Journal of Aquaculture 13 (1) : 21~27 (2000)

Transient Expression of Transgene Introduced by Lipofected Sperm in Olive Flounder (*Paralichthys olivaceus*)

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The successful gene transfer and transient expression was demonstrated in olive flounder embryos using lipofected sperm. Olive flounder sperm interacted with foreign plasmid DNA encapsidated by positively charged liposome. The maximum plasmid copy number that associated with the sperm was 5 copies/ sperm, based on the examination of DNA blot assay. The foreign DNA was transferred into fertilized eggs without any adverse effect on fertilization, and survival of embryos (P>0.05), and retained in embryos until at least 42 hours with successful expression. The maximal expression was detected in 18 hours after fertilization at 18℃, and gradually decreased with development of embryo. Most of DNA transferred into embryos persisted extrachromosomally without significant sign of integration or replication.

Key words : Lipofection, Gene transfer, Expression, Olive flounder

Introduction

Transfer of a gene construct containing novel genetic information into fish has been given much attention to address variety of biological questions on gene regulation and function in vertebrates and also to generate a genetically improved strain with acquiring the desired phenotypes (Iyengar et al., 1996; Nam et al., 1999a). For these goals, many pervious gene transfer studies have been made in various species. However, most reports have been focused on gene transfer in freshwater species, consequently, there are only limited information on gene transfer to marine species.

Olive flounder is the most important marine species in Korean aquaculture and this species has been the subject of many genetic studies including chromosome-set manipulation (Kim et al., 1993), sex manipulation (Kim et al., 1994), hybridization (Kim et al., 1996) and recent transgenesis (Nam et al., 1997). In our previous study, the expression of pFV4CAT containing carp beta-actin promoter fused to CAT gene in olive flounder muscles has been already demonstrated (Nam et al., 1997). However no information on gene transfer and expression of foreign DNA in developing embryos has been available yet, despite it is prerequisite for addressing an important question on whether the regulatory sequences of fusion construct can be used for development of stable transgenic line. Based on this reason, this study is aimed to extend the information on functional utility of carp beta-actin promoter for development of transgenic olive flounder line by examining the successful expression in olive flounder embryos. In this paper we demonstrated a non-invasive transient transgenic system using lipofection strategy, which can provide economic and simple way enabling us to monitor the success of transgene

This study was conducted by the financial support of Korea Research Foundation made in the program year 1999.

expression in embryonic development without time-requiring laborious procedures.

Materials and Methods

Fish and gamete collection

Induced maturation and spawning were conducted to 2-year-old second generation of gynogenetic diploid males and 4-year-old normal female broodstock. The matured olive flounder females and males were given intraperitoneal injections of human chorionic gonadotropin (HCG) according to the methods as described by Park et al. (1994).

Plasmid preparation

The detailed map and information of expression vector containing reporter CAT construct can be referred to Caldovic and Hackette (1995). Plasmid DNA was isolated by conventional alkaline lysis method and purified with Wizard SV Miniprep Kit (Promega CO., USA). Finally, DNA was resuspended in sterile distilled water at a concentration of 1 $\mu g/\mu \ell$ and kept at 4 ℃ until use.

Generation of liposome/DNA complex and lipofection of olive flounder sperm

Sperm were washed with cold physiological saline (0.85% NaCl) and the concentration of spermatozoa was adjusted to 2×10^8 cells/㎖. Liposome (positive liposome kit, Sigma Co., USA) was prepared according to the method by Nam et al (1999b). Liposome/DNA complex was generated by mixing 50 μg of plasmid DNA with twice amount of liposome. The solution was incubated at 41 ℃ for 10 mins and cooled to room temperature. For lipofection of sperm cells, prepared sperm (1.0×10^7 cells) was mixed with the complex. Final concentrations of DNA and sperm were adjusted to 100 μg plasmid DNA/㎖ and 5.0×10^6 cells/㎖ in physiological saline, respectively. Total volume of reaction mixture was 200 $\mu \ell$. The reaction containing sperm/DNA/liposome was incubated at 20 or 25℃ for 30 or 60 min. When the incubations were completed, the reaction was subjected to DNase I (Promega) digestion (15 untit) to remove remaining unassociated plasmid copies. Sperm were collected by centrifugation at 3,500 rpm for 5 mins at 4℃, and resuspended in 500 $\mu \ell$ of physiological saline for DNA dot blot analysis (250 $\mu \ell$) and fertilization trials (250 $\mu \ell$).

