Volume 287, number 1,2, 118–120 FEBS 09999 August 1991

ADONIS 001457939100712C

The delivery of foreign genes into fertilized fish eggs using high-velocity microprojectiles

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Received 24 May 1991

Fertilized eggs of loach (*Misgurnus fossilis*), rainbow trout (*Salmo gairdneri*) and zebrafish (*Brachydanio rerio*) were bombarded with high-velocity tungsten microprojectiles covered with plasmid DNA containing sequences of β-galactosidase and neomycin phosphotransferase genes. About 70% of the eggs survived the bombardment. The activity of both transferred genes was revealed in the fish developed from the bombarded eggs. Neomycin phosphotransferase gene sequences were detected by means of PCR amplification and Southern hybridization in the total DNA of zebrafish that survived after G418 treatment.

High-velocity mechanical DNA transfer; *β-gal* Gene; *neo* Gene; Fish transformation

1. INTRODUCTION

During recent years the creation of transgenic fish has proved to be a fruitful and promising approach to gene engineering and biotechnology [1]. Foreign genetic information can be introduced into fertilized fish eggs of different species [2–9]. The introduced genes can be genetically integrated [2,3,8,9] and, in some cases, phenotypically expressed [4,8] (for review, see [10,11]).

In all these works, the genetic material was introduced by the microinjection of foreign DNA into egg blastodiscs. Here we have applied the method of high velocity mechanical DNA injection for this purpose. The method involves the bombardment of cells with small tungsten particles (microprojectiles) covered with DNA containing the genes to be introduced [12–16]. The method allows one to deliver foreign DNA directly and promptly into cell nuclei without its exposure to the action of cytoplasmic nucleases.

The method was proposed and successfully used for the transfection and genetic transformation of plant cells in calluses and leaves. These cells have very thick walls, which makes their transfection by conventional methods impossible or very difficult [12–14].

We have recently modified the method to transfect animal cells and demonstrated its applicability to the stable genetic transformation of NIH 3T3 mouse cells in culture [15]. Moreover, the method makes it possible to introduce foreign genes into organ explants of rodents in vitro and also can be applied to rat liver cell transfection in vivo [16].

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2. MATERIALS AND METHODS

2.1. *Fish eggs and embryos*

Experiments were carried out with three fish species: loach (*Misgurnus fossilis* L.), rainbow trout (*Salmo gairdneri Rich*) and zebrafish (*Brachydanio rerio* Ham.-Buch.). Eggs were obtained from females either ready for natural spawning (trout) or after hormone induced maturation (loach) and fertilized artificially. Zebrafish zygotes and early embryos were collected in the course of natural spawning. The eggs were taken for experiments in the period of blastodisc formation and up to the fourth division of cleavage.

Immediately before the bombardment, the embryos were placed into a plastic dish and, in the case of trout, turned manually blastodisc up. For one experiment, 600–1000 fish embryos were taken in the case of loach, 300–400 in the case of rainbow trout, and 200–1400 in the case of zebrafish.

2.2. *Plasmid DNA*

Plasmids containing genes of galactosidase (pRSV-β-gal) and aminoglycoside 3′-phosphotransferase II (pSV3-neo) were taken for transfection. Plasmid DNA was prepared by standard lysozyme-alkaline lysis followed by CsCl gradient centrifugation. Tungsten particles 0.3–3.0 μm in diameter were coated with plasmid DNA by calcium-phosphate precipitation as in [14–16]. 10 μl of a DNA solution (1 mg/ml) added to 10–15 mg of tungsten particles was taken per shot.

2.3. *Bombardment*

The shooting technology was mainly similar to that used previously for transfection of a mouse culture [15] and rodent tissues in explants and in situ [16]. The distance between the end of the barrel and the eggs to be bombarded varied from 10 to 25 cm depending on the species whose eggs were taken for the experiments.

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2.4. *Analysis of β-galactosidase activity*

The activity of β-galactosidase was detected by a standard technique [6] using X-gal as a substrate. The intensity of staining was detected microspectrophotometrically at 450 nm with the aid of a 'Microelisa autoreader' (Dynatech Instruments, USA).

2.5. *Selection of G418-resistant fish*

Zebrafish larvae and alevins (two-months-old) were placed in 600–800 mg/ml solution of the antibiotic geneticin (G418, Gibco), kept there for 3–5 days, and then put into fresh water. This concentration of G418 was chosen for the selection of the antibiotic resistant organisms on the basis of preliminary experiments. The embryonic and postembryonic development of the fish specimens and their death were followed up in the experiments.

The DNA of control fish specimens and those survived upon the G418 treatment was used in subsequent analysis.

2.6. *Fish DNA amplification by PCR*

Genomic DNA was separated by a standard phenol-chloroform method and studied using amplification by the polymerase chain reaction [17] with a slight modification. The reagent mixture contained: 10 mM Tris-HCl, pH 9.3 at 22°C, 50 mM KCl, 2.5 mM MgCl, 1 μM of the direct primer, 1 μM of the reverse primer, 0.1 μg/ml of gelatin, 100 mM of each dNTP, 4 U of Tth polymerase for each probe, 1 μg of DNA and 100 μl of mineral oil. Single-stranded 20-b.p. sequences were used as direct and reverse primers. Temperature: 1 min at 94°C, 1 min at 55°C, 2 min at 72°C. The aliquot of the mixture per probe was 50 μl without mineral oil. Amplification products were analyzed in 10% PAAG with subsequent ethidium bromide staining. 10 μl of the reagent mixture was applied to one electrophoretic line.

