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Sperm as a carrier to introduce an exogenous DNA fragment into the oocyte of Japanese abalone (*Haliotis divorsicolor suportexta*)

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We investigated gene transfer in abalone via electroporated sperm. The mobility of sperm electroporated either in seawater or in marine invertebrate physiological solution was as good as that of the control group. The fertilization rate reached as high as 94.7–99.6% (93.0–99.7% for the control group) when 200 eggs were fertilized by 10^6 or 10^7 sperm treated with electroporation at 10 kV and 2^7 pulses for six cycles. Moreover, the fertilization rate of sperm electroporated in the presence of foreign DNA (opAFP-2000CAT) ranging from 0.1 to 3.2 ìg and at voltages ranging from 2 to 10 kV, at 2^7 or 2^{11} pulses for six or 12 cycles showed no differences from the control sperm. After DNase digestion, the genome of the electroporated sperm was analysed by polymerase chain reaction, and it was shown that a 138-bp product was amplified, corresponding to the transgene's amplification product. Southern blotting also showed that a positive band located at the same position as that of opAFP-2000CAT was found in the electroporated sperm after DNase treatment. Analysis by PCR of the genome isolated from a trochophore-stage abalone larva, derived from sperm electroporated with 3.2 ìg opAFP-2000CAT, showed the existence of foreign DNA in 13 out of 20 examined samples (65%). The integration of the transferred DNA into the genome of transgenic abalone was also shown by Southern blot analysis. Furthermore, CAT activity was positive for the experimental larvae, but the level of CAT expression was lower than that of larvae derived from sperm electroporated with pCAT-Control vector, driven by SV40 promoter and enhancer sequences. These results demonstrate the potential for the use of sperm as mass gene transfer strategy in marine mollusks such as abalone.

Keywords: abalone; gene transfer; sperm-electroporation.

Introduction

Transgenic animals provide a powerful system for *in vivo* study of gene regulation, expression, and function. Many approaches have been developed to introduce different types of DNA molecules into zygotes with varying degrees of success. In fish, traditionally these methods have been (1) microinjection of foreign DNA into oocyte nuclei (Ozato *et al.*, 1986; Tsai *et al.*, 1995b), and into cytoplasm of fertilized eggs (Chourrout *et al.*, 1986; Dunham *et al.*, 1987; Zhang *et al.*, 1990; Fletcher *et al.*, 1988; Dunham *et al.*, 1992; Hew *et al.*, 1992; Lu *et al.*, 1992) and (2) electroporation of DNA fragments into eggs (Inoue *et al.*, 1990; Powers *et al.*, 1992; Tsai and Tseng 1994).

Electroporation of fertilized eggs helps overcome some gene transfer problems in cultured fish, namely, the invisible pronuclei and tough chorion. Compared with the traditional microinjection technique, electroporation is a simple, convenient and efficient method to transfer exogenous DNA molecules into fertilized eggs. However, the foreign DNA may well be transferred into a place other than the animal pole since the volume of the animal pole is very much smaller in the fertilized egg. Furthermore, although the efficiency of gene transfer by electroporation for fertilized eggs is 10–100 times greater than that of microinjection (Powers *et al.*, 1992), transfer efficiency is still not high enough to treat the

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tremendously large number of eggs spawned within a very short time by cultured fish and shellfish species. Therefore, sperm-mediated DNA transfer is an important approach since it offers the most efficient mass gene transfer system. The use of sperm cells as a vector for DNA transfer in the production of transgenic fish has previously been attempted. Although Chourrout and Perrot (1992) reported failure using sperm-incubation, some successes have been reported: sperm-incubation for zebrafish (Khoo *et al.*, 1992), sperm-electroporation for common carp, catfish, tilapia (Muller *et al.*, 1992), salmon (Symonds *et al.*, 1994a,b), loach (Tsai *et al.*, 1995a) and zebrafish (Patil and Khoo, 1996). Nevertheless, there are few reports regarding gene transfer in aquacultured shellfish (Powers *et al.*, 1995).

This study describes the use of sperm as a carrier to transfer an exogenous gene into oocytes of a marine gastropod Japanese abalone (*Haliotis divorsicolor supertexta*).

Materials and methods

Experimental animals

Two-year-old aquacultured Japanese abalone, 5.5 ± 0.2 cm in length, 20.6 ± 2.8 (male) and 23.4 ± 2.0 (female) g in body weight, were maintained on a natural light cycle in an aquaculture pond.

Gamete collection

Mature female and male abalone were collected separately and maintained in 5l natural seawater treated with Millipore filtration and UV light irradiation. In each trial, seven females and four males were used to induce ovulation and milt by the temperature-shifting method (Chen and Yang, 1979). Whenever a male began to produce milt, it was isolated and its sperm were collected in a separate glass container filled with 500 ml seawater or marine invertebrate physiology solution (MIPS: 0.4 M NaCl, 10 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 25 mM $NaHCO_3$, 28 mM Na_2SO_4, 0.6 mM NaBr, Hoar and Hickman, 1975). Eggs from one individual female were collected when fresh (no more than 30 min after they were spawned) also in a separate container. The number of gametes were estimated using a haematocytometer slide.

Electroporation solution and sperm preparation

Seawater and MIPS were used as the electroporation solutions. Their osmotic pressures were measured using an Advanced Micro-Osmometer (Massachusetts, USA).

Sperm resuspended in a volume of 100 ìl seawater or MIPS to contain 10^6 cells were electroporated in the presence of opAFP-2000CAT (experimental group), in the absence of opAFP-2000CAT (mock-treated group), and with no treatment (control group).

