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Functional Evaluation of the Rockbream (*Oplegnathus fasciatus*) Beta-actin Promoter as a Candidate Regulatory Element for DNA Vaccination

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The potential utility of the rockbream (*Oplegnathus fasciatus*) β-actin 5′-upstream sequence as a regulatory element for DNA vaccination was evaluated based on *in vitro* and *in vivo* heterologous expression assays. In the *in vitro* transfection experiment, the efficacy of the rockbream β-actin promoter to drive the expression of a downstream *lac*Z gene was significantly higher (more than fourfold) than that of the human cytomegalovirus (hCMV) promoter in two fish cell lines (grunt *Haemulon plumierii* fin and bluegill *Lepomis macrochirus* fry cell lines). In contrast, the functional activity of the rockbream β-actin promoter was hardly detectable in a mammalian mouse embryonic fibroblast cell line. Rockbream skeletal muscles injected *in vivo* with a GFP reporter construct driven by the β-actin promoter displayed the significantly higher expression of a GFP protein (more than threefold) than did those injected with hCMV promoter driven construct. Data from this study suggest that the homologous rockbream β-actin promoter could be used as a potential regulator for DNA vaccination in this species.

Key words: Rockbream (*Oplegnathus fasciatus*), β-Actin regulator, DNA vaccine, Heterologous expression

Introduction

DNA vaccination has been considered as a promising approach to protect farmed fish against a specific pathogen due to its presumed ability to stimulate long-lasting systematic humoral and cellular immune responses without the risk of infection or high costs for vaccine production (Heppell and Davis, 2000; Garver et al., 2005). The development of an expression vector system allowing the efficient expression of the antigen-coding sequence in cells or tissues of the recipient fish species is a prerequisite for successful and cost-effective immunization using DNA vaccines. Despite their importance, the regulators for DNA vaccination of aquaculture relevant fish species have not been exploited extensively, and most previous studies on the DNA vaccination of fish species have used viral regulators, especially a human cytomegalovirus (hCMV) imme-diate early promoter/enhancer (Mikalsen et al., 2005; Sanchez et al., 2007). Although this viral promoter has yielded high levels of expression in a wide range of vertebrate species including fish and mammals (Verri et al., 2003; Pasnik and Smith, 2005), it raises a conceptual concern with regard to the potential risks associated with the introduction of human viral sequences into fish for human consumption (Loren-zen and LaPatra, 2005; Robertson and Griffiths, 2006). Recently, the use of fish promoters for DNA vaccination of farmed fish has been suggested as an alternative to the hCMV element (Ruiz et al., 2008). The use of fish promoters for DNA vaccines might also be supported by the many successful results from previous studies, in which transgene constructs driven by homologous or own piscine promoters were efficiently expressed in various transgenic fish strains (for reviews, see Hackett and Alvarez, 2000; Nam et al., 2008). Furthermore, the use of homologous piscine promoters can offer a favorable opportunity to minimize the public concern about the risks that are often encountered with the use of human viral sequences.

Cytoskeletal β-actin is an evolutionarily conserved and ubiquitously expressed housekeeping protein. It is known to play critical roles in various cellular acti-

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vities especially in maintaining cell shape and cellular mobility (Reece et al., 1992). Regulatory regions of the β-actin genes have been reported to be able to drive a strong expression of its downstream sequences in various fish species (Nam et al., 2001; Hwang et al., 2003; Nam et al., 2008). Experimental evidence has essentially led to the proposed use of the β-actin promoter as a candidate regulatory element for the DNA vaccines (Ruiz et al., 2008).

