Journal of Aquaculture 12 (1) : 71~77 (1999)

Injection Media Affecting Expression of Transgene Introduced by Direct *in vivo* Injection into Olive Flounder (*Paralichthys olivaceus*) Muscle

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The potential utility of injection media (sucrose, PEG, and liposome) was demonstrated for direct gene transfer into olive flounder (*Paralichthys olivaceus*) muscles. Based on the use of sucrose (final conc. 20%), PEG 8,000 (final conc. 10%) or liposome (twice ug of DNA injected), the present injection strategy significantly improved the level of transgene expression as well as persistent duration of expression. The increased amounts of expression in DNA injection with sucrose, PEG, and liposome were as high as from 2.1 to 4.9-folds of conventional TE-based DNA injection. The best result was obtained from injections of liposome-encapsulated DNA in which the expression was detectable at least 32 days after injection when compared to only 8-16 days from TE-based injections.

Key words : Direct gene transfer, Injection buffer, CAT expression, Oliver flounder

Introduction

Direct injection of DNA into the somatic tissues (especially into skeletal muscles) of animal is a powerful strategy for genetic immunization and gene therapy against a wide range of pathogens (Anderson et al., 1996a; Tan and Chan, 1997). This relatively simple technique has also been given much attention as an useful tool for monitoring promoter strength in transgenic studies (Rahman and Maclean, 1992).

Our previous study has already demonstrated the successful expression of reporter transgene introduced by *in vivo* intramuscular injection of purified plasmid DNA into olive flounder (*Paralichthys olivaceus*) muscles (Nam et al., 1997). Although this gene transfer study using naked DNA has been encouraging with regard to transgenic experiment in flounder species, the amount expressed foreign protein in muscles transfected *in vivo* have been very low, and the period for detectable transgene expression was as short as only 8-16 days after injection. This low efficiency and such a short duration of expression might hinder efforts to achieve successful genetic immunization and to study the clinical effect of expression of transfected genes in intact fish.

The objective of this study is to examine the potential utility of several injection media as means improving the level and period of transgene expression in direct *in vivo* gene transfer into olive flounder.

*This study was conducted by the financial support of the Korea Research Foundation made in the Program Year 1998.

Materials and Methods

Vector and plasmid preparation

The recombinant vector (pFV4CAT) containing the chloramphenicol acetyl transferase (CAT) reporter construct has been kindly given by Dr. Hackett (University of Minnesota, USA) and the plasmid preperation was followed as previously described (Nam et al., 1998 & 1999). Briefly, the vector consists of CAT sequence driven by carp beta-actin promoter and regulatory region. Plasmid DNA was isolated by conventional alkaline lysis method and purified using Gene Clean Kit (Bio101, USA). The purified DNA was finally dissolved in double distilled water and the amount was spectrophotometically measured for direct injection.

Injection media

To examine the functional utility of selected chemicals including sucrose, polyethylene glycol, and liposome as an injection buffer for direct gene transfer into this species, we designed the injection experiments as below:

Control injections : TE (10 mM Tris, 1 mM EDTA, pH 8.0), 20% sucrose (Sigma Co., USA), 10% PEG 8,000 (Sigma Co., USA), or 100 μg liposome (liposome positive kit, Sigma Co., USA) alone

TE injection : TE buffer+25, 50 or 100 μg of plasmid DNA

Sucrose injection : 20% sucrose+25, 50 or 100 μg of plasmid DNA

PEG injection : 10% PEG+25, 50 or 100 μg of plasmid DNA

Liposome injection : 50, 100 or 200 μg of liposomes+25, 50 or 100 μg of plasmid DNA, repectively.

Fish and direct injection

All experimental fish were maintained in a same tank for 2 weeks until injection procedure. The randomly taken 12 juvenile fish (average body weight = 50.2±6.5g) per each injection treatment were given an intramuscular injection. Fish were anesthetized using lidocaine (200 mg/ ℓ) and immediately the 0, 25, 50 or 100 μg of circular pFV4CAT in each injection buffer (50 $\mu\ell$) were delivered into the skeletal muscles to a depth of 5 mm. Control fish also treated identically except that they were injected with each buffer alone. On completion of injection procedure, the fish were allowed to recover with well-aerated fresh sea water and maintained at 20℃ until analyzed.