DNA preparation

Plasmid opAFP-2000CAT (6.4 kb) was a gift from Professor C. Hew, University of Toronto, and consists of the antifreeze protein (AFP) promoter of ocean pout fused with the coding region of chloramphenicol acetyltransferase (CAT) and a 39-untranslated region of SV40 ligated in pUC18 (Gong *et al.*, 1991). Plasmid pCAT-Control vector (Promega) (4.8 kb) contains SV40 promoter and enhancer, CAT coding region and an SV40 39-untranslated region ligated in pUC19, resulting in strong expression of CAT activity in eukaryotic cells. They were prepared by the caesium chloride-ethidium bromide ultra-centrifugation method (Sambrook *et al.*, 1989). opAFP-2000CAT and pCAT-Control were linearised with *Aat*II. Linearised DNA was resuspended in the electroporation solution at the desired concentration.

Electroporation

A noncontact mode instrument (Baekon 2000, CA) was used to carry out electroporation. The electric field strength was varied for different tests from 2, 3, 3.5, 4, 6, 7, 8 to 10 kV, 2^7 and 2^{11} pulses per cycle for six and 12 cycles and with transgene concentrations of 0.1, 0.2, 0.3, 0.4, 0.8, 1.6 and 3.2 ìg. The other parameters were kept constant: a burst time of 1.6 s; a pulse time of 120 ìs; an electrode distance of 1 mm; and a solution volume of 100 ìl in a 1 ml cuvette.

Aliquots of 100 ìl of diluted sperm suspension (10^7 cells) with opAFP-2000CAT in electroporation solution (experimental group) or with electroporation solution alone (mock-treated group) were used for electroporation. The control group consisted of sperm without treatment.

Sperm mobility

Sperm mobility was examined microscopically (100×) and categorized into three groups: (1) sperm move vigorously around large area; (2) sperm move quickly around small area; (3) sperm move slowly, remain still but vibrate flagella, or appear dead.

DNA analysis of sperm treated with DNase

After 100 ìl of 10^6 sperm suspension was mixed with 0.1 or 3.2 ìg foreign DNA in seawater, 10 ìg of DNase (Promega) was added and then incubated at 37 8C for 5, 10, 20, 30, 60 and 120 min, respectively. Each treatment was performed in duplicate. The genomic DNA of sperm was extracted and analysed by PCR to optimize the concentration of DNase capable of completely digesting exogenous DNA fragments persisting outside of the sperm.

After complete digestion by DNase, the electroporated sperm were washed several times with solution and their genomic DNA was analyzed by PCR and by Southern blot using a ^{32}P-labelled pUC18 probe. Direct incubation

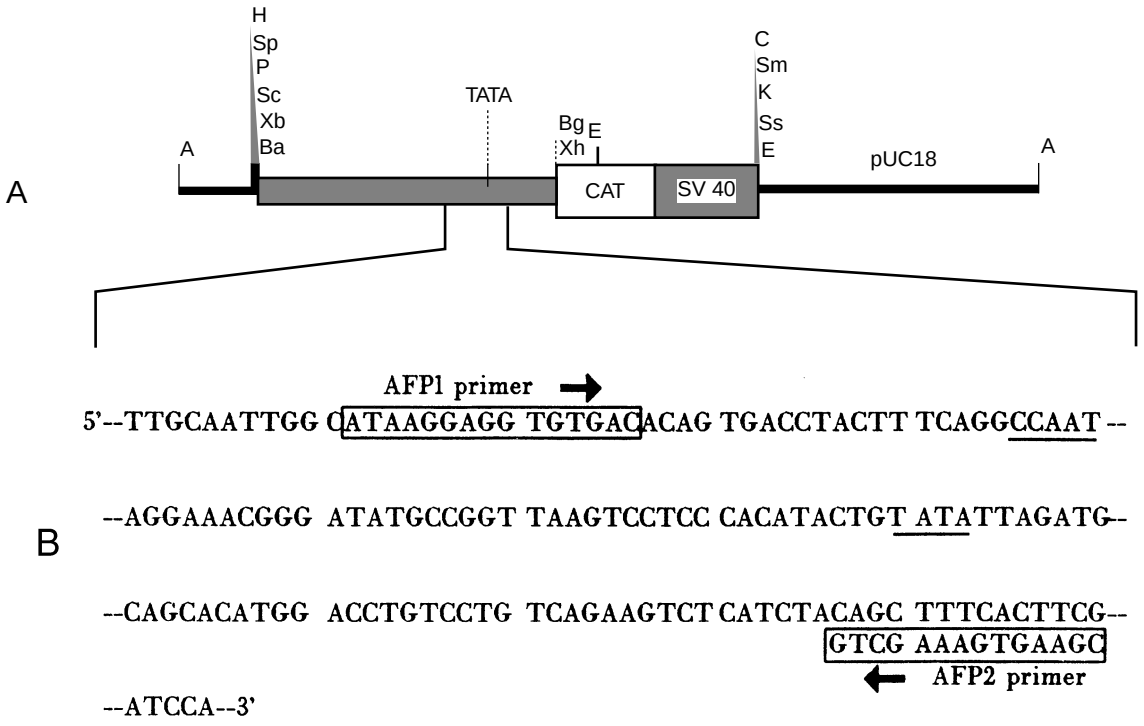

Fig. 1. Panel A, the structure and restriction map of opAFP-2000CAT (Gong *et al.*, 1991). The regulatory segment (hatched box), coding region of chloramphenicol acetyl transferase (CAT; open box) and the transcriptional terminator of SV40 (dotted box) are indicated. A, *Aat* II; Ba, *Bam* HI; Bg, *Bgl* II; C, *Cla* I; E, *Eco* RI; H, *Hin* dIII; K, *Kpn* I; P, *Pst* I; Sc, *Sca* I; Sm, *Sma* I; Sp, *Sph* I; Ss, *Sst* II; Xb, *Xba* I and Xh, *Xho* I. Panel B, the partial polynucleotide sequence of antifreeze protein gene (Du *et al.*, 1992). Two synthesized primers, AFP1 (from 1972 to 1986 of sense strand) and AFP2 (from 2110 to 2097 of antisense strand), used for PCR amplification of opAFP-2000CAT are indicated by boxes.

of abalone sperm in electroporation solution containing foreign DNA for 90 min at room temperature was also studied. The genomic DNA of the incubated sperm was assayed after DNase digestion and washing.