An aliquot of sperm were fertilized with 3,000± 250 floated eggs to examine whether the lipofected sperm could transfer the plasmid copies to embryos by fertilization process. Three replicate fertilization trials were made in order to determine the possible effects of lipofection treatment on the capacity of the sperm to penetrate into eggs. In addition hatching success of the eggs fertilized by such sperm were monitored. The difference in fertilization and hatching success was considered to be significant at the level of $P<0.05$.

DNA isolation, PCR typing and Southern blot hybridization

DNA was isolated from sperm and whole embryos. Sperm collected were digested in a solution containing 50 mM Tris-Cl, 100 mM EDTA, 150 mM NaCl, 1% SDS and 100 $\mu g/$㎖ proteinase K, pH 8.0 at 55℃ for 12 hours. The reaction mixture was extracted with phenol, phenol/chloroform, chloroform/isoamyl alcohol, and precipitated with ethanol. The DNA fiber was washed once with 70% ethanol and resuspended in 1×TE (10 mM Tris, and 1 mM EDTA, pH 8.0). Embryo samples were digested in 50 mM Tris-Cl, 10 mM EDTA, 100 mM NaCl, 0.5% SDS and 100 $\mu g/$㎖ proteinase K, pH 8.0 at 55℃. The samples were processed identically as described above.

PCR detection of transgene was performed using AmpliTaq polymerase (Perkin Elmer, Cetus) according to the protocol described by Nam et al, (1999a). Briefly, the reaction was carried out at 94 ℃ for 1 min, 60℃ for 30 sec, 72℃ for 1 min for 30 cycles with initial denaturation step of 94℃ for 2 min. A 562 bp fragment of CAT sequence was amplified using the following primers (FVC-1) 5′-CTATAACC

AGACCGTTCAGC-3' and (FVC-2) 5'-CGCCCCGCC-CTGCCAC TCATCGCAG-3'. Southern blot hybridization was carried out using 5 ㎍ of genomic DNA digested with Bam HI, Dra II or Eco RV restriction enzymes. The membrane was hybridized with linearized pFV4CAT that had been labeled with digoxygenine-11-dUTP. Prehybridization, hybridization, stringent washes and detection procedures were carried out using Non-Isotopic Labeling and Detection Kit as recommended by manufacturer (Boehringer Mannheim Co., Germany).

Expression assay using RT-PCR and CAT-ELISA

Total RNA was used for reverse transcription- PCR. RNA was prepared by homogenization of embryos using motor-driven homogenizer (Tissue tearer, Biospec Prod. Inc.) under 3 volumes of TriPure Isolation Reagent (Boeringer Mannheim Co., Germany). Purification of RNA was performed according to the manufacturers recommendation. The possible contaminated DNA was removed by teatment of RNA-free DNase I (Progema) for 1 hour. The DNase was inactivated by heating the samples to 72℃ for 10 mins. One ㎍ of DNA-free RNA (confirmed by electrophoretic gel) was used for RT-PCR using Titanic One-Step RT-PCR Kit (Boeringer Mannheim Co.). Primers used in the RT-PCR were the same as ones used in PCR detection.

Transgene expression in embryonic development was examined by immunosorbant enzyme linked assay (ELISA). The CAT-ELISA Kit (Boeringer Mannheim Co.) containing polyclonal antibody against bacterial CAT enzyme was used. Samples (pooled sample of randomly taken 20 embryos) were homogenized in a solution containing 0.25 M Tris-Cl pH 7.8 containing 1 mM phenylmethyl- sulfonyl-fluoride. After homogenization, the debris was removed by centrifugation and the top fraction of supernatant was used for assay. All subsequent procedures were followed by the protocol of manufacturer's. The level of CAT expression was calculated as ng CAT/mg total protein using the bovine serum albumin standard.