DNA isolation

DNA was isolated from the muscles of injected and non-injected fish as described by Nam et al. (1997). Briefly, the tissue was sliced in 2-3 mm pieces and digested in a solution containing 50 mM Tris-Cl, 5 mM EDTA, pH 8.0, 150 mM NaCl, 0.5% SDS, and 200 μg/ml proteinase K at 55℃ for 16 hours. Disgested samples were extracted using TE-saturated phenol (pH 8.0), phenol/chloroform (1:1=v:v) and chloroform/isoamyl alcohol (24:1=v:v), and then DNA was precipitated by 2 volumes of ethano. The DNA pellet was washed using 70% ethanol wash, and finally resuspended in double distilled water.

Polymerase chain reaction (PCR)

A 0.5 μg of DNA was boiled for 2 min and immediately cooled on ice until used for PCR assessment. Reaction mixture contained 20 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 25 mM KCl, 100 μg/ml gelatin, 20 pmoles each primer 50 μM

each dNTPs, and 2.5U Tag DNA polymerase (Perkin Elmer Co., USA). A 2-min initial 94℃ denaturation step was followed by 30 cylcles of 94℃ for 1 min, 60℃ for 30 sec and 72℃ 1 min 30 sec. Two oligonucleotide primers (FV-1 & FV-2) were designed in order to amplify an internal CAT sequence as described by Nam et al. (1999). The PCR product amplified was resolved on a 1.2% agarose gel and the its size were assessed using ethidium bromide staining.

Reverse transcriptase-PCR

To confirm the expression of transgene introduced, the reverse transcriptase-PCR was performed. Total RNA was extracted from muscle tissues using the Tripure Reagent (Boeringer Mannheim Co., Genrmany) accoriding to manufacturer's instructions. One μg of total RNA was reverse transcribed using RNA PCR kit Ver. 2 (Takara Shuzo Co., Japan). To amplify the CAT sequence from reverse transcribed cDNA pool, two oligonucleotide primers (FV-1 and FV-2) were used in PCR reaction. Thermal cycling condition was same as that used in transgene detection described above. The amplified PCR products were separated on a 1.2% agarose gel. The gel was processed for transfer of amplified DNA to positively charged nylone membrane as described by instruction manual of DIG Non-isotopic Labelling and Detection Kit (BM, Co.). Internal CAT fragment was labeled with 11-digoxygenin-dUTP and used for hybridization probe. Prehybridization, hybridization, stringent washes and signal detection were also carried out according to the manual of the kit.

Enzyme-linked immunosorbant assay of CAT

The expression pattens of injected vector in olive flounder muscles were monitored based on CAT-ELISA using rabbit polyclonal antibody specific to CAT protein. Muscles around the injected sites of each individual were surgically removed and homogenized in a buffer containing 250 mM Tris-Cl pH 7.8 and 1 mM phenyl-methylsulfonylfluoride (PMSF). Cell debris were removed by centrifugation (X 1,000g for 10 min) and the supernatant were subjected to ELISA procedure. Protein concentration was determined using Protein Assay ESL Kit (BM Co.,) and 200 μg of total protein was applied in each well of micoplate. Preparation of antibody, washing, substrate reaction, and calculation were made as described by manufacturer's instruction of CAT ELISA Kit (5Prime-3Prime, Inc., USA). The concentration of CAT protein expressed was deterimined from a standard curve.

Results

Detection of transgene by PCR and transgene expression by RT-PCR

The presence of pFV4CAT in muscles injected was clearly evidenced by amplified fragments of which size was exactly matched to that of positive plasmid (pFV4CAT). All muscle tissues from DNA-injected individuals represented the positive signal of identical molecular weight size at 72 hours post injection (Fig. 1).