In vitro fertilization

Sperm with a count of 10^6 cells electroporated in the presence of foreign DNA (experimental group), electroporated in the absence of foreign DNA (mock-treated group), and with no treatment (control group) were used to fertilize 200 eggs. The fertilized eggs were washed 3–4 times with seawater after which they were fertilized for 30 min. Then, the embryos were dispersed in a container at 26 ºC for development.

Fertilization rate and hatching rate

The fertilization rate was calculated on the basis of eggs which developed into the 2-cell or 4-cell stage (1 h after fertilization). The hatching rate was calculated on the basis of eggs which developed to the trochophore stage (9 h after fertilization).

Genomic DNA extraction

Genomic DNA was extracted from sperm and larva at the trochophore stage by the SDS-phenol method (Inoue *et al.*, 1990): sperm were incubated in lysis buffer (10 mM Tris-HCl, pH 7.5; 10 mM EDTA; 150 mM NaCl; 0.5% SDS; and 0.3 mg ml^{-1} proteinase K) at 37 ºC for 1 h, followed by phenol/chloroform extraction.

Dot blot analysis

Genomic DNA of larvae was extracted, and 0.5–1.0 µg was spotted on a nylon membrane (Amersham). After air drying, the DNA was cross-linked to the membrane by UV irradiation and then hybridized to a ^{32}P-labelled opAFP-2000CAT probe. Prehybridization was carried out in a solution of 50% formamide, 5 × Denhart's solution, 0.5% SDS, 100 µg ml^{-1} denatured calf thymus DNA and 6 × SSC (1 × SSC: 0.15 M NaCl and 15 mM sodium citrate) at 42 ºC overnight. Hybridization was carried out in the same solution but containing a denatured probe at 42 ºC overnight. Membranes were washed sequentially in the following solutions before autoradiography: 2 × SSC and 0.1% SDS for 30 min at 37 ºC; 1 × SSC and 0.5% SDS for 30 min at 42 ºC; 0.5 × SSC and 0.1% SDS for 30 min at 68 ºC; and 0.1 × SSC and 0.1% SDS for 30 min at 68 ºC.

PCR analysis

Two oligonucleotide primers, AFP1 and AFP2, were synthesized for PCR analysis. Their nucleotide sequences (Du *et al.*, 1992) and locations at the upstream end of the antifreeze protein gene are illustrated on Fig. 1. PCR was carried out in 20 ìl of a solution consisting of 10–20 ng templates, 10 pmol of each primer, 125 ìM of each dNTP, 2 mM $MgCl_2$ and 2.5 U Taq DNA polymerase in a 1 × PCR buffer (Promega). Amplification was performed using a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). PCR consisted of 35 cycles denaturing at 94 8C for 1 min, annealing at 37 8C for 1 min and extension at 72 8C for 2 min with a 7 min extension at 72 8C. Each PCR sample was subjected to electrophoresis on a 3% NuSieve GTG agarose gel (FMC BioProducts, USA).

Southern blot analysis

Genomic DNA isolated from sperm after DNase treatment and isolated from larvae at the trochophoric stage with *Aat*II digestion were analysed on an agarose gel and transferred onto a nitrocellulose membrane. Hybridization was carried out as for the dot blot analysis but using radiolabelled pUC19 as a probe. Membranes were sequentially washed in solutions containing 4 × SSC and 1% SDS for 30 min at 37 8C, and 0.1 × SSC and 0.1% SDS for 30 min at 50 8C, before autoradiography for 2 days. For analysis of the PCR products, the membranes were sequentially washed by 2 × SSC and 0.1% SDS for 30 min at 37 8C, and 0.1 × SSC and 0.1% SDS for 30 min at 42 8C, for 1 week.

Transient CAT assay

The CAT assay was performed as described by Gong *et al.* (1991). Generally, batches of 400 trochophore-stage larvae were pooled, washed three times with phosphate buffer saline, sonicated in 80 ìl of 250 mM Tris (pH 7.8) and lysed with 80 ìl of lysis buffer for 15 min at room temperature. Supernatant was saved after centrifugation and then incubated at 60 8C for 10 min. The CAT reaction mixture included 160 ìl cell extract, with 2.5 ìl ^{14}C-chloramphenicol (55 mCi mmol^{-1}, 25 ìCi ml^{-1}, Amersham) and 5 ìl 4 mM acetyl Coenzyme A added. The mixture was incubated at 37 8C overnight and then extracted with 400 ìl of ethyl acetate. Acetyl chloramphenicol and chloramphenicol were separated by thin-layer chromatography.

Results

Electroporation conditions and sperm activity

Variable amplitudes (2, 3, 3.5, 4, 6, 7, 8 and 10 kV) were examined for their effect on sperm mobility while other

Table 1. Effect of electroporation voltage for Japanese abalone sperm on the fertilization rate and the hatching rate.

	Control			*Voltage (kV)*											
				2	*3*	*3.5*	*4*	*4*	*6*	*7*	*8*	*10*	*10*	*10*	
Trial	I	II	III	I	I	I	I	II	I	II	I	I	II	III	
Fertilization (%)	97.0	93 ± 1.5	99.7 ± 0.2	93.3	96.8	96.3	96.0	97.1 ± 0.5	92.9	96.7 ± 1.2	94.7	96.8	97.8 ± 0.4	99.6 ± 0.4	
Hatching (%)	96.3	93 ± 0.9	99.5 ± 0.3	95.8	96.1	97.1	96.9	97.6 ± 0.2	94.0	98.2 ± 0.7	93.6	95.4	98.3 ± 0.2	99.1 ± 0.5	

The fertilization rate and the hatching rate are based on eggs which developed into the two- or four-cell stage (1 h after fertilization) and into the trochophore stage (9 h after fertilization), respectively. Values are means ±SD ($n = 1$ for trial I, $n = 3$ for trial II, $n = 10$ for trial III).

