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Gene Transfer and Transient Expression of Foreign DNA in *Limanda yokohamae*

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The utility of RSV-LTR and carp beta-actin promoters was evaluated in a marine flatfish species, *Limanda yokohamae* by examining successful expression of transgenic DNA in muscles (transfected by direct injection) and in early embryos (transformed by lipofected sperm). The expressed pattern of injected DNA in skeletal muscles was dependent on the DNA amount injected. The activity reached to maximal level at 48 hours post injection, and persisted up to 1 month transiently. Gene transfer into early embryo of this species was successfully achieved using lipofected sperm with the efficiency ranging 36.8 to 48.1%. The expression of transgene during embryonic development was shown as stage-specific and transient.

Key words : Gene transfer, Expression, *Limanda yokohamae*

Introduction

Transfer of a gene construct containing novel genetic information into fish has been considered as one of the powerful methods to address variety of fundamental questions on gene regulation and function in vertebrates (Iyengar et al., 1996). Although the stable transgenic line should be established in order to achieve the final goal of transgenesis with facilitating the desired expression and transmission (Nam et al., 2000), however, such a transgenic project should inevitably require an essential information on the functional utility of fusion gene construct in a given species before generating transgenic line.

Based on this reason, the prerequisite study with transient transgenic system should be made to demonstrate whether the fusion construct can be expressed in cells of fish species concerned (Rahman and Maclean, 1992). Furthermore, if the transformation treatment could be performed by non-invasive mass gene transfer, this system will be more valuable by circumventing the traditional microinjection methodology where the number of transformant is limited and skillful techniques are needed.

This study was performed as an initiation of transgenesis of flatfish. The objective of this study is to examine functional expression of transgenic constructs of different promoters (RSV-LTR and carp beta-actin promoters) in *Limanda yokohamae* by direct gene transfer techniques, and also to demonstrate the success of expression of these transgenic constructs in developing embryos of this species.

Materials and Methods

Fish and gamete collection

Experimental juvenile fish for direct gene transfer were maintained in a same tank for 1 weeks until injection procedure. The randomly

taken 12 juvenile fish (average body weight = 45.2±4.8 g) per each injection treatment were given an intramuscular injection. For gene transfer into early embryos, induced spawning was conducted with fully matured females (2.0-3.5 kg body weight) according to the slightly modified procedure described by Park et al. (1994). The good quality of eggs judged based on the examining identical size and color was used for fertilization process.

Plasmid preparation

The plasmid vectors used were pFV4CAT containing carp beta-actin promoter fused to CAT reporter construct, pRSVCAT and pRSVZ consisting rous sarcoma virus promoter fused to CAT, and bacterial beta-galactosidase (lacZ), respectively. Detailed map and information of these expression vectors can be referred to Caldovic and Hackett (1995) for pFV4CAT, Wolff et al. (1990) for pRSVCAT and pRSVZ. Plasmid DNA was isolated by alkaline lysis method (Nam et al., 1999b) and purified with Gene Clean Kit II (Bio 101, USA). Finally, DNA was resuspended in sterile distilled water at a concentration of 1 $\mu g/\mu \ell$.

Direct gene transfer into skeletal muscles

Fish was given 0, 25, 50 or 100 μg of circular plasmid in 50 $\mu \ell$ of injection buffer (10 mM Tris and 1 mM EDTA, pH 8.0). For DNA delivery, a 26-gauge needles connected with 1 $m\ell$ tuberculin syringe was used. Injection was performed with a depth of 50 mm (Nam et al., 1999a). Control fish also treated identically except that they were injected with buffer alone. On completion of injection procedure, the fish were allowed to recover with well-aerated fresh sea water and maintained at 16℃ until analyzed.