Rockbream (*Oplegnathus fasciatus*) is a highly valued marine food fish in Korea, and its market demand has increased gradually over the last decade. However, the aquacultural production of this species has been hindered by frequent outbreaks of mass mortality caused by infections associated with rockbream iridovirus (RBIV). Obviously, RBIV disease is now the major hurdle to enhancing the productivity of rockbream culture in Korea (Choi et al., 2006). The objective of this study was to evaluate the potential utility of the rockbream β-actin promoter as a regulatory element in the heterologous expression vector for DNA vaccination of this species. We examined the functional ability of the β-actin promoter to drive the expression of foreign sequences both *in vitro* and *in vivo* in teleost and mammalian systems.

Materials and Methods

Fish specimens and β-actin gene

The experimental fish used in this study were purchased from a local farm. Fish were transferred to the laboratory aquarium and acclimated for at least one week prior to use in the heterologous expression assay. Isolation of the rockbream β-actin regulatory region was based on our previous work (Lee et al., 2009). The sequences of the rockbream β-actin gene and its promoter are available from GenBank (accession no. FJ975146).

Construction of heterologous expression vector

LacZ expression vectors were constructed for the *in vitro* transfection experiment using fish or mammalian cell lines. The rockbream β-actin promoter fragment was obtained by PCR isolation using a pair of oligonucleotide primers, pRB β-ACT FW1 (5'-AGATCTCGCTGGACCAATCAGAGGGC-3') and pRB β-ACT RV1 (5'-AAGCTTGAACTGTTAAAG AGGAGAAA-3') containing *Bgl*II and *Hin*dIII restriction sites (underlined), respectively. After TA cloning into pGEM-T easy vector, the β-actin promoter fragment was excised using *Bgl*II and *Hin*dIII in order to replace the *Bgl*II-*Hin*dIII hCMV promoter in pcDNA3.1 plasmid (Invitrogen, USA). Next, the *Hin*dIII-*Bam*HI fragment containing the full-length β-galactosidase gene open reading frame (ORF) fragment, which was obtained from a pSV40-lacZ plasmid (Promega, USA), was ligated to the *Hin*dIII-*Bam*HI site downstream of the rockbream β-actin promoter. Consequently a *LacZ*-expression vector driven by rockbream β-actin promoter (pRBβA-lacZ) was constructed. The *Hin*dIII-*Bam*HI β-galactosidase gene fragment was also ligated to the *Hin*dIII-*Bam*HI site of the pcDNA3.1 plasmid to generate a pcDNA 3.1-lacZ vector driven by the hCMV promoter.

Alternatively, green fluorescence protein (GFP) reporter constructs driven by either CMV or β-actin regulators were also constructed for an *in vivo* direct injection experiment. The rockbream β-actin regulatory fragment was isolated by PCR using two primers, pRB β-ACT FW2 (5'-ATTAATGTGAGTGACGCTG GACCAATCAG-3') and pRB β-ACT RV2 (5'-GC TAGCGGCTGAACTGTTAAAGAGGAG-3'), which contain *Ase*I and *Nhe*I restriction sites (under-lined), respectively. The PCR product was TA cloned into pGEM-T easy vector (Promega, USA) and then excised from the vector using *Ase*I and *Nhe*I. Because the rockbream β-actin promoter sequence contained one *Ase*I restriction site in the middle, two restriction fragments (0.9 kb of *Ase*I-*Ase*I fragment and 0.7 kb of *Ase*I-*Nhe*I fragment) were included together in a ligation reaction containing the *Ase*I-*Nhe*I restricted pEGFP-C1 plasmid backbone (Clontech Laboratories Inc., USA). From this ligation, the hCMV promoter in the pEGFP C-1 plasmid was replaced with the rockbream β-actin promoter in order to generate the pRBβA-GFP vector. Correct orientation of the β-actin promoter fragments in the resultant plasmid was confirmed by sequence analysis. As a control construct driven by hCMV promoter, pEGFP-C1 was used for the *in vivo* injection experiment.

