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Production of Transgenic Olive Flounder (*Paralichthys olivaceus*) I. *In vivo* Gene Transfer in Olive Flounder by Direct Intramuscular Injection

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외래 유전자가 이식된 넙치(*Paralichthys olivaceus*) 생산 I. 근육내 유전자 직접 주입법을 통한 *in vivo* 유전자 이식

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The carp β-actin regulatory sequences and RSV/LTR promoter were tested whether they are functional to express linked structure gene (chloramphenicol acetyltransferase, CAT) in olive flounder (*Paralichthys olivaceus*) by determining the patterns of gene expression following intramuscular in vivo direct injection. The injection experiments with various concentrations of both pRSVCAT and pFV4CAT clearly revealed the effectiveness of DNA dosage on expression of CAT. The increase of CAT activity was linear in both plasmids, and maximal CAT activity was obtained with 100 μg of pFV4CAT injection. The amounts of CAT expression with pFV4CAT-injected fish were higher than those with pRSVCAT-injected fish. CAT activity was readily detectable as early as one day after injection, slightly increased at day 2, and declined over time. Most amount of DNA intramuscularly injected into olive flounder muscles persisted extrachromosomally without showing any integrated or replicated form in vivo.

Key words : Olive flounder, Direct intramuscular injection, CAT, Expression

Introduction

Direct injection of purified DNA into animal muscle has been given much attention not only as an useful method for assaying promoter strength and transient expression but also as a potential mean for genetic immunization and gene therapy (Tan and Chan, 1997). One of major advantages of this technology is its simple and easy proce-

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dure to express foreign DNA construct, thus could obviate the time-consuming and laborious procedures involved in making transgenic animal (Rahman and Maclean, 1992).

Since Wolf et al. (1990) developed this technique in mice, a lot of experiments of direct gene transfer have been made in various fish species including carp (Hansen et al., 1991), tilapia (Rahman and Maclean, 1997), rainbow trout (Anderson et al., 1996 a), and zebrafish (Tan and Chan, 1997), as well as in a number of mammalian systems (Davis et al., 1993 ; Wolff et al., 1991, 1992).

It is necessary to use gene constructs in which the promoter will drive expression of the linked protein coding sequence in the chosen fish. The selection of appropriate promoter and regulatory part is prerequisite for developing transgenic line in a species concerned (Rahman and Maclean, 1992).

Carp β-actin gene including its promoter and regulatory region was isolated by Liu et al. (1990), and several types of construct containing its regulatory sequences have been prepared as an all-fish transgenic vector (Caldovic and Hackett, 1995). The Rous sarcoma virus long terminal region (RSV /LTR) is the most versatile promoter used in transgenic studies including mammal, plants and fish. Although these sequences have been successful to express transgene in several fish species so far (Caldovic and Hackett, 1995 ; Iyengar et al., 1996), they have not been tested in olive flounder (*Paralichthys olivaceus*). Thus, the validity of usefulness of these regulatory sequences should be challenged.

The objective of this study is to test if carp β-actin regulatory sequences and RSV/LTR promoter are effective for expression of linked structure gene (CAT) in olive flounder by determining the patterns of gene expression introduced *in vivo* by intramuscular injection.

Materials and methods

Fish maintenance

The 4-month-old olive flounder (average body weight : 23.4± 4.3g) maintained in Genetic Engineering Laboratory, Pukyong National University were used for gene transfer experiments. Each group of fish were reared in 500-ℓ of well aerated aquaria in which the temperature was 23℃.

Plasmids

The gene construct pFV4CAT were generously provided by Dr. Perry B. Hackett, University of Minnesota, USA. The construct pRSVCAT was kindly provided by Dr. Jon A. Wolff, University of Wisconsin, USA. Plasmids were extracted by conventional alkaline lysis method, and purified using Gene Clean Kit (BIO 101, USA). The purified plasmids were concentrated into 1xTE buffer (10 mM Tris-Cl pH 8.0 and 1 mM EDTA), and the amount of DNA for injection was measured spectrophotometrically.

Intramuscular injection

The DNA in aliquots equivalent to 25, 50 and 100 μg was suspended in 30 μl of 1xTE buffer. For delivery, a 26-gauge needle and a 1 mℓ tuberculin syringe were used. In case of pRSVCAT injection, the linear form of plasmid were also prepared by *Eco*RI digestion. Fish were anesthetized by immersion in 200 ppm of lidocaine solution, and then the DNA was injected immediately into the muscle to a depth of 50 mm. Control fish were also treated identically except that they were injected with 1xTE buffer alone.