Results and Discussion

Fertilizing ablility of lipofected olive flounder sperm

Mean fertilization success of the group treated for 60 mins showed a slightly decreased fertilization rate, however the difference was not significant ($P > 0.05$). All the groups including non-treated control sperm were found to have similar fertilizing capacity higher than 90% of success ($P>0.05$) (Fig. 1). In hatching success and early survival rates up to yolk sac absorption, similar phenomenon was observed. Mean hatching success and early survival rates of eggs (and hatched larvae) fertilized with lipofected sperm showed no difference, when compared to control groups, including non-treated group and the groups treated with liposome or DNA alone ($P > 0.05$). In addition, the incubation period revealed no effect on the fertilization and survival of embryos regardless of treatment ($P>0.05$) (Fig. 1). It suggest that sperm of this species might have high endurance against nucleic acid as well as positively charged liposome. Consequently the strategy of transient gene transfer in this study can be used for non-invasive method enabling us to ensure large number of Fo transformants for expression assay, although it is believed this system can provide only transient traits. The sustaining ability of sperm in lipofection treatment has been previously reported in other species (Nam et al., 1996, 1999b)

Sperm-DNA association

DNA blot analysis revealed that olive flounder had the capacity to associate with both naked DNA and liposome-encapsulated DNA. However, the efficiency was significantly different: only less than 1 copy of naked DNA can be associated with a sperm, while the sperm membrane can interact positively charged liposome/DNA complex with the efficiency ranging 2 to 5 copies per sperm (Fig. 2). Our data support the previous observations in mammal (Lavitrano et al., 1992) as well as fish (Nam et al., 1996, 1999b), in which the efficiency of gene transfer to sperm could be increased by use of positively

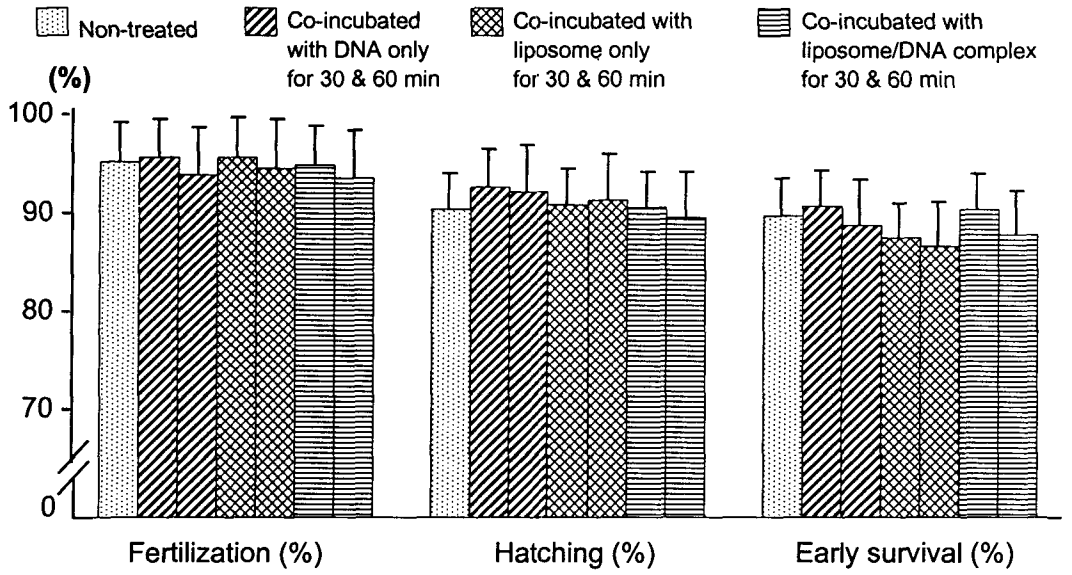

Fig. 1. Fertilization, hatching and early survival rates of the eggs fertilized with lipofected and control sperms.