In vitro transfection assay

The *in vitro* transfection experiment was carried out in two fish cell lines and one mammalian cell line. Grunt *Haemulon plumierii* fin (GF) and bluegill *Lepomis macrochirus* fry (BF) cell lines were grown in L-15 medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and the appropriate antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Mouse embryonic fibroblast cell line (NIH/3T3 cell line) was grown in RPIM1640 medium (Sigma, USA) supplemented with 10% FBS and the appropriate antibiotics. GF and BF cells were cultured at 25°C, and NIH/3T3 cells were cultured at 37°C under a humidified atmosphere containing 5%

CO_2. On the day before transfection, cells were subcultured on either 24-well plates for fish cell lines (1×10^5 cells/well) or 96-well plates for the mouse cell line (1×10^4 cells/well) in appropriate media without antibiotics. Cells were grown to about 80% confluence. Transfection with pRBβA-lacZ or pCMV-lacZ was carried out using FuGENE 6 transfection reagent (Roche Applied Science, Germany) according to the manufacturer's instructions. After 48 h of transfection, cells were harvested, and then β-galactosidase activity in each transfected group was determined from triplicate samples using an o-nitrophenyl-β-D-galactopyranoside (ONPG) assay. Briefly, cells were lysed with Triton X-100, mixed with ONPG solution, and incubated at 37°C for 1 h. The reaction was stopped by adding a sodium carbonate solution and immediately measured at 420 nm using a microplate reader (Model 680; Bio-Rad, USA). After subtracting the basal background level obtained from non-transfected cells, relative levels for β-galactosidase expression between groups were compared using arbitrary values.

***In vivo* intramuscular injection assay**

For the *in vivo* injection experiment, juvenile rockbream (average body weight = 115 ± 24 g) purchased from a local farm were transferred to the laboratory aquarium. Fish (n = 12 per group) were injected intramuscularly with 40 μg of circular plasmid (pRBβA-GFP or pEGFP-C1) resuspended in 60 μL of phosphate buffered saline (PBS; pH 7.6). Injection was carried out 1/26G needle-equipped syringe. The control group was also prepared in the same manner, except for the plasmid DNA. After injection, individuals belonging to each group were transferred to one of three 100-L tanks containing 80 L of well-aerated sea water at 22 ± 1°C. Fish were maintained for 96 h and the daily water exchange rate was 50%. At 96 h after injection, eight individuals were chosen randomly from each tank and the muscle tissue (about 0.8 g) around the injection site (1 cm below at the last dorsal fin ray) was sampled individually. Each muscle sample was homogenized in extraction buffer (250 mM Tris-Cl [pH 7.8], 1 mM phenylmethylsulfonylfluoride) using a motor-driven tissue homogenizer at 4°C, and centrifuged at 2000×g for 10 min. Then, the supernatant containing the soluble fraction was subjected to GFP analysis using a VersaFluor fluorometer (Bio-Rad, USA). For each sample, the total protein content in the supernatant was measured using a protein assay kit (Bio-Rad, USA). The arbitrary reading per mg soluble protein was converted to pg GFP per mg soluble protein, based on the standard curve generated using serial dilutions of recombinant GFP protein standards (Clontech Laboratories Inc., USA). The relative GFP expression levels in muscles injected with either pRBβA-GFP or pEGFP-C1 were compared.

Results and Discussion

Based on the ONPG assay, β-galactosidase activity was detectable in both GF and BF cells transfected with either pRBβA-lacZ or pcDNA3.1-lacZ. However, the β-galactosidase expressed levels in these fish cell lines differed between the two constructs. The β-galactosidase activities were significantly higher in the groups transfected with pRBβA-lacZ than those with pcDNA3.1-lacZ ($P<0.05$ based on student's *t*-test). Based on triplicate experiments, the mean values in pRBβA-lacZ-transfected GF (0.566± 0.070) and BF cells (0.550 ± 0.033) were more than fourfold greater than those in cells transfected with pCMV-lacZ (0.129 ± 0.012 for GF and 0.127 ± 0.006 for BF cells) (Fig. 1a and b). On the other hand, the mammalian NIH/3T3 mouse cell line transfected with pRBβA-lacZ showed no significant β-galactosidase activity, whereas, the successful expression of β-galactosidase (0.099 ± 0.006) was achieved by transfection with pcDNA3.1-lacZ (Fig. 1c).

