Molecular Characterization of Cytoskeletal Beta-Actin and its Promoter in the Javanese Ricefish *Oryzias javanicus*

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Abstract

We characterized the cytoskeletal beta-actin (β-ACT) gene (**actb**) and its 5'-upstream regulatory region in the Javanese ricefish *Oryzias javanicus*. The gene and protein structures were deduced from amino acid sequences of the **actb** gene and conserved in the teleost lineage. The *O. javanicus* **actb** gene has common transcription factor binding motifs in its regulatory region found in teleostean orthologues. Following quantitative reverse transcription-PCR, **actb** gene transcripts were detected in all tissues examined; however, the basal expression levels were different. During early development, *O. javanicus* **actb** mRNA levels showed a gradual increase and peaked between late somitogenesis and the heartbeat stage. Microinjection of *O. javanicus* embryos with the **actb** gene promoter-driven red fluorescent protein (RFP) gene reporter vector showed a ubiquitous distribution of RFP signals, although most exhibited a mosaic pattern of transgene expression. A small number of microinjected embryos displayed a wide distribution of RFP signals over their entire body, which resembled the expression pattern of endogenous **actb**. Data from this study provide a basis to develop a transgenic system with ubiquitous expression of foreign genes in *O. javanicus*.

Key words: β-actin, Gene and promoter structure, Microinjection, *Oryzias javanicus*, Javanese ricefish

Introduction

The Javanese ricefish *Oryzias javanicus* is closely related to the Japanese ricefish *O. latipes*, a popular model organism. However, unlike *O. latipes*, *O. javanicus* is capable of hyperosmoregulation and can tolerate saltwater (Koyama et al., 2008). Due to this ability, previous studies have suggested the use of this euryhaline ricefish species as a potential indicator species for the ecotoxicology of brackish and marine environments (Yu et al., 2006; Woo et al., 2006, 2009). This model fish species has the same traits observed in most small, egg-laying *Oryzias*; year-round spawning, easy to culture in a laboratory, and a short-generation time (Koyama et al., 2008; Song et al., 2010). In addition to this, the Javanese ricefish is mostly transparent throughout its lifecycle, which is especially useful in transgene-based heterologous expression assays using fluorescent or colorimetric reporters. However, despite having all of these useful traits, the Javanese ricefish has not been widely used in transgenic investigations.

The cytoskeletal beta-actin (β-ACT) gene (**actb**), a member of the actin multigene family, encodes a non-muscle, cytoskeletal actin protein that is ubiquitously distributed in most animal cell types. The β-ACT is highly conserved in the animal kingdom and plays essential roles in maintaining cytoskeletal structure, cellular mobility, cell division, and contractile processes (Reece et al., 1992). In addition, **actb** is a housekeeping gene and due to its essential role, **actb** mRNA expression has often been used as a versatile invariant control for gene expression studies (Andreassen et al., 2005; Cao et al., 2007). More importantly, previous studies have demonstrated that the regulatory region of **actb** has a high capacity to drive efficient expression of downstream foreign gene(s) both in vivo and in...
Materials and Methods

Fish maintenance and nucleic acid preparation

The Javanese ricefish specimens used in this study were a laboratory strain maintained at the Institute of Marine Living Modified Organisms (IMLMO), Pukyong National University, South Korea. Fish were maintained in water at a concentration of 15 g salt/L water (15 ppt) and water temperature was kept at 25-27°C throughout the experiments. Spawning conditions, egg collection, and embryonic development monitoring were as outlined in previous reports (Song et al. 2010; Cho et al., 2011). Genomic DNA was purified from the fin or whole fry following a conventional SDS/proteinase K method (Cho et al., 2011). Total RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Reverse transcription from purified total RNA was performed using the Omniscript Reverse Transcription Kit (Qiagen) according to the manufacturer’s manual.