Expression of injected DNA sequence was demonstrated by reverse-transcriptase PCR and hybridization of RT-PCR products to internal CAT fragment. pFV4CAT plasmid vector injected using different injection media (TE, sucrose, PEG and liposome) could successfully express the CAT mRNA in olive flounder muscles. The amplified product from reverse transcribed CAT cDNA showed an eaqual molecular

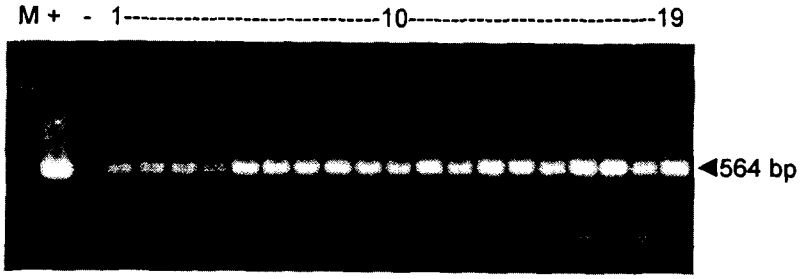

Fig. 1. Ethidium bromide-stained agarose gel (1.2%) showing PCR detection of transgene (pFV4CAT) in olive flounder muscles. This photograph shows an example of PCR assay with muscles injected with 25 ug DNA. M, 1kb ladder molecular weight size marker; +, postive control (pFV4CAT); -, non-injected control muscle; 1-4, muscles injected with pFV4CAT in TE; 5-9, muscles injected with pFV4CAT in sucrose; 10-14, muscles injected with pFV4CAT in PEG; 15-19, muscles injected with pFV4CAT encapsulated in positive liposome. The expected size of PCR product is 564 bp.

size regardless of the injection media. However the signal intensity of RT-PCR product was slightly different among the injection buffer in which the liposome-mediated injection revealed the strongest intensiy (Fig. 2).

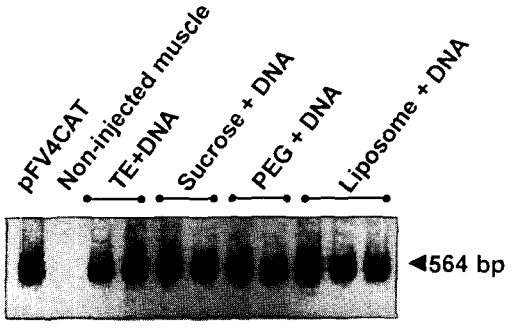

Fig. 2. Southern blot of RT-PCR products. Five ul of amplified PCR products were resolved on a 1.2% agarose gel, transferred onto positively charged nylon membrane and hybridized with internal CAT fragment which had been labeled using digoxygenin-11-dUTP.

Expressed amount of CAT depending on injection buffer

The level of CAT expression at 48 hous after injection of 0, 25, 50 or 100 μg DNA in TE buffer showed clear dose-dependent pattern of expression. The background signal from control injections (except DNA) injection were negligible. Injections with e 25, 50 and 100 μg DNA with TE revealed the average 18.1, 22.3 and 68,2 pg CAT/mg protein, respectively.

The amounts of CAT expression in injections including sucrose, PEG and liposome were also dose-dependent, however, the actual amounts of CAT protein expressed were significantly higher when compared to those of TE-based injections in each DNA dose level. In case of sucrose-based injections, 38.3 pg CAT (for 25 μg pFV4CAT), 52.8 pg CAT (for 50 μg pFV4CAT), and 151.7 pg CAT (for 100 μg pFV4CAT) were detected per mg total protein. PEG-mediated injections showed more higher level of CAT expression than sucrose-based injections in every dose of DNA (25, 50 and 100 μg). The range of improved amounts were from 2.8 to 3.7-folds of those of TE-based injections. Injection of liposome-encapsulated DNA induced the highest improvement of expression among other injection media. Increased amount of CAT protein expressed is 2.8-folds (for 25 μg DNA), 4.6-folds (for 50 μg DNA), and 4.9-folds (for 100 μg DNA) of TE-based injections (Table 1).

Table 1. Effects of injection buffers on expression of transgene (pg CAT/mg protein) introduced by direct in vivo injection into olive flounder muscles, based on CAT-ELISA. Each average value (ranges) was estimated from analysing of 12 fish

Amount of DNA injected	Injection buffer*			
	TE	Sucrose	PEG	Liposome
0**	1.6 (0.3 ~ 2.1)	0.9 (0.4 ~ 2.0)	1.1 (0.2 ~ 2.0)	1.3 (0.2 ~ 2.2)
25	18.1 (13.7 ~ 22.9)	38.3 (29.7 ~ 44.4)	50.0 (41.8 ~ 62.1)	51.5 (42.1 ~ 60.7)
50	22.3 (16.6 ~ 28.1)	52.8 (47.1 ~ 60.0)	78.9 (66.6 ~ 87.2)	101.9 (89.7 ~ 121.0)
100	68.2 (51.9 ~ 77.3)	151.7 (143.9 ~ 163.5)	253.2 (221.1 ~ 287.5)	334.2 (291.4 ~ 368.5)

* The composition of injection buffer is given in Materials and Methods.
** Basal background value in ELISA assay.