As a result of the *in vivo* injection experiment, the background fluorescence reading observed from the PBS-injected control was subtracted for the normalization of GFP signals observed in the plasmid construct-injected groups. Both pRBβA-GFP and pEGFP-C1 induced *de novo* synthesis of GFP protein in injected rockbream muscle, although significant variation was found in the levels expressed among individuals. Significantly higher amounts of GFP (more than threefold) were detected in muscles injected with the construct driven by the β-actin promoter (pRBβA-GFP) than in those injected with the hCMV-driven construct (pEGFP-C1). Average levels of GFP (expressed as pg GFP per mg soluble protein) were 157.6 ± 33.4 and 53.2 ± 18.5 for groups injected with pRBβA-GFP and pEGFP-C1, respectively ($P<0.05$ based on student's *t*-test; Fig. 2).

In this study, the rockbream β-actin regulatory sequence was able to drive the transcription of downstream reporter sequences in both *in vitro* and *in vivo* assays. From the *in vitro* transfection experiment, the rockbream β-actin promoter was highly active in fish cell lines but silent in the mammalian mouse cell line. Differential *in vitro* activities of a given piscine regulator depending upon fish or mammalian cell

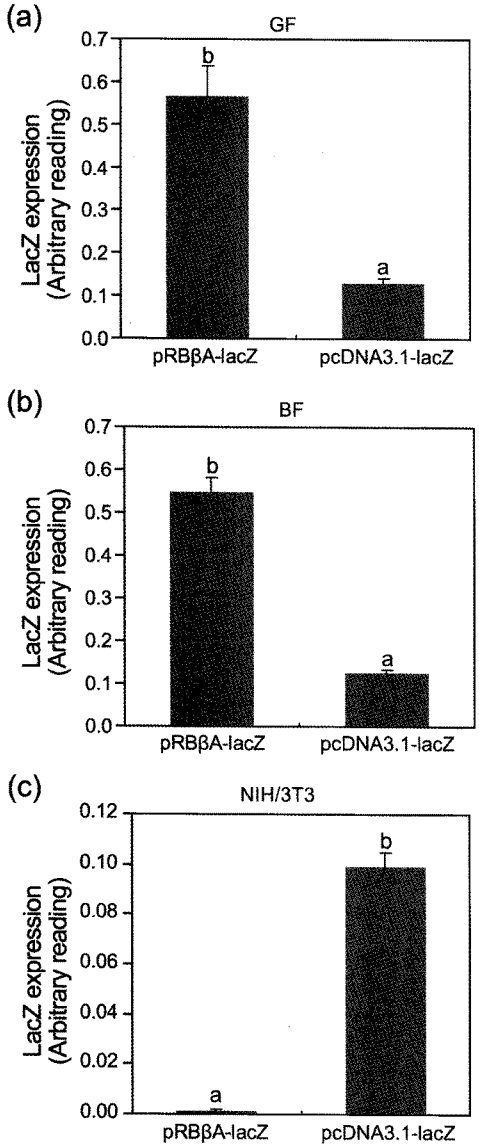

Fig. 1. Expression of reporter constructs driven by rockbream *Oplegnathus fasciatus* β-actin promoter or hCMV promoter in transfected fish and mammalian cell lines. (a) Grunt fin (GF) cells. (b) Bluegill fry (BF) cells. (c) Mouse embryonic fibroblast (NIH/3T3) cells. The cells were transfected with pRBβA-lacZ or pcDNA3.1-lacZ, and the relative levels of β-galactosidase expression were determined using the ONPG assay with arbitrary reading at 420 nm. Means with different letters were significantly different based on student's *t*-test at $P=0.05$. T bar on each histogram indicates standard deviations from triplicate readings.