Isolation of β-ACT cDNA, the genomic gene, and the promoter

We performed an expressed sequence tag (EST) analysis, using a Javanese ricefish whole-body cDNA library (constructed with a Lambda ZAP cDNA Synthesis Kit; Stratagene, La Jolla, CA, USA) to isolate clones showing significant homology with previously known vertebrate β-ACTs (unpublished data). Selected ESTs (9 out of a total 4,500 ESTs examined) were assembled into contigs using Sequencher 4.2 (Gene Codes, Ann Arbor, MI, USA), and a full-length, continuous sequence of the actb cDNA was validated by reverse transcription-PCR (RT-PCR) isolation from whole-body total RNA, using RT-PCR primers OJβ-ACTc FW (5´-GTCACA-

CAGCTTGTGCGGATA-3´) and OJβ-ACTc FW RV (5´-CAAGTCGGAACACATGTCAGC-3´). Amplified RT-PCR product was purified using a QIAEX II Gel Extraction Kit (Qiagen) and cloned into the pGEM T-easy vector (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Inserts of six randomly chosen recombinant clones were sequenced in both directions by primer walking to determine the representative cDNA sequence of each gene.

Real-time RT-PCR assay to assess mRNA distribution in embryos and adult tissues

For adult tissues and developing embryos, the distribution patterns as well as basal expression levels of actb transcripts were assessed by real-time RT-PCR. Ten tissue samples (from the brain, eye, fin, gill, heart, intestine, kidney, liver, skeletal muscle, and spleen) were taken from 12 healthy individuals (average body weight, 1.4 ± 0.3 g), frozen on dry ice, and stored at -85°C until used. A total of 300 embryo samples were taken at 13 different developmental stages (just fertilized, eight-cells, morula, blastula, gastrula, neurula, four-somite stage, heartbeat stage, retinal pigmentation stage, gill
blood vessel formation stage, visceral blood vessel formation stage, spleen development until total RNA extraction. For the control sample, the 18S rRNA gene (GenBank accession no. KC020115) of O. javanicus was cloned by PCR isolation. An aliquot (2 μg) of total RNA for each sample was reverse transcribed into cDNA using an oligo-(dT)$_{30}$ primer and an O. javanicus 18S rRNA reverse primer (OJ18S-RV) at final concentrations of 1.0 μM and 0.05 μM, respectively. The RT product from each sample was diluted 4-fold (for actb) and 40-fold (for 18S rRNA control) with sterile distilled water, and 2 μL diluted cDNA template was used in a real-time PCR. Real-time PCR was performed using the 2× SYBR Supermix (Bio-Rad, Hercules, CA, USA) and the iCycler (Bio-Rad, Hercules, CA, USA) Real-Time Detection System (Bio-Rad). The β-ACT segment (ampiclon, 253 bp) was amplified using the following primers: qOJβ-ACT 1F (5´-CAACTCTTTGAGCATGGCTTC-3´) and qOJβ-ACT 1R (5´-GCCCTCATCAGAGCCAAATAC-3´). The 18S rRNA control (amplicon, 253 bp) segment was amplified using the following primers: qOJ18S RNA 1F (5´-TCCAGCTCAGAGCCAAATAC-3´) and qOJ18S RNA 1R (5´-AGAACGGTGCTCTATTCCA-3´). Based on the standard curve of each gene, using four log-dilutions of positive cDNA samples, PCR efficiencies were higher than 91%. Expression levels of each gene, using four log-dilutions of positive cDNA samples, were normalized against the levels of the 18S rRNA control according to Kubista et al. (2006). We performed triplicate assays per cDNA sample, and differences in expression levels of actb genes, ranged from 89 to 92 bp, and the 4-translated exon (NTE1, 90 bp) with O. javanicus non-translated exon (NTE1, 90 bp) with Oryzias javanicus β-Actin promoter.