Improved time course of transient expression

The duration of CAT expression in olive flounder muscles was monitored up to 32 days after injection of 50 μg plasmid for each injection buffer. Muscles injected DNA resuspended in TE initiated CAT expression at 24 hous post injection (14.8 pg CAT/mg protein), and showed a peak at 2 days (18.1 pg CAT). This CAT expression was rapidly decreased to background level with days and no difference was detected at 16 days post injection (Fig. 3a).

The initiation of CAT expression in muscles injected with DNA in 20% sucrose (2 days post injection) was different from those of other injection media (1 day post injection). The expressed amount of CAT showed a peak at 2-4 days (47.8~55.3 pg CAT), decreased with time and then most CAT activity were diminished at 24 days after DNA delivery (Fig. 3b).

The level of CAT expression was more improved by substituting 10% PEG 8,000 for 20% sucrose. The peak of CAT activity (78.9 pg) was achieved at 2 days post injection. Although the expressed pattern of PEG-based injection was also transient, the time course of expression was more sustainable; the CAT activity could be detectable until at 32 days post injection (Fig. 3c).

Most successful expression was obtained in liposome-based direct gene transfer. The actual amount of CAT in olive flounder muscles injected with DNA in liposome was higher than those of injections with any other injections media. The highest amount CAT expression (100 pg CAT/mg protein) was detected in 2-4 days post injection, and the considerable amount of expression was retained at 32 days after DNA delivery. However, it was not exception for transient expression even in this case (Fig. 3d).

Discussion

The use of gene therapy and genetic immunization for commercially important fish species such as olive flounder require an efficient gene

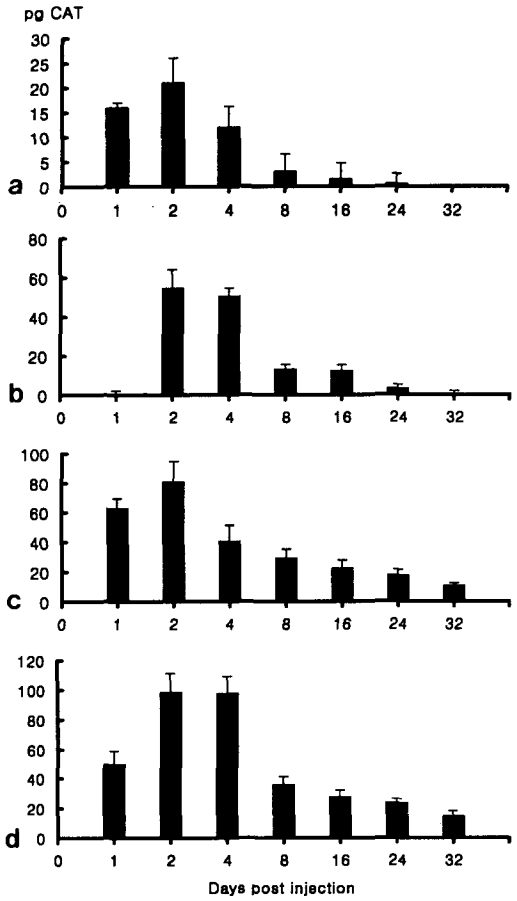

Fig. 3. Analysis of time course of expression (pg CAT/mg protein) based on CAT-ELISA. Fifty μg of circular pFV4CAT were injected into skeletal muscles with a) TE (10 mM Tris, 1 mM EDTA, pH 8.0), b) 20% sucrose, c) 10% PEG, and d) 100 μg of positive liposome.

expression as well as a safe and practical delivery system (Guzuman et al., 1993; Verma and Somia, 1997). Our previous reports have suggested that direct gene transfer of purified DNA led to detectable gene transfer and expression in muscle cells of olive flounder. Although this provided a practical and relatively convenient delivery system, the reported efficiency and duration of CAT activity after delivery appeared to be too low and too short to allow sufficient biological effects of heterologous gene expression for genetic immunization or gene therapy (Nam et al., 1997). In the present study, the potential utility of injection media (sucrose, PEG, and liposome) that could improve transgene expression in direct gene transfer into olive flounder muscles was demonstrated.