Lipofection of sperm and artificial fertilization

The pFV4CAT and pRSVCAT were transferred into *Limanda yokohamae* spermatozoa by liposome treatment to examine the expression vector can also be expressed in embryos of this species. Also to confirm the developmental stage showing the expression of foreign DNA, the expression was visualized by transfection of pRSVZ and immunohistochemical in situ analysis. Milt were washed with cold physiological saline (0.85% NaCl) by centrifugation (3,500 rpm for 5 mins at 4℃) and the concentration of spermatozoa was adjusted to 1.5 x 10^7 cells/$m\ell$. Liposome (positive liposome kit, Sigma Co., USA) was resuspended in sterile distilled water at a concentration of 1 $\mu g/\mu \ell$. To make liposome/DNA complex, 50 μg of plasmid DNA was added to 100 μg of liposome solution and incubated at 41℃ for 10 mins. The DNA/liposome complex formed was cooled to room temperature and sperm (1.0×10^6 cells) was mixed with the complex. Final concentrations of DNA and sperm was 100 $\mu g/m\ell$ and 2.0×10^6 cells in physiological saline, respectively. The mixture (sperm/DNA/liposome) was incubated at 20℃ for 60 min. After completion of incubation, sperm were collected by centrifugation (3,500 rpm for 5 mins at 4℃). Finally the treated sperm were resuspended in 500 $\mu \ell$ of physiological saline used for fertilization. An aliquot of sperm were fertilized with 3,000 eggs, and fertilization, and hatching success were monitored with 3 replicate treatments. The difference in fertilization success was considered to be significant at the level of $P<0.05$.

DNA isolation

DNA was isolated from muscles, sperm and whole embryos as described by Nam et al

(1999b). Muscles were digested in a solution containing 50 mM Tris-Cl, 100 mM EDTA, 150 mM NaCl, 1% SDS and 100 ㎍/㎖ 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). Sperm and embryo samples were digested in 50 mM Tris-Cl, 10 mM EDTA, 100 mM NaCl, 0.5% SDS and 100 ㎍/㎖ proteinase K, pH 8.0 at 55℃. The samples were processed identically as in the case of DNA isolation from muscles. The concentration of DNA was measured by spectrophotometry for the use of polymerase chain reaction and restriction digestion.

Detection of transgene by PCR

Detection of transgene was performed using PCR. PCR was performed using AmpliTaq polymerase (Perkin Elmer, Cetus) and 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. For amplification of CAT segment, two set oligonucleotide primers (FVC-1) 5′-CTATAACCAGACCGTTCAGC-3′ and (FVC-2) 5′-CGCCCCGCCCTGCCACTCATCGCAG-3′ (Nam et al., 1999b). On the other hand, the primers was designed as described by Ueno et al. (1994) for amplifying the lacZ segment (Nam et al., 1998).

RT-PCR analysis

Total RNA was prepared from embryos using the TriPure Isolation Reagent (Boeringer Mannheim Co., Germany) according to the manufacturers recommendation. Purified RNAs were treated for 1 hour with 10 units of RNase-free DNase I per ㎍ of RNA at 37℃. The DNase was inactivated by heating the samples to 72℃ for 10 mins. One ㎍ of RNA so treated was used for reverse transcriptase- PCR (RT-PCR) using RNA PCR Kit Ver. 2 (Takara Shuzo, Japan). Primers used in the RT-PCR were the same as described above.

CAT assay using ELISA

The level of CAT expression was determined using CAT-ELISA Kit (5Prime-3Prime, Inc., USA). Tissues were surgically removed and homogenized in extraction buffer (0.25 M Tris-Cl pH. 7.8 containing 1 mM phenylmethyl- sulfonylfluoride). Two hundred ㎍ of total protein from each tissue were used for ELISA reaction. All procedures including preparation of antibodies and substrates, washing, and color development were followed according to manufacturer's instructions.

X-gal histochemistry for lacZ expression

The specimens were fixed with 1.25% glutaraldehyde in PBS pH 7.4 for 1 hour. Fixed samples were washed with PBS three times at room temperature and incubated in a staining solution containing 1.2 mM X-gal (5-bromo-4-chloro-3-indolyl beat-D-galactopyranoside, 0.1 % Triton X-100, 1 mM $MgCl_2$, 6 mM $K_4[Fe(CN)_6]$, and 6 mM $K_3[Fe(CN)_6]$ at 37℃ for 3 hours. Reaction was stopped by adding 1/10 volume of 10 mM EDTA/PBS solution. The stained samples was washed once with distilled water and twice with PBS.

Results

Detection of transgene by PCR in injected muscles

PCR was performed to confirm that the

muscles injected with plasmid DNA retained the transgene construct when the tissues were sampled for expression assay. The presence of injected DNA was successfully evidenced by the amplification of PCR products with exactly matched size when compared to that of positive amplification using plasmid DNA. The transgene was detectable in all specimens regardless of DNA dosage (Fig. 1).

