Molecular characterization of the giant freshwater prawn (*Macrobrachium rosenbergii*) *beta-actin* gene promoter

Junjun Yan [1, 2] , **Qiang Gao** [3] , **Zongbin Cui** [1] , **Guoliang Yang** [Corresp., 4] , **Yong Long** [Corresp. 1]

[1] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China

[2] University of the Chinese Academy of Sciences, Beijing, China

[3] Zhejiang Institute of Freshwater Fisheries, Huzhou, China

[4] Huzhou University, Huzhou, China

Corresponding Authors: Guoliang Yang, Yong Long
Email address: ygl0572@163.com, longyong@ihb.ac.cn

Constitutive promoters are important tools for gene function studies and transgenesis. The *Beta-actin* (*actb1*) gene promoter has been isolated from many species but remains to be cloned from the giant freshwater prawn (*Macrobrachium rosenbergii*) . In this study, we cloned and characterized the *Mractb1* gene promoter. Two alternative promoters were identified for the *Mractb1* gene , which direct the generation of two transcripts with different 5’ untranslated region . Three CpG islands were predicted in the upstream sequence, which are intimately related to transcription initiation and promoter activity. In addition to the CCAAT-box and CArG-box, molecular dissection of the flanking sequence revealed the existence of one negative and two positive elements in the upstream region and the first intron. Finally, the *Mractb1* promoter demonstrated comparative activity to the carp (*Cyprinus carpio*) *actb1* promoter. Our investigations provide a valuable genetic tool for gene function studies and shed light on the regulation of the *Mractb1* gene.

Molecular characterization of the giant freshwater prawn (*Macrobrachium rosenbergii*) *beta-actin* gene promoter

Junjun Yan[1, 2], Qiang Gao[3], Zongbin Cui[1], Guoliang Yang[4*], Yong Long[1*]

[1] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

[2] University of the Chinese Academy of Sciences, Beijing, China

[3] Zhejiang Institute of Freshwater Fisheries, Huzhou, China

[4] Huzhou University, Huzhou, China

*Corresponding authors

Yong Long[1]

longyong@ihb.ac.cn (YL)

Guoliang Yang[4]

ygl0572@163.com (GY)

Abstract

Constitutive promoters are important tools for gene function studies and transgenesis. The *Beta-actin* (*actb1*) gene promoter has been isolated from many species but remains to be cloned from the giant freshwater prawn (*Macrobrachium rosenbergii*). In this study, we cloned and characterized the *Mractb1* gene promoter. Two alternative promoters were identified for the *Mractb1* gene, which direct the generation of two transcripts with different 5′ untranslated region. Three CpG islands were predicted in the upstream sequence, which are intimately related to transcription initiation and promoter activity. In addition to the CCAAT-box and CArG-box, molecular dissection of the flanking sequence revealed the existence of one negative and two positive elements in the upstream region and the first intron. Finally, the *Mractb1* promoter demonstrated comparative activity to the carp (*Cyprinus carpio*) *actb1* promoter. Our investigations provide a valuable genetic tool for gene function studies and shed light on the regulation of the *Mractb1* gene.

Introduction

Giant freshwater prawn is the biggest freshwater prawn in the world. The males can grow up to 320 mm in body length and weigh over 200 g (Ling 1969). It has a wide distribution throughout the world and is particularly favored for farming in the tropical and subtropical developing countries in Asia, such as China, India and Thailand. Owing to its fast growth rate and higher profit realization per unit area, it now has become the most widely aquacultured freshwater prawn species and contributed 51.7% to the total global production of freshwater prawns (Banu

& Christianus 2016; New 2005; New & Nair 2012). However, outbreak of diseases is a major limitation facing aquaculture industry and several diseases such as white spot disease and white tail disease have been reported to cause mass mortality for giant freshwater prawn farming worldwide (Bonami & Widada 2011; Saurabh & Sahoo 2008).

Transgenic technology has been considered as a powerful and effective way to improve economic traits of aquaculture species (Bennett & Jennings 2013; Rasmussen & Morrissey 2007; Sin 1997), through which pathogen-resistant strains of prawn could be generated to reduce or even avoid the disease-related economic losses. So far, successful gene transfer in freshwater prawn and several marine shrimp species have been reported (Arenal et al. 2000; Liu et al. 2001; Preston et al. 2000). However, most of the researches used expression vectors based on heterologous promoters such as CMV (cytomegalovirus) and SV40 (simian vacuolating virus 40) promoter, hobo transposable element (HFL1) and carp *beta-actin* gene promoter. Studies about fish transgenesis have shown that promoters of fish origin were more efficient in driving expression of the target genes than those from mammalian or viral origins (Alam et al. 1996; Hanley et al. 1998). Moreover, negative concerns about the use of viral promoters to express transgenes have led to the generation of "all-fish" transgene constructs (Rasmussen & Morrissey 2007). In accordance with the "all-fish" concept, promoters originated from prawn species have strong potential for use in prawn transgenesis and gene function studies.