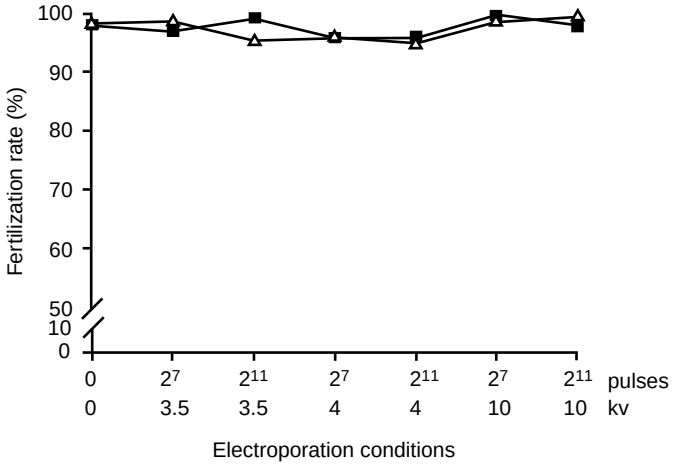

Fig. 2. Effect of electroporation conditions on fertilization rate. Abalone sperm were electroporated at various combinations of voltages (3.5, 4 and 10 kV), numbers of pulses (2^7 and 2^{11}) and cycles (six and 12). The solid square and empty triangle indicate six and 12 cycles, respectively.

parameters were kept constant: a 120 ìs pulse time, a 1.6 s burst time, and 2^7 pulses per cycle for six cycles in a volume of 100 ìl seawater contained in a 1-ml cuvette with a 1-mm electrode distance. Natural seawater and MIPS were the two solutions chosen for electroporation because their osmotic pressures are 920 and 870 mOsm, respectively, which are quite close to that of the body fluid of abalone which ranges from 800 to 900 mOsm. Abalone

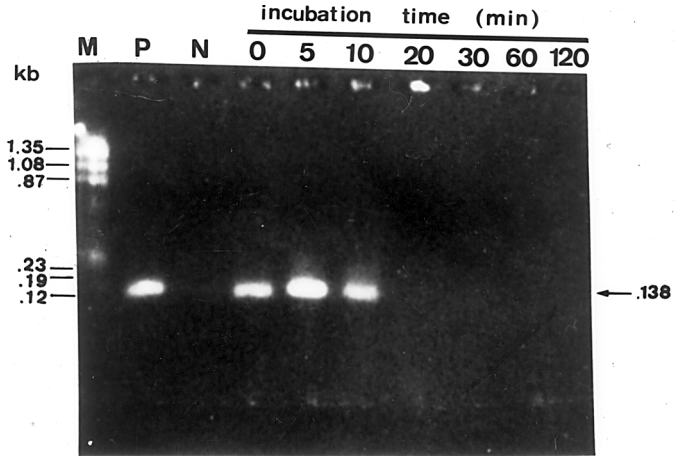

Fig. 3. Detection of opAFP-2000CAT after DNase digestion by PCR analysis. An aliquot of 0.1 ìg opAFP-2000CAT was incubated with 10 ìg DNase in a volume of 100 ìl seawater at 37 8C. P, 10 ng opAFP-2000CAT template added (positive control); N, no template DNA added, negative control. The incubation time is indicated on the top row. Arrow indicates the PCR-product (138 bp).

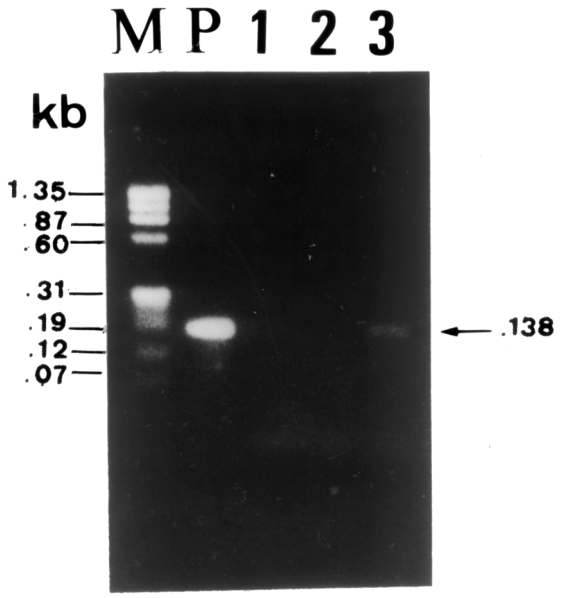

Fig. 4. PCR analysis of genomic DNA isolated from electroporated and DNase-treated sperm. After sperm were electroporated at 10 kV, 2^{11} pulses and 12 cycles, sperm were incubated with 20 ìg DNase at 37 8C for 1 h. Genomic DNAs of sperm were then extracted and analyzed. Lane M, molecular markers, *Hae* III-digested öX-174-RF DNA; lane P, from 10 ng opAFP-2000CAT as a template (positive control); lane 1, from sperm electroporated in the absence of opAFP-2000CAT (mock-treatment); lane 2, from sperm incubated with 3.2 ìg opAFP-2000CAT; and lane 3, from sperm electroporated in the presence of 3.2 ìg opAFP-2000CAT. Arrow indicates the 138-bp PCR product.

sperm electroporated in either seawater or MIPS solution retained normal activity. However, if abalone sperm were electroporated in lower osmotic pressure solutions, such as Hepes (281 mOsm), sperm activity decreased dramatically.

We found that sperm mobility was not lost when the amplitude was increased from 2 up to 10 kV under the electroporation parameters described above. The activity of the electroporated sperm remained in group 1 (moving vigorously) as did that of the non-electroporated sperm – even sperm electroporated at 10 kV.