Expressed levels of different promoters (RSV-LTR vs carp beta actin promoters) with various DNA dosages

Both promoters (RSV-LTR and carp beta-actin promoters) can express their fused structure gene (CAT) in *Limanda yokohamae* muscles, when determined by reverse transcriptase-PCR (RT-PCR) and CAT-ELISA. The clear positive signal obtained in RT-PCR analysis in all three dose levels of DNA injected (25, 50 and 100 μg) at 2 days post injection. The size of RT-PCR product was identical to that of positive amplification (expected size is 562 bp) (figure not shown).

Different promoters induced different levels of expression with DNA dose-dependent patterns in both plasmids. Carp beta-actin promoters revealed higher amount of CAT expression (average 77, 181, and 241 pg/mg total protein in 25, 50 and 100 μg DNA injections, respectively) than RSV-LTR promoters (average 35, 83, and 121 pg/mg total protein in 25, 50 and 100 μg DNA injections, respectively) (Fig. 2).

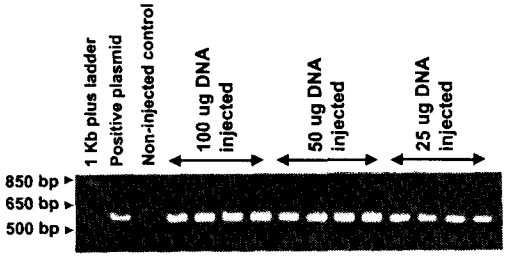

Fig. 1. PCR gel showing CAT segments amplified from DNA of injected and non-injected muscles.

Time course of expression in injected muscles

Figure 5 showed duration of CAT activity in muscles injected with 50 μg of pFV4CAT. The activity was detectable at 24 hours post injection and reached to the highest level (250 pg CAT/mg protein) at 2 days after injection and slightly decreased at 4 day. The activity was markedly decreased at 8 days where only one-third amount of CAT at 2 days post injection was detected. Remaining activity sustained up to 32 days after injection, although the amount was significantly reduced (Fig. 3).

Fertilizing ability of lipofected sperm and efficiency of gene transfer

The fertilizing capacity of sperm did not significantly affected by liposome treatment. The mean fertilization success of untreated

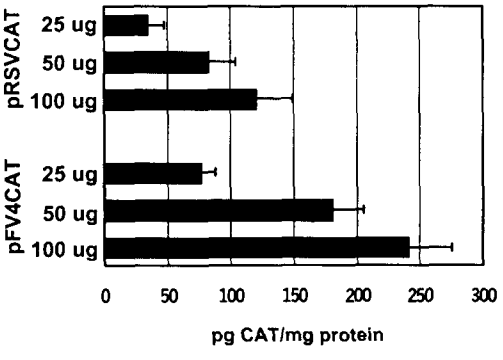

Fig. 2. Expression response of *Limanda yokohamae* muscles to RSV-LTR and carp beta-actin promoters linked to CAT reporter construct. CAT-ELISA was performed at 2 days post injection. Non-injected muscles showed negligible level of background.

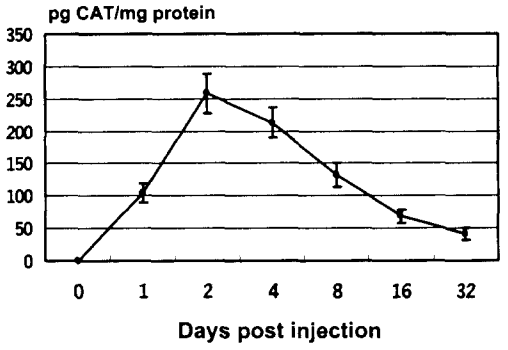

Fig. 3. Time course of expression after direct intramuscular injections of pFV4CAT.

control sperm (91.3±4.8%), sperm treated with only liposome (91.0±5.5%) and treated with only DNA (90.5±4.1%) was similar, and no significant difference was detected (P>0.05). Also the sperm treated with liposome/DNA complex showed no difference in fertilization (89.9±6.7%) success (P>0.05). The lipofected sperm could successfully delivered transgene to embryos via fertilization process. The PCR assessment for detection of transgene in hatched larvae revealed that the overall efficiency of gene transfer was slightly variable among the gene constructs (48.1% for pFV4CAT, 41.2% for pRSVCAT and 36.8% for pRSVZ in average), however, no direct relationship was observed between incidence of gene transfer and transgene construct.

