# Direct Gene Transfer into Skeletal Muscle of Seabass (*Lates calcarifer*) and Black Tiger Prawns (*Penaeus monodon*)

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**ABSTRACT** Gene expression after injection of plasmid pCMVβ-gal into muscle of seabass (*Lates calcarifer*) and black tiger prawns (*Penaeus monodon*) was followed for 14 days. It was found that β-gal expression was first detected 1 day after injection in seabass and 2 days after injection in prawns. Expression persisted up to 7 and 14 days after injection in seabass and prawns, respectively. Persistence of β-gal expression appears to be short-term in seabass, but this is probably due to high variability of gene expression within samples. The relatively low intensity of β-gal expression in prawns may suggest specificity of the CMV-1E promoter to vertebrates. Incidence of samples positive for β-gal expression in seabass and prawns was 33.3% (7/21) and 20% (6/31), respectively.

KEYWORDS: direct gene transfer, β-gal expression, *Lates calcarifer*, *Penaeus monodon*.

## INTRODUCTION

Direct gene transfer into skeletal muscle is a simple technique, requiring only a hypodermic syringe and genes of interest for injection. It is less expensive and much faster than the other techniques of gene transfer such as microinjecting DNA into fertilized egg,[1] or using high electric field pulses to transfer DNA from the surrounding media into host cells,[2] Gene transfer and expression in skeletal muscle have been shown successfully in mice,[3] rats,[4] cats and rhesus monkeys,[5] cattle,[6] *Xenopus*,[7,8] and fish.[9,-13] Similar success of direct gene transfer in rat cardiac muscle has also been reported.[14-16]

Direct gene transfer into muscles may have implications on somatic gene therapy[9] and vaccine development.[3] The latter would certainly be useful in controlling diseases in fish and prawn farms. The preliminary study described here may therefore facilitate such future applications.

## MATERIALS AND METHODS

### Plasmids

The plasmid pCMVβ-gal contains the reporter gene β-galactosidase (β-gal) which is under the control of CMV-IEP (human cytomegalovirus immediate early promoter/enhancer). The gene construct was purchased from Clontech Laboratories and amplified in the laboratory following the procedure by Sambrook *et al.*[17] Plasmid DNA was quantified using DyNA Quant Fluorometer (Hoefer, USA).

### Experimental Seabass and Prawns

The nose to tail length of seabass for the experiment was 8.9 to 14.7 cm and the length of black tiger prawns from rostrum to uropod was 14.5 to 20 cm. The seabass and prawns were transferred to 30‰ aerated sea water and 10‰ aerated pond water, respectively. The temperature of water in laboratory aquaria was maintained at 26°C.

### Plasmid Injection

The seabass and prawns were anaesthetized in 100 µg/ml of tricaine methanesulphonate before injection. Using a 1 ml disposable Terumo syringe and a 26.5 gauge needle, seabass were injected with 5 µg (in 100 µl phosphate-buffered saline) and prawns with 7.5 µg (in 100 µl Tris-EDTA buffer, pH 8.0) of plasmid pCMVβ-gal. Control seabass and prawns were injected with corresponding amounts of phosphate-buffered saline and Tris-EDTA buffer solutions, respectively.

Seabass were injected in the muscle below the trailing edge of the dorsal fin, above the lateral line. The angle of injection was 45° to a depth of approximately 2 to 5 mm.

The point of injection for the prawns was muscle of the second abdominal segment under the exoskeleton. The angle of injection was 45° to a depth of approximately 10 mm.

Both seabass and prawn samples were sacrificed at various days after injection by immersing them in 2% phenoxyethanol.

### β-Gal Assay

Using sterile scalpel blades, approximately 100 to 200 $mm^3$ tissues at site of injection were recovered from seabass and prawn samples. The tissue was homogenised in 50 µl 250 mM Tris-HCl (pH 7.85) and the homogenate was analysed by fluorometric determination using methylumbelliferyl-β-D-galactoside as a substrate following the procedure described by Braell.[18]

### Statistical Analysis

The 99% upper confidence limit was calculated for the controls. Test values which were above the 99% upper limit and of maximum control value were considered significant.