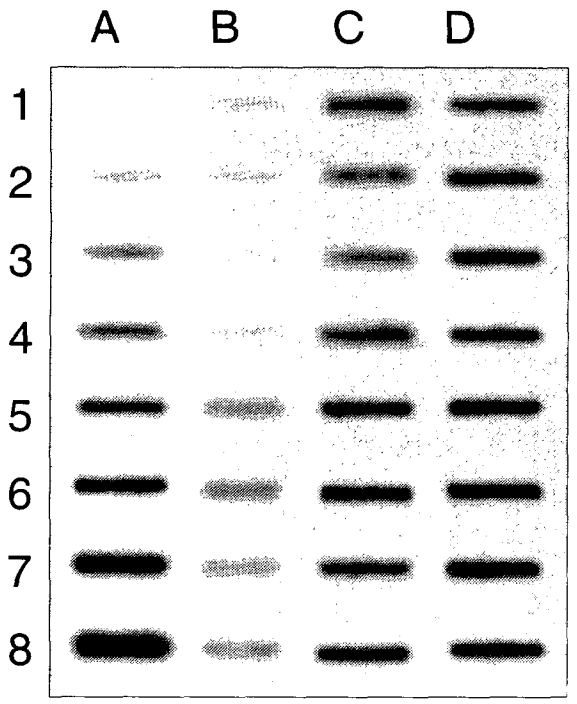

Fig. 2. DNA slot blot analysis showing positive standards of 0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 copies/sperm (A), control sperms (B1-B4), sperms incubated with naked DNA (B5-B8) and lipofected sperms (C and D).

charged liposome. Usefulness of positively charged liposome as an enhancing agent for transferring negatively charged macromolecules such as DNA has been claimed based on the principle that the positively charged liposome/DNA complex can more efficiently interact with negatively charged cell membrane. However, the copy number of plasmid associated with a olive flounder sperm in present study was lower than that reported in bovine sperm (Lavitrano et al., 1992). It might be mainly due to the small size of olive flounder sperm, compared to that of bovine, ultimately leading the decreased outer surface area that can associate with DNA. PCR analysis also confirmed the sperm-DNA association, evidenced by amplification of exactly matched PCR product from only the DNA-included treatment. No positive signal from any of control treatments was detected. (Fig. 3).

Transfer of transgene into embryo by fertilization process

The transgenic copies of associated with sperm could successfully be trasferred to olive flounder embryo via fertilization procedures. No transgene retention was detected in hatched larvae developed from the eggs fertilized with sperm treated with plasmid DNA only, however, all of groups fertilized with lipofected sperm resulted larvae containing

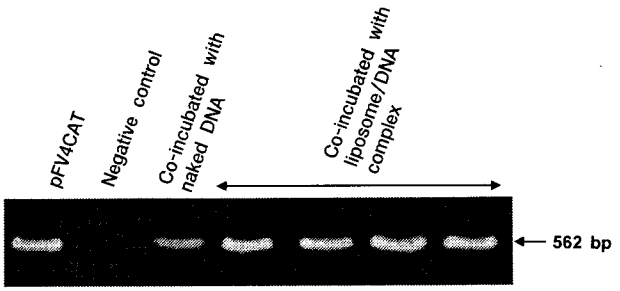

Fig. 3. PCR detection of lipofected and control sperms.

transgene copies (Table 1). The finding of failure in co-incubation of sperm with naked DNA only support previous observations made in mud loach (Nam et al., 1996) and rainbow trout (Chourrout and Perrot, 1992). On the other hand, successful generation of transient transgenic system using lipofected sperm was similar with our previous result on *Limanda yokohamae* (Nam et al., 1999b). Of two parameters (incubation temperature and duration) in the present gene transfer, incubation period was more effective on the efficiency of gene transfer than temperature: longer incubation was more efficient than incubation in higher temperature. In all three replicate treatments, 60 min-incubation resulted higher incidence of transgene when compared to 30 min-incubation. The maximal efficiency up to 82% was detected based on PCR assessment of hatched larvae (Table 1). The incidience of transgene in hatched larvae assessed by PCR was well matched to the results of dot blot analysis: higher association between DNA and sperm would result higher incidence of transgene in hatched larvae. This high incidence of transformant is comparable to any previous transgenic studies (Pandian and Marian, 1994) and makes it possible to use the group for expression studies without tedious further screening procedures if the expression assay would be performed in early embryonic development. In spite of high transformation efficiency, the major weakness of lipofection in fish transgenesis, the lack of integration into host chromosomes couldn't be overcome. Most transgene copy was proven to exist as extrachromosomal status as in other cases (Szelei et al., 1994; Nam et al., 1996): based on Southern blot hybridization, only a week signal of high molecular weight fragment was detected in DNA digested by Dra II (Fig. 4), which was believed to be lost in subsequent cell division.