3. RESULTS AND DISCUSSION

The shooting technology was mainly similar to that used previously to transfect a mouse culture [15] and rodent tissues in explants or in vivo [16].

The destruction of fish eggs by bombardment depended to a great degree on the distance between the end of the barrel and the plate which contained the material to be bombarded as well as on the size of microparticles and their number in a pencil. The following conditions for egg bombardment and efficient cell transfection were chosen by varying these parameters: the distance between the end of the barrel and the Petri dish 20–25 cm for small eggs (loach and zebrafish) or 10–12 cm for bigger eggs (rainbow trout). A mixture of tungsten particles 0.1–1.5 μm in diameter was taken to shoot small eggs and of 1.0–3.5 μm for bigger eggs. The total weight of particles per shot was the same in both cases.

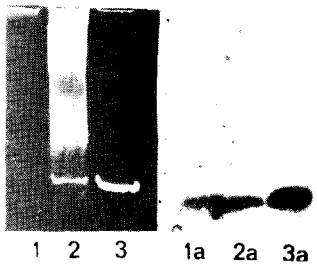

Fig. 1. Zebrafish larvae DNA amplification. (a) Confirmation of the PCR results by blot hybridization. Lanes 1 and 2, fish DNA (positive results); lane 3, positive control (plasmid DNA pSV2-neo).

When the cells were bombarded under these conditions, about 70% of fertilized eggs of all three fish taken for experiments (loach, rainbow trout, and zebrafish) survived the bombardment.

In the second series of experiments, individual developing loach embryos were analyzed for the activity of β-galactosidase 3 days after the bombardment with microprojectiles which had carried the corresponding gene. In about 5% of the larvae studied, a positive reaction for the enzyme activity was revealed (50 times exceeding the control).

Thus the gene for β-galactosidase was shown to be transferred by high velocity microprojectiles into fish fertilized eggs and expressed in their larvae.

In the third series of experiments, we studied how a neo gene introduced into zebrafish influenced its resistance to geneticin (G418). During the first two days of the incubation with G418, we found no differences either in the behaviour or in the state of the control (non-bombarded) and experimental (bombarded) fish. Differences appeared only by the 3th–5th days.

At the end of 3–5 days, destruction commenced both in the control and experimental (bombarded) organisms. At that period a G418 solution was replaced by running or repeatedly changed water. The destruction of fish embryos went on but its dynamics differed in the control and in the experiment (Table I). As one can see from Table I the survival of specimens with an introduced *neo* gene was higher than in control ones.

Table I

Survival of zebrafsih larvae and alevins in a G418 solution after egg transfection with pSV3 *neo* plasmid DNA

No. of experiment	Stage of development	G418 concentration (mg/ml)	Time of incubation with G418 (days)	Cumulative lethality (%) Hours after treatment with G418				
				24	48	72	96	120
1	larvae	600	5	5/0*	15/7	50/20	75/45	90/67
2	"	800	3	12/1	30/11	65/27	99/50	100/80
3	alevins	600	6	1/0	17/5	53/18	80/40	100/70
4	"	600	9	5/1	12/2	40/20	80/35	95/40

*control experiment

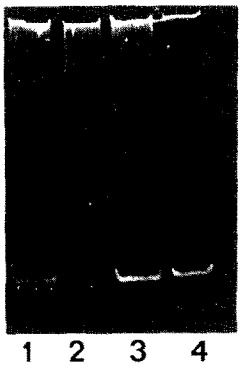

Fig. 2. Zebrafish alevin DNA amplification. Lanes 1-4, results of the amplication of fish DNA; lane 2, negative control.

These experiments have thus demonstrated that the introduction of the neomycin phosphotransferase gene makes some of the developing embryos resistant to the action of G418. It means that the introduced gene is expressed in their cells. Our results are consistent with the data of experiments in which the *neo* gene was introduced into goldfish eggs by a microinjection technique [9].

The fourth series of experiments was concerned with detecting the neomycin phosphotransferase gene in the DNA of zebrafish larvae resistant to G418. Sixteen larvae were analyzed by PCR technique in this series. In two cases fish were shown to contain sequences similar to those of the *neo* gene (Fig. 1). The results of amplification experiments were confirmed by electroblotting and a standard hybridization technique (Fig. 1a).

In the last series of experiments, transfected alevins which had survived after the G418 treatment were analyzed for a *neo* DNA sequence. In all the three cases, the presence of a *neo* DNA sequence was revealed by PCR (Fig. 2).

Our experiments have thus shown the applicability of high-velocity microprojectiles to the introduction of foreign DNA into developing fish embryos. The reasonable survival, the expression and the presence of the introduced genes during a rather long time of development indicate that the method can be used for the creation of transgenic fish.

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