## Results and Discussion

It was found that β-gal was first expressed at the site of injection 1 day after injection in seabass and 2 days after injection in prawns (Figures 1A and B). Expression of plasmid DNA within 1 day after injection has been reported with other studies using rainbow trout[11] and zebrafish.[12] Likewise, transgene expression 2 days after injection has also been reported[3,10] and such observations have been explained to result from the time required for injected plasmid DNA to find its way to muscle nuclei[10] and from DNA degradation of some injected sequences.[3] The maximum level of expression for seabass and prawns was detected after 1 day and 2 days of injection, respectively.

Expression of β-gal could still be detected 7 days after injection in seabass and 14 days after injection in prawns (Figures 1A and B). Examples of long term transgene expression in fishes are 115 days in rainbow trout[11] and 1 year in zebrafish.[12] The short-term persistence of β-gal expression in seabass may be explained by high variability of gene expression. This has been reported in other studies as well.[3,5,7,8,19,20] In a recent study on zebrafish, a second reporter gene was used successfully to reduce variability of gene expression.[12]

CMV-IEP has been shown to be a strong viral promoter in rainbow trout,[11] zebrafish[12] and mice,[20] but in this study, strong β-gal expression was obtained only from muscle extracts of seabass and not from prawns. Seabass required 5 µg of plasmid pCMVβ-gal to elicit strong expression whereas plasmid dosage of 7.5 µg injected into prawn muscle did not elicit a similar intensity of expression. One possible explanation is that the promoter may be specific to vertebrate systems, as has been demonstrated in other studies.[11,12,20] However, more experiments are needed using other promoters to confirm this finding. The low β-gal expression may also result from low amount of injected plasmid DNA. In rainbow trout, 50 µg of injected DNA were required for maximum gene expression.[11] Other factors that may affect efficiency of gene transfer in prawns would be age and gender.[21]

Incidence of samples positive for expression in seabass and prawns was 33.3% (7/21) and 20% (6/30), respectively. The fluorometer readings were

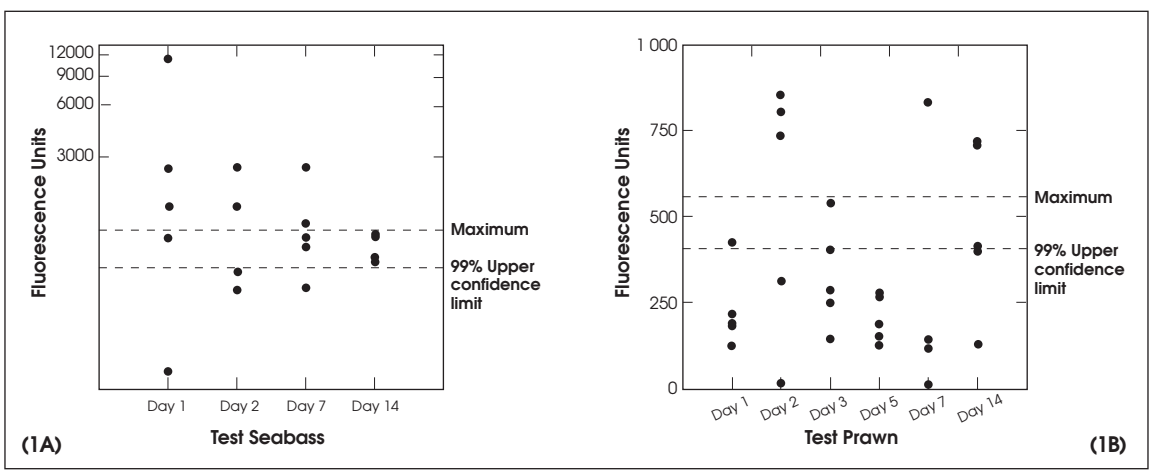


**Fig 1.** Fluorometer readings of muscle samples taken from test seabass (Fig 1A) and prawns (Fig 1B) at different days after injection and subjected to assay as described. Fluorometer reading is in fluorescence units [1 pmol 4MU (β-galactosidase) = 5 fluorescence units]. Dashed lines represent maximum value and 99% upper confidence limit, respectively, of control group. Test values above these lines were considered significantly different from the control values.

above the 99% upper confidence limit and of maximum value, making them significantly different from the controls (Figures 1A and B).