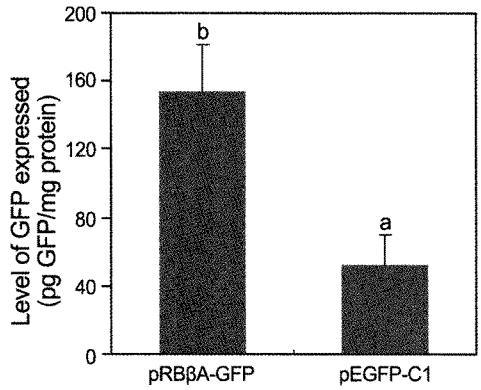

Fig. 2. Potency of the rockbream *Oplegnathus fasciatus* β-actin promoter (pRBβA-GFP) and hCMV promoter (pEGFP-C1) to drive heterologous expression of the downstream reporter gene in the rockbream skeletal muscles. Plasmid constructs were introduced into juveniles through a direct intramuscular *in vivo* injection. Relative GFP expression was fluorometrically measured as pg GFP protein per mg soluble protein). Mean ± SDs with different letters were significantly different based on student's *t*-test at $P=0.05$.

types have been reported previously, and in general, fish regulators would be more active in homologous or closely related fish cell lines than in distantly heterologous ones (Winkler et al., 1992; Chan and Devlin, 1993). The close taxonomic positions of rockbream (*O. fasciatus*), grunt (*H. plumierii*) and bluegill (*L. macrochirus*), which all belong to the same suborder (*Percoidei*), may support of the present data. Although the β-actin genes are highly conserved in their coding regions, relatively large structural differences exist in the 5′-flanking upstream regions among fish species belonging to different taxa (Noh et al., 2003; Lee et al., 2009).

In addition to the *in vitro* assay, the *in vivo* intramuscular injection of reporter constructs also revealed the functional ability of the present β-actin regulator to induce heterologous expression in muscles harboring the β-actin driven plasmid constructs. The potency of the rockbream β-actin promoter was much stronger than that of the hCMV promoter, coinciding with the results of the *in vitro* assay in this study. Our data are also congruent with a previous claim that the common carp (*Cyprinus carpio*) β-actin promoter may function as an appropriate regulatory element for DNA vaccination in salmonids, although the overall potency of the carp β-actin promoter was not higher than that of CMV regulator (Gómez-

Chiarii and Chiaverini, 1999). This may be due to the large distance in the phylogenetic relationship between the β-actin promoter-donating species (common carp) and the recipient species (Atlantic salmon, *Salmo salar*). More recently, *in vitro* evidence for the functional utility of the carp β-actin and zebrafish (*Danio rerio*) long terminal repeats (LTR) promoters to express the G-protein in the transfected *epithelial papulosum cyprini* (EPC) cell line (Ruiz et al., 2008). Hence, a comparative examination of the strengths of different fish promoters would be a good subject for future study concerning the DNA vaccination of rockbream.

Improved antigen expression efficiency through the use of a stronger regulator in DNA vaccination is important in the sense of vaccination cost, which is a key determinant of the potential application of a DNA vaccine to commercial fish farms, because unlike the case with mammals, the immunization of great numbers of individuals in aquaculture operation might often be unavoidable (Heppell and Davis, 2000). Apart from the improved efficiency, the use of non-viral homologous piscine promoter will be favorable in addressing the increasing public demand for safer constructs in the vaccination of fish for human consumption (Lorenzen and LaPatra, 2005). To meet this requirement, further design of vector constituents may be needed to improve the capacity of the regulator, as well as to eliminate or replace unwanted elements such as non-piscine polyadenylation signals and antibiotics-resistant genes (see Adams and Thompson, 2006). Based on this study, immunization experiments with the antigenic sequences should follow to validate the efficacy of β-actin promoter-based DNA vaccination in this species.

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