DNA isolation

DNA were isolated from muscles around areas of injected site. The tissue was sliced in 2-3 mm pieces, and incubated in a solution of 50 mM Tris, 5 mM ethylendiaminetetracyclicacetic acid, sodium salt (EDTA-Na), pH 8.0, 150 mM NaCl, 0.5% sodium dodecyl sulfate (SDS), 200 μg/mℓ proteinase K. Samples were digested at 55℃ for 16 hours, extracted once with TE-saturated phenol (pH 8.0) and twice with phenol/chloroform (1 : 1). The DNA then precipitated with equal volume of isopropanol, and wa-

shed with 70% ethanol. DNA pellets were dissolved in 1xTE (10 mM Tris, 1 mM EDTA-Na, pH 8.0).

Polymerase chain reaction

About 0.5 μg of boiled genomic DNA were used in a PCR reaction. PCR reaction mixture contained 20 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 25 mM KCl, 100 μg/mℓ gelatin, 20 pmoles each PCR primer, 50μM each dNTPs, 2.5 U *Taq* DNA polymerase (Perkin Elmer Co.). The reaction was carried out at 94℃ for 1 min, 60℃ for 30 sec, 72℃ for 1 min for 30 cycles with 2-min initial 94℃ denaturation step. Primer design was as same as that described by Nam et al. (1997) in order to amplify the internal fragment of CAT sequence.

Southern blot

For southern blot analysis, 20 μg of muscle DNA was digested with *Eco*RI (for pRSVCAT injected fish) or *Eco*RV (for pFV4 CAT injected fish). The Digested DNA was separated by an electrophoresis on a 0.8% agarose gel. The gel was de-purinated, denaturated and neutralized were done as described by manufacturer's recommendations of non-isotopic labeling and detection kit (BM Co., Germany). DNA on a gel was transferred to a positive nylon membrane using a capillary method. The membrane was hybridized with a 11-digoxy-dUTP labeled 0.8 kb CAT fragment. Hybridization and detection were carried out using non-isotopic labeling and detection kit (BM Co., Germany) according to manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed with the muscle tissue from DNA-injected and TE-injected fish. Total RNA was extracted and purified by RNAzol method according to the procedure of manufacturer's recommendations (Biotecx Lab. Inc., USA), and 1 μg of total RNA from each tissue was reverse transcribed. RT reaction and PCR procedures were carried out using RNA PCR Kit Ver. 2 (Takara Shuzo Co., Ltd., Japan) as described in manufacturer's protocol. PCR primers used in RT-PCR were same as ones used in detection of transgenic individuals.

Enzyme-linked immunosorbant assay of CAT

To examine the patterns of CAT expression, enzyme-linked immunosorbant assay (ELISA) was performed using rabbit polyclonal antibody specific CAT protein. Muscles from each experimental fish including control individuals were surgically removed and homogenized in extraction buffer (0.25 M Tris-Cl pH. 7.8 containing 1 mM phenylmethylsulfonylfluoride). Preparation of antibodies, washing, color development and calculation were done as described by manufacturer's instructions of CAT ELISA Kit (5Prime-3Prime, Inc., USA). The concentrations of CAT protein in different tissue extract were determined from the standard curve. The levels of gene expression was evaluated as values of injected fish subtracted by ones of control fish.

Statistics

The differences between means of ELISA were assessed by using ANOVA test. $P<0.05$ was considered to be statistically significant.

Results

Comparison of circular and linear form of plasmid pRSVCAT

The plasmid pRSVCAT was digested with *Eco*RI to obtain the linear construct. And the effect of plasmid conformation on gene expression following direct injection of either 50 μg of circular or linear DNA was compared at 2 days after injection. As shown in Table 1, there was no significant difference in CAT activity between circular and linear form, although circular DNA revealed slightly higher activity than linear construct.

Comparison of different promoters (RSV-LTR and carp β-actin promoters) and DNA dosage

Table 1. Comparison of plasmid form on gene expression following intramuscular injection in olive flounder

Plasmid form	Number of fish examined	Average amount of CAT expression (pg/mg protein)	Range
Circular	4	8.46	(6.84-9.87)
Linear	4	8.26	(4.81-11.94)

The expression was assessed by ELISA of muscles injected with 50 μg of pRSVCAT at 2 days after injection.