To construct a fluorescent reporter vector driven by the O. javanicus promoter, a 4.3 kb, 5´-upstream fragment, including the non-translated exon 1 and intron 1, was isolated by PCR using the following primers: OJβ-ACTp 2F (5´-ATGTCAGAGAGGCTATGACGCTTC-3´) and OJβ-ACTp 2R (5´-ATACCGGTTGCTAACTGGAAAGAAAC-3´). The 5´-ends of these primers were designed to include SalI (recognition sequence, GTCGAC) and AgeI (recognition sequence, ACCGGT) restriction sites, respectively, to facilitate downstream cloning. Amplified product was TA-cloned, spliced from the T-easy vector (Promega) by digestion with the SalI and AgeI restriction enzymes (New England Biolabs, Ipswich, MA, USA), and unidirectionally ligated upstream of the translation initiation codon (ATG) of the red fluorescent protein (RFP) gene in the pre-digested pDsRed2-1 plasmid vector (Clontech Laboratories Inc.). The resultant RFP vector was called pOJβ-actRFP (8.4 kb). Transient, heterologous expression assays were performed with microinjection of pOJβ-actRFP into O. javanicus embryos. Circular pOJβ-actRFP was resuspended in an injection buffer (10 mM Tris-Cl, 0.1 mM EDTA, pH 8.0; supplemented with 0.01% phenol red; Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 50 μg/mL. Fertilized eggs were collected from egg-laying females and immediately placed in an incubator at 15 °C until microinjection. Microinjection was conducted on one-celled embryos using a Narishige MMN-330 micromanipulator (Narishige Scientific Instrument Lab, Tokyo, Japan). After injection, the embryos were transferred to a 25 °C incubator containing 5 μm filtered water of 15 ppt salinity, adjusted with synthetic sea salt (Kent Marine, Acworth, GA, USA). Transient expression of RFP was monitored in developing embryos using NIS-Elements Microscope Imaging Software and AZ100 Epifluorescence microscopes (Nikon Co., Tokyo, Japan).

Results and Discussion

Structural characteristics of O. javanicus actb cDNA and the genomic gene

O. javanicus actb cDNA is 2283 bp long as follows: an 81 bp 5´-UTR; 1,125 bp for a single ORF encoding a putative polypeptide of 375 amino acids (aa); 673 bp for the 3´-UTR, including stop codon (TAA); and a 29 bp poly(A)+ tail. A putative polyadenylation signal (AATAAA) is located 27 bp before the poly A+ tail (GenBank accession no. JQ905607). The predicted molecular weight and theoretical pl value of the β-ACT polypeptide are 41.7 kDa and 5.29, respectively. Overall, O. javanicus β-ACT shared common structural characteristics with vertebrate orthologues at both the nucleotide and amino acid levels. Multiple sequence alignments between the O. javanicus β-ACT polypeptide and teleostean orthologues show a high degree of secondary structure homology, sharing a high number of amino acid sequences with species belonging to the Oryzias genus (alignment not shown).