Expression of transgene in early embryos

The expressed pattern of foreign DNA during embryonic development of *Limanda yokohamae* was stage-specific. In our assay conditions of time course (0, 6, 12, 18, 24, 30, 36, 48, and 60 hours post fertilization at 16℃), the initiation of considerable CAT expression was detected at 18 hours after fertilization (52 pg CAT for pRSVCAT and 71 pg for pFV4CAT), reached at the highest expression at 24 hours (98 pg CAT for pRSVCAT and 164 pg for pFV4CAT), decreased at 30 hours (49 pg CAT for pRSVCAT and 88 pg for pFV4CAT), and the most activity was diminished after 36 hours post fertilization (Fig. 4a, histogram of pRSVCAT is not shown). The expression within narrow window during developmental stage was confirmed by immunohistochemical analysis of pRSVZ-transfected group (Fig. 4b).

Discussion

The successful expression of foreign DNA driven by carp beta-actin promoter and RSV-LTR was examined in *Limanda yokohamae* muscles and embryos. The present study demonstrate that these two promoters (and regulatory sequences) can induce the expression of linked structure genes in *Limanda yokohamae*, thereby suggest they can be used as functional transgenic vectors for transgenesis in this species.

The present direct gene transfer into skeletal muscles showed the results of general coincidence with the many previous reports on other

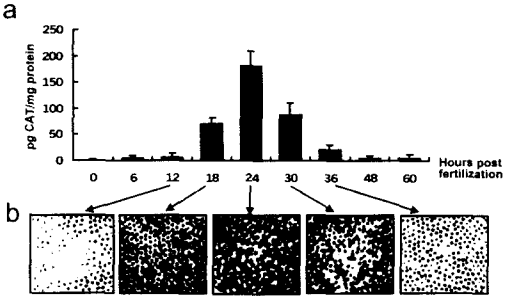

Fig. 4. ELISA of expressed CAT (a) and immunohistochemical analysis of expressed LacZ proteins (b) in early embryos of *Limanda yokohamae* developed from the eggs fertilized with lipofected sperm.

fish species in aspect of different responses to different promoters (Anderson et al., 1996a; Nam et al., 1997), DNA dose expression (Davis et al., 1993), and transient status of expression (Anderson et al., 1996b; Tan and Chan, 1997). The different expressed level depending on the promoters in fish muscles is well known in many of direct gene transfer studies and this capacity has made this simple technique as one of powerful method for evaluating functionality and strength of promoters (Verma and Somia, 1997). The DNA dosage-dependent data in present study support the saturable mechanism of DNA uptake in muscles cells, as suggested by Wolff et al. (1990). The injected DNA can persisted with detectable expression as long as 1 month, and it suggest that the present transgenic vector can be used for DNA therapeutic approach in this species.

The lipofected sperm showed no difference in ability to fertilize the eggs, and the survival of resulted embryos was also equal to that of control groups. This result was similar with the previous finding that has been made in lipofection of mud loach (Nam et al., 1996). Association rate between sperm and DNA was enhanced by liposome treatment. The present result demonstrate the main principle of lipofection: the negatively charged DNA would be coated by large amount of positively charged liposome and should form positively charged liposome/DNA complex. Then positively charged complex will react the negatively charged cell membrane. Increase of association with DNA by liposome treatment has already been reported in mammalian sperm (Lavitrano et al., 1992), and also in our previous report on mud loach (Nam et al., 1996).

Lipofected sperm successfully delivered the plasmid DNA into oocytes via fertilization. Even though the transformation efficiency was readily high up to 50% under our condition, the the status of delivered DNA was thought to be mainly restricted to extrachromosomal copies. It is well known that the major limitation of lipofection is lack of integration in fish (Szelei et al., 1994).

Expression was detected by CAT-ELISA and X-gal histochemistry. Transgene expression detected from 18 to 36 hours after fertilization (corresponding to morula stage to early gastrula), and this finding was in accordance with many previous reports where the expression was detected in this developmental phase. However, the exact mechanism responsible for high expression only in narrow developmental phase has not been understood yet. One possible and plausible explanation is the copy number dependent expression of extrachromosomal plasmid where the delivered plasmid was replicated in early phases, and degraded and diluted with cell division. Replication of injected plasmid DNA in early phase and rapid degradation during subsequent cell division has also been reported in many transgenic reports in fish (for review, see Pandian and Marian, 1994).

From the study, it is demonstrated that structure gene coding the desired proteins can be expressed in *Limanda yokohamae* by linking with the carp beta-actin promoter (and also with RSV-LTR). Based on the present study, stable transgenic line ensuring the stable expression and transmission should be generated with various purposes including the research for developmental genetics and establishment of economically favorable flatfish strain.

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