A comparison of strength of different promoters and regulatory parts to express the CAT was made with 0, 25, 50 and 100 μg of DNA injected into skeletal muscle of olive flounder. Considerable amount of CAT expressions were observed for injections of both plasmid constructs based on ELISA at 2 days post injection, even though relatively high variations were found. The only little negligible background was detected in TE-injected fish (Fig. 1). The injection experiments with varying concentrations of both pRSVCAT and pFV4CAT clearly revealed the effectiveness of DNA dosage on expression of CAT. The increase of CAT activity was linear in both plasmids, and maximal CAT activity was obtained with 100 μg of pFV4CAT injection. The amounts of CAT expression with pFV4CAT-injected fish were higher than those with pRSVCAT-injected fish in all 3 different DNA dosage levels.

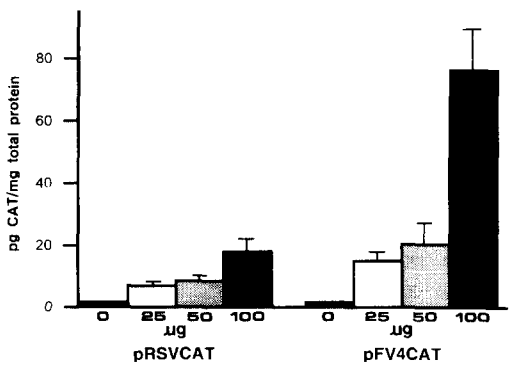

Fig. 1. Comparison of different promoters and DNA dosage. The CAT activity in muscle tissue was determined at 48 hours post injection. Each bar represents the average of four fish, and the standard deviation is shown for each data by T bars.

Duration of CAT activity in DNA-injected fish

The time course of pFV4CAT expression in olive flounder skeletal muscle were monitored up to 24 days after injection. As shown Fig. 2, CAT activity was readily detectable even as early as day 1. The activity was slightly increased at day 2, and gradually declined over time. The CAT activity had diminished to approximately 10% of maximum by 16 days post injection, and almost lost by 24 days after injection to background level.

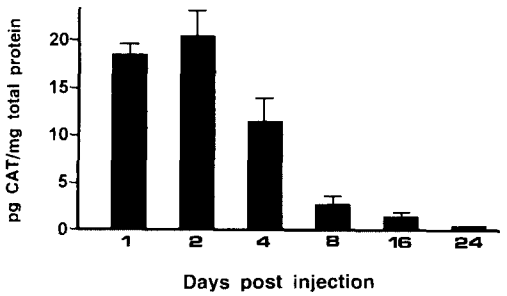

Fig. 2. Duration of CAT expression. Fish were injected with 50 μg of pFV4CAT, and sampled on the indicated days to determine the activity based on ELISA.

Confirmation of mRNA synthesis by RT-PCR

The capacity of the plasmids pRSVCAT and pFV4CAT to express the CAT mRNA in olive flounder cells was assessed by RT-PCR. It was found that the size of RT-PCR products from DNA-injected fish was exactly matched to that from positive plasmid containing CAT sequence. No positive signal was detected in muscle tissue injected with 1xTE buffer alone (Fig. 3).

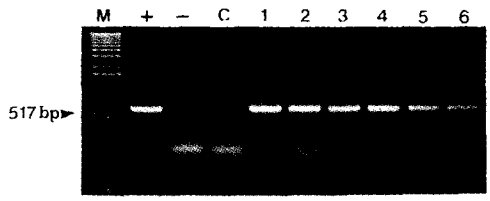

Fig. 3. Results of RT-PCR of muscles injected with pRSVCAT or pFV4CAT. M, 1kb ladder ; −, negative blank ; +, pRSVCAT plasmid ; C, muscle injected with TE alone ; 1-3, muscles injected 25, 50, and 100 μg of pRSVCAT, respectively ; 4, blank ; 5-7, muscles injected 25, 50, and 100 μg of pFV4CAT, respectively.

PCR and Southern blotting analysis

The presence of plasmid injected was confirmed by PCR. The presence of injected DNA containing CAT sequence in muscle DNA was clearly evidenced by the expected sizes of amplified fragments at 72 hours post injection. The injected DNA was detected in all specimens injected regardless of plasmid type and DNA dosage (Fig. 4).