The O. javanicus actb gene consists of five translated exons (exons 2-6; 130, 240, 182, and 144 bp, respectively), interrupted by four introns (introns 1-4; 92, 321, 74, and 81 bp, respectively) (GenBank accession no. JQ905606). As in other major actin genes in vertebrates, it also possesses a non-translated exon (91 bp) beginning at -1,241 bp upstream from the ATG initiation codon, 8 bp after the 5´end of exon 2 (Fig. 1A). The intron, which follows the non-translated exon 1, is 1,144 bp long. The GT-AG exon/intron splicing rule is well conserved in all the boundary regions. Alignment of the putative O. javanicus non-translated exon (NTE1, 90 bp) with those from 12 representative teleosts showed that the fish actb genes shared considerable homology in their NTE1 sequences. The lengths of NTE1 in the other teleosts, calculated from the teleostean actb genes, ranged from 89 to 92 bp, and the overall similarity agreed with the known taxonomic appraisal (Fig. 1B). It is consistent with the known evolutionary history of the teleostean actin multigene family, consisting of gene
putative TSS, respectively. A further three CAAT boxes (CCAAT) signals are located at -29 bp and -92 bp from the TATA (consensus sequence, TATAAA) and CAAT boxes (Fig. 1C). TATA (start site; TSS) and the 1.1 kb intron 1 predicts the presence of various TF binding sites including TATA and CAAT boxes or coding regions, as actb (β-actin) gene also had common features in its regulatory region, including intron 1 following the NTE1. Bioinformatic analysis of the 5′ flanking region (i.e., the 3 kb upstream sequence from the putative transcription start site; TSS) and the 1.1 kb intron 1 predicts the presence of various TF binding sites including TATA and CAAT boxes (Fig. 1C). TATA (consensus sequence, TATAAAA) and CAAT (CCAAAT) signals are located at -29 bp and -92 bp from the putative TSS, respectively. A further three CAAT boxes were predicted in distant promoter regions, at -1,635 bp, -2,180 bp, and -2,415 bp from the TSS. Two CarG boxes (CC[\(W=A/T\)],GG) were identified: one in the proximal promoter region (CCTTTATGG; -62 bp from the TSS) one in intron 1 (CCT-TATATGG; -183 bp upstream from the ATG initiation codon) (Liu et al. 1990; Kim et al. 2008; Lee et al. 2009). Intron 1 of the vertebrate actb can function as an enhancer, and previous studies on fish actb regulators have shown its β-ACT regulatory role when it forms part of the transgene construct (Liu et al. 1991; Noh et al. 2003). A canonical E-box (CACGTTG) is predicted 4 bp after the TATA box, and five non-canonical E-boxes (CANNTG) are identifiable in the distal promoter region, in a non-canonical form. Alongside these essential motifs, several TF binding sites related to the stress response are also expected. They include the following: sites targeted by nuclear factor for activated T-cells (NF-AT; consensus sequence, WGGAAAA); activator protein 1 (AP-1; TGA[S=G/C][T[M=A/C]) A); CCAAT-enhancer binding protein (C/EBP; TT[D=A/G/T] NGNAA); glucocorticoid receptor (GR; half site, AGAACCA or TGTTCCT); and metal regulatory transcription factor 1 (MTF-1; TGC[R=A/G]CNC). Previous studies on fish β-ACT promoters have also reported similar TF profiles containing response factor binding sites (Kosuke et al., 2009; Cho et al., 2011), although functional characterization of the motifs have not been performed to date. Much of the literature claims that the fish actb gene might be modulated by stimulation and/or the physiological states of the fish (Filby and Tyler, 2007; Small et al. 2008), and this may indirectly support our findings in the O. javanicus actb promoter.

Expression of β-ACT transcripts in adult tissues and developing embryos

As expected, actb transcripts were ubiquitously detected in all tissues examined, based on the real-time RT-PCR assay (Fig. 2A). However, the basal expression levels were variable among tissue types when normalized against the 18S rRNA level for each tissue. The spleen showed the highest actb transcript level, with the liver and skeletal muscle exhibiting the lowest levels (\(P < 0.05\)). The kidney, heart, and intestine had
During development is not yet fully understood, the expression pattern observed in *O. javanicus* is similar not only to that in the related ricefish, *O. dancena* (Cho et al., 2011), but also to distantly related teleost species, the Atlantic halibut *Hippoglossus hippoglossus* (Fernandes et al., 2008) and the European seabass *Dicentrarchus labrax* (Mitter et al., 2009).

**Transient expression of reporter constructs in microinjected embryos**

A total of 795 one-celled embryos were injected with pOJβ-actRFP in two independent microinjection trials. The average viability of injected embryos during early development until the gastrula stage was 52%, and the viability from gastrula to hatching was 61%, resulting in an average 30% hatching success, which was significantly lower than the hatchability of the non-injected control group (85%). In the pOJβ-actRFP-microinjected group, the onset of RFP expression was observed in less than 5% of the embryos at the neurula stage; however, the expression strength was not very high at this stage. When the embryos reached the somite formation stage, about 30% of the surviving embryos started to exhibit active RFP signals, and RFP intensity became stronger as development progressed. In general, these findings concur with those from previous studies on microinjection of the *actb* promoter-driven transgene construct, although onset of expression of exogenously introduced genes are reported to differ among fish species (Gibbs and Schmale, 2000; Cho et al., 2011).