Initiation of transgene expression and time course

The transgene expression in early embryo was detected by reverse transcription-PCR (RT-PCR). RT-PCR assessment of total RNA isolated from 0, 2, 4, and 6 hours post-fertilization revealed that initiation of transgene expression in embryonic development of olive flounder was 6 hours post fertilization (Fig. 5). No positive signal was detected in any of control treatment. The expressed level of transgene at the

Table 1. Efficiency of gene transfer depending on the lipofection condition of olive flounder sperm

Exp. group	Incubation temperature (℃)	Duration (min)	Incidence of transgene (%)*
Non-treated	25℃	60	-
Co-incubation with naked plasmid	20℃	30	0.0±0.0
		60	0.0±0.0
	25℃	30	0.0±0.0
		60	0.0±0.0
Co-incubation with liposome-coated DNA	20℃	30	31,3±5.8[a]
		60	78.5±9.8[b]
	25℃	30	40.2±9.5[a]
		60	82.1±6.8[b]

*Incidence of gene transfer was determined by PCR of hatched larvae.
Means with different superscript are significantly different (P<0.05).

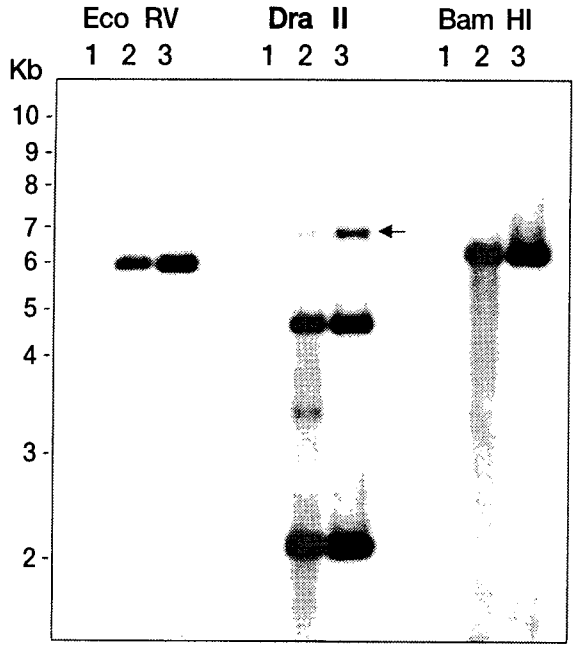

Fig. 4. Southern hybridization of embryos developed from the eggs fertilized with control (lane 1) and lipofected sperm (lane 2). Lane 3 in each enzyme digestion shows positive detection of pFV4CAT. Arrow indicates linearized size of pFV4CAT.

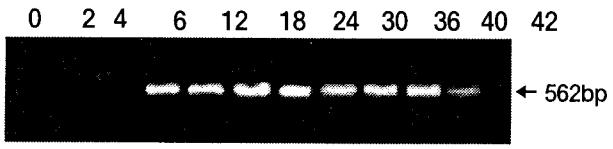

Fig. 5. RT-PCR gel showing the initiation of transgene expression in early embryos tranformed by lipofected sperm. Figures indicate the times (hours) post fertilization at 18℃.

point of initiation (6 hours post fertilization) was 21.2 pg of CAT/mg protein, when determined by CAT-ELISA Kit (5'Prime-3'Prime). The level was slightly increased at 12 hours post fertilization (31.4 pg) and reached to maximal level (174.2 pg) at 18 hours post fertilization. However, the expressed level was gradually decreased with development of embryo and diminished down to background level at 48 hours post fertilization (Fig. 6). The initiation of transgene expression at around morula stage (6 hours post fertilization in this species) and transient mode with narrow phase showing maximal expression

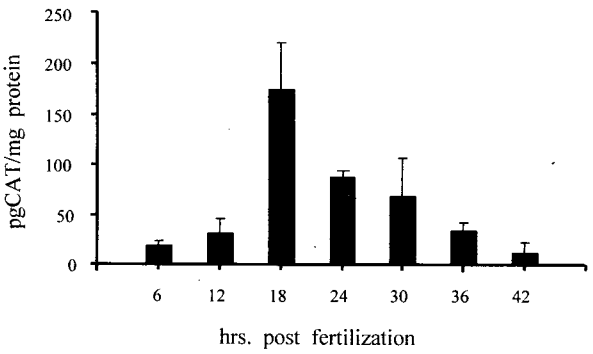

Fig. 6. CAT-ELISA showing the expressed level and time course of transgene expression in early embryos tranformed by lipofected sperm.

were similar to previous reports in medaka (Tsai et al., 1995) and flatfish (Nam et al., 1999b). Further study should be needed to develop an improved transient transgenic system allowing the prolonged expression enabling the monitoring transgene expression in post-hatched larvae.

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