Based on the substitution of sucrose, PEG or liposome for our previous injection buffer (TE, 10 mM Tris, 1 mM EDTA, pH 8.0), the present direct injections successfully accelerated transgene expression. The increased amounts of expression in injection with sucrose, PEG 8000, and liposome were as high as from 2.1 to 4.9-folds of TE-based injection, respectively. However in contrast, our results are inconsistent with previous reports by Wolf et al. (1991) who concerned the effects of various composition of injection buffer including sucrose on direct gene transfer into rodent muscle. It is difficult to compare directly the present data of olive flounder with those of rodent, although it is believed the species difference (fish to mammal) might play a major role for the reason responsible for the different effect of sucrose buffer.

The persistance of expression could also be significantly improved in olive flounder muscles by use of present injection media. The best result was obtained by injection of liposome-encapsulated DNA in which the expression was detectable at least 32 days after injection when compared to only 8-16 days of persistent expression in our previous TE-based injection. The sustainable period of DNA sequences introduced via direct injection into skeletal muscles were quite variable from only 2 days in tilapia (Rahman and Maclean, 1992) to as

long as 1 year in zebrafish (Tan and Chan, 1997). From existing data, it is not clear whether the remaining CAT activity at 32 days post injection (in PEG and liposome-based injections) could show a long-term stability as in the case of zebrafish (Tan and Chan, 1997). Thus our on-goining study is to test several potential construct containing viral or glycoprotein genes in order to verify that expression of possible antigen persisting to 32 days post injection would be sufficient for activating the biological pathway for genetic immune response.

Further studies will be carried out to make a fine tuning of chemical concentrations and also to illuminate the molecular mechanism of such chemicals for expression of transgene in direct gene transfer.

References

Anderson, E. D., D. V. Mourich, and J. C. Leong, 1996a. Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. Mol. Mar. Biol. Biotechnol., 5 : 105-113.

Anderson, E. D., D. V. Mourich, S. C. Fahrenkrug, S. Lapatra, J. Shepherd and J. A. Leong, 1996b. Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Mol. Mar. Biol. Biotechnol., 5 : 114-122.

Guzman, R. J., Lemarchand, P., Crystal, R. G., Epstein, S. E. and Finkel T., 1993. Efficient gene transfer into myocardium by direct injection of adenovirus vectors. Circulation Res., 73 : 1202-1207.

Nam, Y. K., C. H. Jeong and D. S. Kim, 1998. A simple clearing method for improved visualization of lacZ expression in fish larvae. Transgenic Res., 7 : 397-399.

Nam, Y. K., C. H. Noh, and D. S. Kim, 1999. Transmission and expression of an integrated reporter construct up to three generations of transgenic mud loach, *Misgurnus mizolepis*. Aquaculture, 172 : 229-245.

Nam, Y. K., S. D. Chu, C. H. Jeong, I. C. Bang, S.-B. Hur and D. S. Kim, 1997. Production of transgenic olive flounder (*Paralichthys olivaceus*). I. In vivo gene transfer in olive flounder by direct intramuscular injection. J. Aquacult., 10 : 409-415.

Rahman, A. and N. Maclean, 1992. Fish transgene expression by direct injection into fish muscle. Mol. Mar. Biol. Biotechnol., 1 : 286-289.

Tan, J. -H. and W. K. Chan, 1997. Efficient gene transfer into zebrafish skeletal muscle by intramuscular injection of plasmid DNA. Mol. Mar. Biol. Biotechnol., 6 : 98-109.

Verma, I. M. and N. Somia, 1997. Gene therapy - promises, problems and prospects. Nature, 389 : 239-242.

Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani and P. L. Felgner, 1990. Direct gene transfer into mouse muscle *in vivo*. Science, 247 : 1465-1468.

Wolff, J. A., P. Williams, G. Acsadi, S. Jiao, A. Jani and W. Chong, 1991. Conditions affecting direct gene transfer into rodent muscle *in vivo*. Biotechniques, 11 : 474-485.