Effect on fertility and hatchability of electroporated sperm

No differences were observed between the fertility of untreated sperm (control group) and that of electroporated sperm in the absence of DNA fragments (mock-treated group) treated under voltages varying from 2 to 10 kV, with constant 2^7 pulses for six cycles and other parameters (Table 1). Similarly, no difference of hatchability was shown between these 2 groups (Table 1).

The fertility of sperm electroporated in the presence of various concentrations from 0.1 to 3.2 ìg of linear opAFP-2000CAT at 10 kV and 2^7 pulses for six cycles

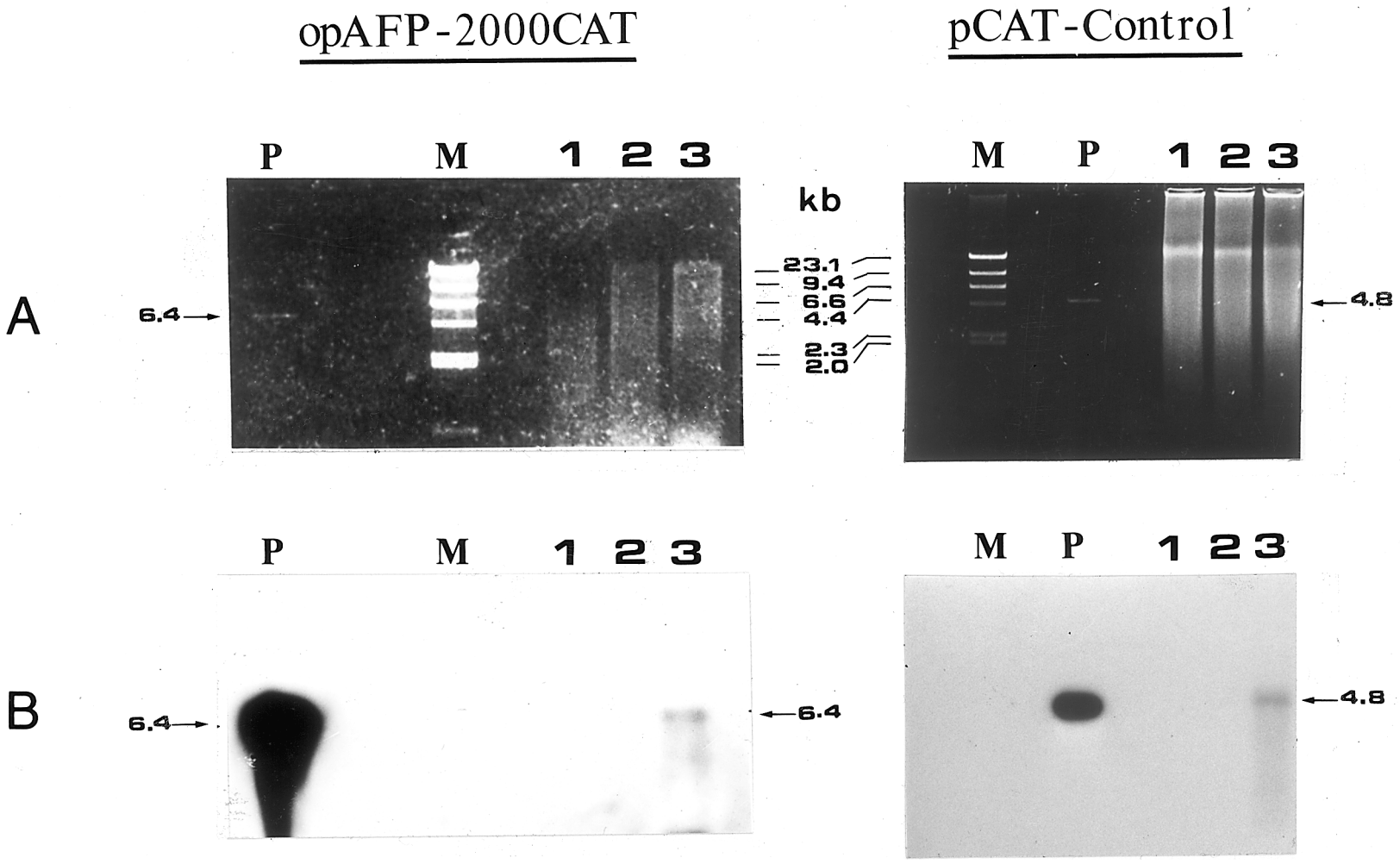

Fig. 5. Southern blot analysis of genomic DNA isolated from sperm electroporated in the presence of opAFP-2000CAT and pCAT-Control. Panel A, *Aat*II-digested genomic DNA were separated on the gel; and panel B, transferred to a membrane and hybridized to ^{32}P-labelled pUC18 probe. Lane P, plasmid with *Aat*II-digestion used for transferring (positive control); lane M, molecular marker, *Hin*d III-digested lambda; lane 1, sperm electroporated in the absence of DNA; lane 2, sperm incubated with 3.2 ìg DNA solution for 60 min, then digested with DNase; lane 3, sperm electroporated with 3.2 ìg DNA solution, then digested with DNase. Arrows indicate the molcular sizes of transferred DNAs.

was also examined. The fertility rates ranged from 99.14% to 99.87%, similar to that of the control group at 99.01%.

The fertilization rates derived from sperm electroporated under 12 combinations of voltages (3.5, 4 and 10 kV), pulse number (2^7 and 2^{11}) and cycles (six and 12) ranged from 95.1% to 99.2%, which appeared similar to that of the control group at 97.2% (Fig. 2).

DNA uptaken by sperm after electroporation

No positive band was found on PCR analysis of DNA isolated from sperm (10^6 cells in 100 ìl seawater) incubated with opAFP-2000CAT (0.1 ìg) after digestion with 10 ìg DNase at 37 8C for periods longer than 20 min (i.e., 30, 60 and 120 min) (Fig. 3). However, a 138-bp band was shown for groups treated for 0 (control), 5 and 10 min (Fig. 3). These results were also observed by digesting 3.2 ìg opAFP-2000CAT with 10 ìg DNase.