RFP signal distribution was not tissue-specific, as RFP signals were detectable at many sites, including the yolk and embryonic body, as expected from the ubiquitous nature of the cytoskeletal *actin* gene. Although all of the RFP-positive embryos had a mosaic RFP signal distribution, the expression patterns in pOJβ-actRFP-microinjected embryos could be broadly categorized into three groups. First, more than 50% of RFP-positive embryos displayed RFP expression only in very restricted area(s) of the embryonic body or the yolk. This could be attributed to the mosaic status of the transgene, which is a common phenomenon seen in most microinjection-based gene transfer studies in fish embryos (Nam et al., 1999; Hackett and Alvarez, 2000; Cho et al., 2011). Second, a considerable number of embryos showed significant expression of RFP in the yolk without any apparent RFP signals in the embryonic body (Fig. 3A and 3B). This yolk-exclusive (or dominant) pattern could be explained by the crucial roles of cytoskeletal *actin* proteins in the yolk syncytial layer (YSL) epiboly during fish embryogenesis (Carvalho and Heisenberg, 2010). YSL is a transient embryonic syncytial tissue, which persists until the larval stage, and it undergoes dynamic movements that are important in morphogenesis and conformational changes in the developing embryos (Carvalho and Heisenberg, 2010). Third, a few embryos (less than 10% of RFP-positive embryos) had a ubiquitous distribution of high RFP expression in almost the entire embryonic body, including the lens of the eye (Fig. 3C),...
which is a known pattern of actb gene expression, suggesting that the integration of the microinjected DNA occurs in early cleavage, and therefore persists in subsequent cell divisions (Cho et al., 2011). In several hatchlings, strong RFP expression in the muscles was observed, especially in dorsal and peduncle muscles (Fig. 3D), which was not consistent with the low expression of endogenous actb transcripts in skeletal muscle of adult fish. This suggests that the transcriptional regulation of the actb gene in muscles might differ between early larvae and adults. Several RFP-positive larvae developed from pOJβ-actRFP-microinjected embryos displayed a wide distribution of RFP signals over nearly their entire body (Fig. 3E and 3F), potentially resembling the expression pattern of the endogenous actb gene.

To summarize, we characterized the genetic determinant of O. javanicus β-ACT and evaluated its 5’-upstream regulatory region, using microinjected embryos in heterologous expression assays. O. javanicus β-ACT shares conserved features with its vertebrate orthologues at both the nucleotide and amino acid levels. The actb gene transcripts were widely distributed in all adult tissues with differential levels of basal expression. During development, the O. javanicus actb mRNA was also differentially modulated. In embryos microinjected with the O. javanicus actb gene promoter-driven RFP reporter
vector, a ubiquitous distribution of RFP signals was observed, although all of the embryos and resultant hatchlings did not have a uniform expression due to the mosaic nature of the introduced transgene. Data from this study could provide a useful basis on which to develop a transgenic platform representing strong and ubiquitous expression of various foreign genes in *O. javanicus*.

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**References**


Lin CY, Yang PH, Kao CL, Huang HI and Tsai HJ. 2010. Transgenic zebrafish eggs containing bactericidal peptide is a novel food supplement enhancing resistance to pathogenic infection of fish. Fish Shellfish Immunol 28, 419-427.


for alternative promoters to the human immediate early cytomegalovirus (IE-CMV) to express the G gene of viral haemorrhagic septicemia virus (VHSV) in fish epithelial cells. Vaccine 26, 6620-6629.