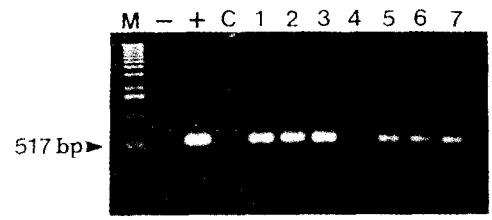

Fig. 4. Detection of transgenic constructs by PCR. M, 1kb ladder ; +, pRSVCAT plasmid ; −, negative blank ; C, muscle injected with TE alone ; 1-3, muscles injected 25, 50, and 100 μg of pRSVCAT, respectively ; 4-6, muscles injected 25, 50, and 100 μg of pFV4CAT, respectively.

The fate of injected plasmids in muscle cells of olive flounder was examined by Southern blotting analysis. The appearance of hybridized bands in expected positions (2.3 kb in pRSVCAT and 6.2 kb in pFV4CAT) were obtained in all specimens tested (Fig. 5).

Discussion

The expressions of foreign DNA constructs (pRSVCAT and pFV4CAT) following

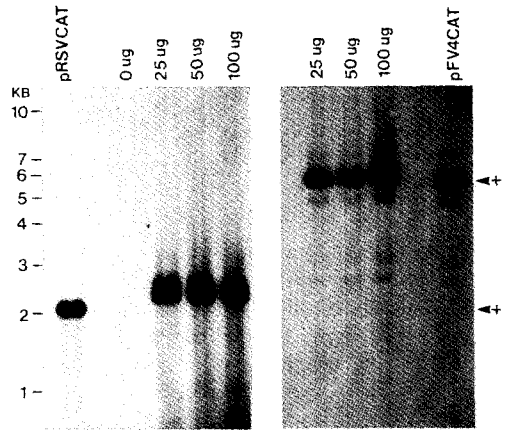

Fig. 5. Southern blotting analysis of DNA isolated from muscles of fish injected with pRSVCAT or pFV4CAT. Arrows marked with + indicate the each expected size yielded by EcoRI and EcoRV digestion of pRSVCAT and pFV4CAT, respectively.

in vivo intramuscular injection were examined in olive flounder. The results obtained suggest that the promoters (RSV-LTR and carp β-actin promoters) challenged in this study could work relatively well in olive flounder to express the linked protein coding sequence based on CAT-ELISA and RT-PCR.

The level of gene expression based on direct injection of DNA was found to be dependent on the quantity of the DNA. The DNA-dosage dependent expression is consistent with other previous reports of direct introduction of DNA in mice (Wolff et al., 1990). carp (Hansen et al., 1991) and rainbow trout (Anderson et al., 1996). Dose-dependent studies suggest that the uptake of injected plasmid DNA by muscles appears to occur through a saturable mechanism (Wolff et al., 1990 ; Davis et al., 1993). Also as in other studies mentioned above, olive flounder cells respond to different promoters with different levels of expression. The carp β-actin promoter showed to have stronger effect in olive flounder than RSV-LTR. Thus, direct gene

transfer technique in fish could provide a powerful and simple approach for functional analysis of untested promoters before the creation of transgenic fish line.

The CAT expression in flounder muscles was proven to be only transient ; the activity was declined over time and completely diminished to background level by the 24 days post injection. It is unclear whether the DNA was slowly degraded over time or the growth of the tissue at the injection was "diluting" the DNA expression per given weight of tissue. The transient expressions of injected DNA have already been reported in most cases of direct injection experiments (Anderson et al., 1996b).

The most DNA introduced by intramuscular injection into olive flounder muscles persist in an extrachromosomal state with nonintegrated and nonreplicated form *in vivo*. The extrachromosomal persistence of injected DNA was not surprising because many reports has been made in other fish species (Anderson et al., 1996a ; Tan and Chan, 1997) as well as mammal (Wolff et al., 1990). Further study should be needed to determine the mechanism for DNA-uptake and DNA stability (rate of degradation of DNA).

In summary, the present study demonstrates that olive flounder muscle has a capacity to take up and express intramuscularly injected plasmid DNA driven by carp β-actin promoter and RSV-LTR. This system may provide a simple experimental tool for the study of transcriptional regulatory promoters *in vivo* andthe functional analysis of proteins.

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