After sperm suspensions were electroporated at 10 kV and 2^{11} pulses for 12 cycles in the presence or absence of 3.2 ìg opAFP-2000CAT, the suspension was treated with 20 ìg DNase at 37 8C for 1 h. The genomic DNA of pooled sperm (10^6 cells) was extracted and analysed by PCR amplification. Results show that a 138-bp PCR product, which originated from the transgene, was present in the DNA sample isolated from the electroporated sperm with opAFP-2000CAT (lane 3 of Fig. 4), whereas no band was found in the DNA sample isolated either from sperm incubated with opAFP-2000CAT (lane 2 of Fig. 4) or from sperm electroporated in the absence of opAFP-2000CAT (lane 1 of Fig. 4).

Sperm electroporated at 10 kV and 2^{11} pulses for 12 cycles in the presence or absence of opAFP-2000CAT were digested with 20 ìg DNase at 37 8C for 1 h. The genomic DNA of pooled sperm (10^6 cells) was extracted and digested with *Aat*II (Fig. 5A). Southern blot analysis showed that there was a positive band with a molecular weight (MW) of 6.4 kb, which was the same size as that of the *Aat*II-digested opAFP-2000CAT when pUC18 was used as a probe (lane 3 of Fig. 5B). This positive band was not detected in samples either from the sperm electroporated in the absence of foreign DNA (mock-treated group; lane 1 of Fig. 5B) or from the sperm incubated with foreign DNA without electroporation (lane 2 of Fig. 5B).

Reproducible results were also observed from sperm electroporated in the presence of 3.2 ìg pCAT-Control vector (column of pCAT-Control in Fig. 5).

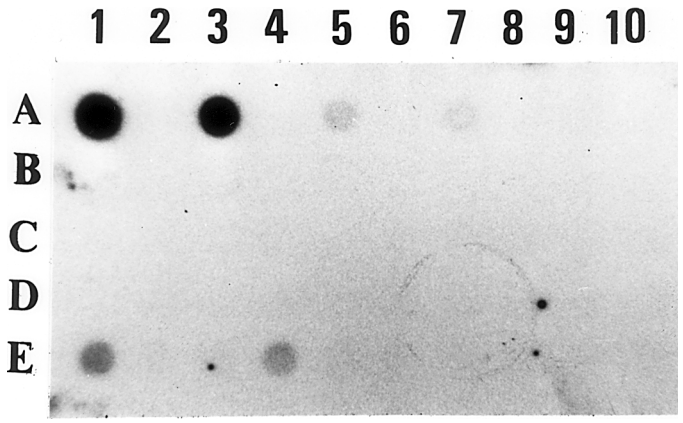

Fig. 6. Dot blot hybridization of genomic DNA isolated from an abalone larva at the trochophore stage. A1, A3, A5 and A7 are 750, 250, 30 and 15 pg opAFP-2000CAT (positive control), respectively; rows B and D are blank; row C is larvae chosen from the untreated control group; row E is larvae randomly chosen from the experimental group.

Analysis of transgenic abalone larva by dot, PCR, and Southern blotting

Genomic DNA was extracted from individual larvae at the trochophore stage derived from sperm electroporated at 10 kV and 2^{11} pulses for 12 cycles in the presence of 3.2 ìg opAFP-2000CAT. The isolated DNA was analyzed by dot blot hybridization. The dot-blot-positive rate was 20% (ten positives out of 50 larvae examined) (E1 and E4 of Fig. 6).

When the larval genome was analysed by PCR, a single positive band of 138 bp was shown which was generated from the transgene (Fig. 7A). The PCR-positive rate was 28.6% (four positives out of 14 examined; lanes 1, 2, 4 and 11 of Fig. 7A). However, when the PCR products were submitted to Southern blot analysis, the rate of positive signal occurrence increased to 65% (13 positives out of 20) (Fig. 7B), including five PCR-false-negative samples (lanes 5, 6, 12, 13 and 14 of Fig. 7B).

Genomic DNA was isolated from trochophore-stage larvae randomly chosen from the experimental and control groups, digested with *Aat*II, and analysed by Southern blot hybridization. Some larvae derived from the sperm electroporated with opAFP-2000CAT showed a positive band with a MW higher than that of the transferred DNA, 6.4 kb (lanes 3, 4 and 7 of Fig. 8). However, some larvae from the experimental group showed a negative result (lanes 1, 2, 5 and 6 of Fig. 8). Moreover, larvae derived from the electroporated sperm in the absence of opAFP-2000CAT (mock-treated group) were negative (lanes 10–12 of Fig. 8).

CAT assay

CAT assays were used to detect the effectiveness of the opAFP promoter and SV40 promoter in the transgenic abalone larvae at trochophore stage. As shown in Fig. 9, CAT activity was clearly detected in the positive control (lane 1) and larvae derived from sperm electroporated with pCAT-Control, which contain SV40 promoter and enhancer sequences (lane 5). Expression of CAT was also shown in abalone larvae derived from sperm electro-

