fish. *Cell Diff Dev* 29:123-128.
- Lavitrano, M., Camaioni, A., Fazio, V.M., Dolci, S., Farace, M.G., and Spadafora, C. (1989). Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. *Cell* 57:717-723.
- Maclean, N., Penman, D., and Zhu, Z. (1987). Introduction of novel genes into fish. *Bio/technology* 5:257-261.
- Magyari, I., Szabó, R., Kovács, T., and Horváth, L. (1991). Effect of different Na citrate concentrations on the motility of carp sperm (in Hungarian). *Halászat* 84:142-144.
- Neumann, E.M., Schafer-Ridder, M., Wang, Y., and Hofschneider, P.H. (1982). Gene transfer into mouse lymphoma cells by electroporation in high electric fields *EMBO J.* 1:841-845.
- Palmiter, R.D., and Brinster, R.L. (1986). Germ-line transformation of mice. *Annu Rev Genet* 20:465-499.
- Potter, H. (1988). Electroporation in biology: methods, applications and instrumentation. *Anal Biochem* 174:361-373.
- Szelei, J., Szlanka, I., and Duda, E. (1992). Detection of the neo gene expression in animal cells after genetic transformation. *Anal Biochem* 203:166-168.