Since this was a preliminary study, more experiments are needed to address some relevant questions of gene transfer in seabass and prawns. Issues that need to be addressed include time span for injected DNA to remain active in muscle, plasmid dosage for maximum efficiency of gene expression and the use of a second reporter gene to reduce variabilty of gene expression.

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## REFERENCES

1. Stuart GW, Vielkind JR and Westerfield M (1988) Replication and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development* **109**, 577-84.
2. Inoue K, Yamashita S, Hata J, Kabeno S, Asada S, Nagahisa E and Fujita T (1990) Electroporation as a new technique for producing transgenic fish. *Cell Diff and Dev* **29**, 123-8.
3. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A and Felgner PL (1990) Direct gene transfer into mouse muscle *in vivo*. *Science* **247**, 1465-8.
4. Thomson DB and Booth FW (1990) Stable incorporation of a bacterial gene into adult rat skeletal muscle *in vivo. Am J Phys* **258**, C578-82.
5. Jiao S, Williams P, Berg RK, Hodgeman BA, Liu L, Repetto G and Wolff JA (1992) Direct gene transfer into nonhuman primate myofibres *in vivo*. *Hum Gene Ther* **3**, 21-33.
6. Cox GJM, Zamb TJ and Babiuk LA (1993) Bovine herpesvirus-1: immune responses in mice and cattle injected with plasmid DNA. *J Virol* **67**, 5664-7.
7. de Luze A, Sachs L and Demeneix BA (1993) Thyroid hormone-dependent transcriptional regulation of exogenous genes transferred into *Xenopus* tadpole muscle *in vivo*. *Proc Natl Acad Sci USA* **90**, 7322-6.
8. Sachs L, de Luze A, Lebrun JJ, Kelly PA and Demeneix BA (1996) Use of heterologous DNA-based gene transfer to follow physiological, T-3 dependent regulation of myosin heavy chain genes in *Xenopus* tadpoles. *Endocrinology* **137**, 2191-4.
9. Hansen E, Fernandes K, Goldspink G, Butterworth P, Umeda PK and Chang KC (1991) Strong expression of foreign genes following direct injection into fish muscle. *FEBS Lett* **290**, 73-6.
10. Rahman MA and Maclean N (1992) Fish transgene expression by direct injection into fish muscle. *Mol Mar Biol Biotechnol* **1**, 286-9.
11. Anderson ED, Mourich DV and Leong JC (1996) Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. *Mol Mar Biol Biotechnol* **5**, 105-13.
12. Tan JH and Chan WK (1997) Efficient gene transfer into zebrafish skeletal muscle by intramuscular injection of plasmid DNA. *Mol Mar Biol Biotechnol* **6**, 98-109.
13. Sulaiman ZH (1998) Transgene expression in seabass (*Lates calcarifer*) following muscular injection of plasmid DNA: a strategy for vaccine development. *Naga* **21**, 16-8.
14. Lin H, Parmacek MS, Morle G, Bolling S and Leiden JM (1990) Expression of recombinant genes in myocardium *in vivo* after direct injection of DNA. *Circulation* **82**, 2217-21.
15. Acsadi G, Jiao S, Jani A, Williams P, Chong W and Wolff JA (1991) Direct gene transfer and expression into rat heart in vivo. *New Biol* **3**, 71-81.
16. Wang J, Jiao SS, Wolff JA and Knechtle SJ (1991) Gene transfer and expression into rat cardiac transplants. *Transplantation* **53**, 703-5.
17. Sambrook J, Fritsch T and Maniatis T (1989) *Molecular Cloning: A Laboratory Manual, 2nd ed*, pp. 1,626. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York..
18. Braell, W.A. (1991) β-Galactosidase assay using the TKO 100 mini fluorometer. *Hoefer Scientific Instruments, Technical Bulletin* No. 129.
19. Wolff, J.A., Williams, P., Acsadi, G., Jiao, S., Jani. A. and Chong, W. (1991) Conditions affecting direct gene transfer into rodent muscle in vivo. *Biotechniques* **11**, 474-485.
20. Manthorpe M, Cornefert-Jensen F, Hartikka J, Felgner J, Rundell A, Mergalith M and Dwarki V (1993) Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice. *Hum Gene Ther* **4**, 419-31.
21. Wells DJ and Goldspink G (1992) Age and sex influence expression of plasmid DNA directly injected into mouse skeletal muscle. *FEBS Lett* **306**, 203-5.