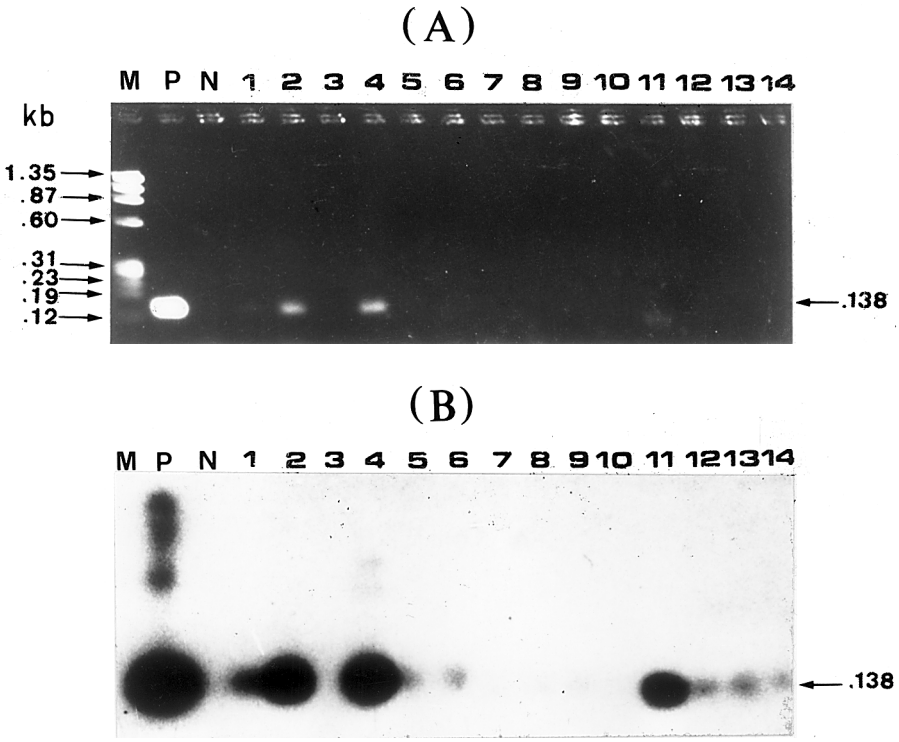

Fig. 7. PCR and Southern transfer analyses of genomic DNA isolated from a trochophore-stage abalone larva. Lane M, molecular markers, *Hae* III-digested öX-174-RF DNA; lane P, from 10 ng opAFP-2000CAT as a template (positive control); lane N, from untreated abalone larva (negative control); lanes 1–14 from the experimental group. Panel A, PCR analysis and panel B, Southern blot analysis of the PCR product from panel A, using opAFP-2000CAT as a probe. Arrows indicate the 138-bp PCR products.

porated with opAFP-2000CAT, which contains the AFP promoter (lane 4). The level of CAT activity resulting from opAFP-2000CAT was around 25% of that of pCAT-Control. Little or no CAT activity was detected in the negative control (lane 2) and the mock-treated larvae (lane 3).

Discussion

Natural seawater and MIPS are two suitable solutions for abalone sperm electroporation because their osmotic pressures are quite close to that of the body fluid of abalone. Thus, abalone sperm electroporated in either seawater or MIPS solution can retain normal activity and maintain their fertility. On the other hand, when sperm were electroporated in solutions with lower osmotic pressures, such as Hepes, the activity and fertility of the sperm decreased dramatically after electroporation.

Tsai *et al.* (1995a) reported that maintenance of normal sperm mobility and normal embryos of loach after electroporation is related to the amplitude during electroporation. If 10 kV was used to treat loach sperm, their mobility was totally lost or only Brownian movement was detectable. Therefore, electroporation of loach sperm should not be too severe, and an amplitude of no higher than 8 kV was suggested. Similarly, Symonds *et al.* (1994b) demonstrated that the activity of salmon sperm decreased from 82% to 2% as sperm were electroporated at voltages increasing from 625 V cm^{-1} to 1000 V cm^{-1}. Xie and Tsong (1992) also showed that the survival rate of cultured cells decreased from 100% to 40% as electroporation voltages increased from 0 kV cm^{-1} to 15 kV cm^{-1}. Unexpectedly, however, we found that amplitude was not an influential variable for sperm of this species of abalone. The motility, fertility and hatching ability of Japanese abalone sperm electroporated at 10 kV (mock-treated group and experimental groups) showed no difference from the untreated sperm. This may be the first report to show that abalone sperm are resistant to high voltages. Whether or not this specificity is related to the structural characteristics of Japanese abalone sperm, i.e., having a rigid truncated cone structure (Sakai *et al.*, 1993), remains to be studied.

We demonstrated that at least 3.2 ìg foreign DNA fragments (opAFP-2000CAT or pCAT-Control) in sperm suspension (100 ìl) prior to electroporation was completely digested by 10 ìg DNase at 37 8C after at least 20 min. Thus, a positive band (138 bp) found in PCR analysis of sperm DNA treated with 20 ìg DNase for

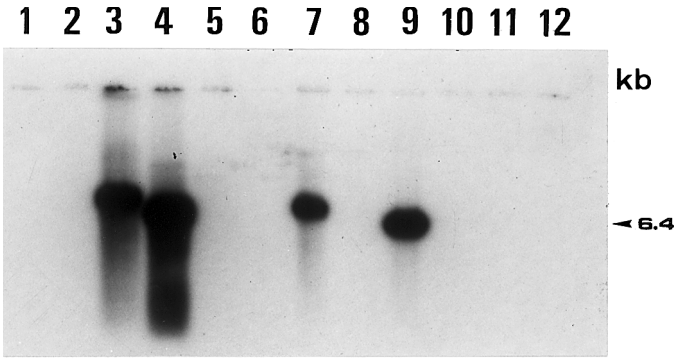

Fig. 8. Southern blot analysis of some presumptive transgenic trochophore-stage abalone larvae derived from ora fertilized with electroporated sperm carrying opAFP-2000CAT. *Aat* II-digested genomic DNA was electrophoresed on an agarose gel and analyzed by Southern blotting. Lanes 1–7, different presumptive transgenic individuals; lane 8, blank (molecular marker on the gel); lane 9, opAFP-2000CAT (positive control) used for transferring; lanes 10–12, different individuals from mock-treated control group. Arrowhead indicates the molecular size of the transgene (6.4 kb).

60 min after electroporation in the presence of opAFP-2000CAT and a positive band with 6.4 kb and 4.8 kb respectively found in Southern blot analysis of sperm genomes extracted from opAFP-2000CAT and pCAT-Control treatment groups suggest that foreign DNA was introduced into sperm using the electroporation conditions outlined in this study. Our findings are in agreement with the results of Gagne *et al.* (1991), who demonstrated that foreign plasmid DNA could be introduced into bovine oocytes by electroporated spermatozoa. However, if abalone sperm were incubated with opAFP-2000CAT, the exogenous DNA was not transferred into sperm. This result is consistent with that of Gavora *et al.* (1991), who failed to transfer DNA into eggs of mice or chickens by using spermatozoa incubated with bacterial plasmid DNA. The result is also in agreement with Chourrout and Perrot (1992), who failed to detect transgenesis by incubated sperm in rainbow trout. These observations strongly support Muller *et al.* (1992) and Tsai *et al.* (1995a), who demonstrated that a foreign gene was introduced into fish with electroporated sperm, but not with incubated sperm. It is, however contrary to the report of Khoo *et al.* (1992), who suggested that foreign plasmid DNA could be introduced into zebrafish by simply using sperm incubated with DNA. It appears, therefore, that the exact mechanism for sperm-mediated DNA transfer needs further study.

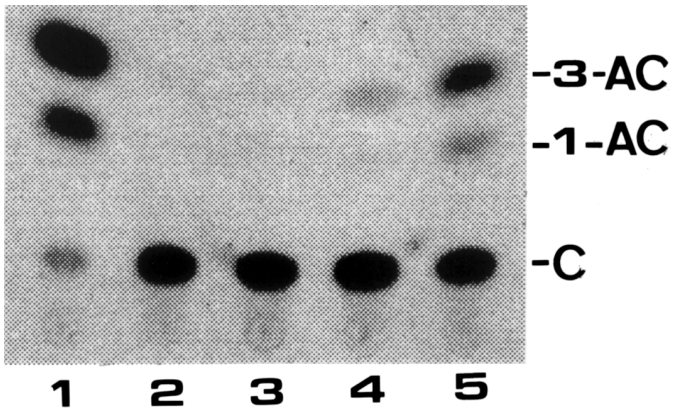

Fig. 9. CAT assay of trochophore-stage abalone larvae. Larvae were derived from ova fertilized with electroporated sperm at 10 kV and 2^{11} pulses for 12 cycles. Lane 1, positive control (1 U CAT enzyme was added); lane 2, negative control (no enzyme was added); and lane 3, larvae from mock-treated group (sperm electroporated in the absence of DNA). Larvae derived from sperm electroporated in the presence of 3.2 ìg linear opAFP-2000CAT (lane 4) and pCAT-Control (lane 5) were assayed. The acetylated (Ac) and nonacetylated chloramphenicol (C) were separated by thin-layer chromatography, followed by autoradiography.

As depicted in Fig. 1, the oligonucleotide primers chosen for PCR amplification of the regulatory segment of the antifreeze protein gene can generate a 138-bp DNA fragment. As expected, a 138-bp PCR product was observed if the opAFP-2000CAT fragment was present either in sperm or in the transgenic larvae. On the other hand, when primers were used for amplifying DNA either from the non-transgenic abalone in the experimental group, from the control abalone larva, or from the control sperm, no PCR product was produced. The PCR products were also positive in Southern blot hybridization when a radiolabelled probe was used.

We suggest that PCR-Southern-blot analysis was more sensitive in detecting the existence of the transgene because some PCR products were of too weak an intensity to be shown on the agarose gel, resulting in PCR-false-negative data. Based on the results from the PCR-Southern-positive samples from trochophore-stage larvae, the success rate of gene transfer was 65%, although we have not yet analyzed all the experimental larvae. This figure is comparable to that reported for fertilized-egg-electroporation in red abalone, i.e., 72% (Powers *et al.*, 1995) and zebrafish embryos, i.e., 35–75% (Powers *et al.*, 1992). Nevertheless, the success rate of this study is not only higher than that reported for microinjection in medaka, i.e., 16–27% (Ozato *et al.*, 1986; Inoue *et al.*, 1990; Lu *et al.*, 1992), zebrafish, common carp and channel catfish, i.e., 0–35% (Powers *et al.*, 1992), but also higher than that reported for electroporation in medaka eggs, i.e., 4–20% (Inoue *et al.*, 1990; Lu *et al.*, 1992) and for electroporation in sperm of carp, tilapia and catfish, i.e., 2.6–4.2% (Muller *et al.*, 1992).

Southern blot analysis of the genome extracted from trochophore-stage larvae showed a positive band with a MW higher than that of the transferred gene opAFP-CAT-2000. This evidence strongly suggests that the transferred

gene may be integrated into the chromosome of abalone via electroporated sperm after rearrangement of DNA molecules. These results agree with those reports which demonstrated a positive hybridization band with a size larger that that of the transferred DNA occurred in transgenic medaka (Inoue *et al.*, 1990) and loach (Tsai *et al.*, 1995a).

The opAFP promoter contains a long, 5′-flanking control region of type III AFP gene from ocean pout (Davis and Hew, 1990). It is a very effective promoter in directing CAT expression in the transgenic medaka, salmonids (Du *et al.*, 1992) and loach (Tsai *et al.*, 1995a). However, in transgenic abalone the opAFP promoter yielded a relatively low level of CAT activity in the trochophore-stage abalone larvae. CAT expression driven by the opAFP promoter was only one-fourth intensity of that driven by the SV40 promoter and enhancer, which is an non-specific and strong regulator segment for eukaryotic systems.

Electroporation has often been used for gene transfer into cultured cells and embryos. In this report we have clearly demonstrated that foreign genes can be transferred into abalone embryos using electroporated sperm as a vector. Compared to conventional microinjection (1–4 eggs min^{-1}; Ozato *et al.*, 1986; Inoue *et al.*, 1990) and electroporation (35–200 eggs min^{-1}; Powers *et al.*, 1992) for aquacultured animals, electroporation of sperm is a relatively simple mass gene transfer procedure, especially for shellfish, because it is efficient enough to enable the processing of several hundred thousand eggs at one time.

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