Beta-actin is a housekeeping gene with ubiquitous expression. The promoter of *beta-actin* gene

has been isolated from and characterized in a wide variety of species such as human (Gunning et al. 1987), chicken (Kosuga et al. 2000), amphioxus (Feng et al. 2014), teleosts (Barman et al. 2015; Hwang et al. 2003; Kong et al. 2014; Liu et al. 1991; Noh et al. 2003) and shrimps (Shi et al. 2015; Shi et al. 2016). Because *beta-actin* gene promoter confers high level of constitutive transcriptional expression, it is widely used for transgenesis in both plants and animals (Cho et al. 2011; Hong et al. 2016; Kosuga et al. 2000). Although the cDNA of the *beta-actin* gene has been cloned from giant freshwater prawn (Zhu et al. 2005), its promoter remains to be characterized. Here, we report the isolation and characterization of the giant freshwater prawn *beta-actin* (*Mractb1*) gene promoter (Mbap). Our results provide insights into the regulation mechanisms of *Mractb1* gene and indicate the potential usage of Mbap in gene function and transgenesis studies for giant freshwater prawn and closely related species.

Materials and Methods

Animals

The animal protocol of this study was approved by the Institutional Animal Care and Use Committee of Institute of Hydrobiology (Approval ID: Y341131501). Adult giant freshwater prawns of both sexes (body weight 20 - 30 g) were acquired from Zhejiang Institute of Freshwater Fisheries, Huzhou, China. After transportation, the prawns were acclimated to laboratory conditions for 2 weeks in a circulating fresh water system at 28 ^{o}C.

89 **Total RNA and genomic DNA extraction**

90 The prawns were immersed in eugenol solution (0.125 mL/L in Phosphate Buffered Saline) until
91 loss of consciousness and then placed into ice-slurry for a 2-Step euthanasia procedure (Leary et
92 al. 2013). After that their bodies were dissected and total RNA was extracted from the gill, liver,
93 muscle and intestine of both sexes, and from testis and ovary, using TRIZOL (Invitrogen)
94 according to the manufacturer's instructions. For genomic DNA extraction, about 100 mg muscle
95 tissue was put into a 1.5 mL Eppendorf tube with 0.5 mL lysis buffer (10 mM Tris-Cl, 1 mM
96 EDTA, 0.1 % SDS, 200 μg/mL proteinase K, pH 8.0). The samples were incubated in 58 ^{o}C
97 water bath for 6 hours with gentle vibration. After that, the lysate was subjected to a traditional
98 phenol/chloroform extraction procedure. The concentration of RNA and DNA samples was
99 measured by a NanoDrop 8000 from Thermo Scientific and the quality of RNA and DNA
100 samples was assessed by agarose gel electrophoresis.
101
102 **Genome walking**
103 Genome walking was performed as previously described (Zhou et al. 2015) to clone the 5′
104 flanking sequence of the *Mractb1* gene. Each walking step contains three rounds of nested PCR
105 using different gene specific primers (GSP). Before genome walking, primers Mractb1-F and
106 Mractb1-R (sequences of all the primers used in this study are listed in Table 1) designed
107 according to the cDNA sequence deposited in GenBank (AY626840) were used to amplify
108 partial genomic sequence. A 1293-bp genomic DNA fragment was obtained and sequenced. The
109 gene specific primers from Mractb1-GSP1 to Mractb1-GSP3 were subsequently designed

110 according to this known genomic sequence. These gene specific primers were sequentially mated
111 with the 4 degenerate primers included in the genome walking kit (Takara) to amplify the
112 unknown 5′ flanking sequence. Similarly, gene specific primers from Mractb1-GSP4 to Mractb1-
113 GSP6 based on the product of the first walking experiment were used for the second walking
114 step. A total of 3 walking experiments were conducted to identify the 5′ flanking sequence of the
115 *Mractb1* gene.
116
117 **5′ RACE**
118 5′ RACE (rapid amplification of cDNA ends) was performed using the Takara 5′-Full RACE Kit
119 according to the manufacturer's instructions. Briefly, the RNA sample was first treated with
120 CIAP (calf intestine alkaline phosphatase) to remove the naked phosphorous from the RNA
121 molecules, followed by TAP (tobacco acid pyrophosphatase) treatment for mRNA decapitation.
122 The decapped mRNA was ligated to the 5′ RACE adaptor using T4 RNA ligase. Reverse
123 transcription was performed using M-MLV (moloney murine leukemia virus) reverse
124 transcriptase and 9-mer random primer. After that, two rounds of nest PCR using 5′-RACE-
125 outer-primer/GSP1 and 5′-RACE-inner-primer/GSP2 primer pairs were conducted sequentially
126 to clone the 5′ cDNA ends. Product of the second round PCR was purified using the Biospin Gel
127 Extraction Kit (BioFlux), subcloned into the pMD18-T vector (TaKaRa) and then sequenced by
128 the Tsingke Biological Technology (Wuhan, China) Co., Ltd.
129
130 **Quantitative real-time PCR**

Quantitative real-time PCR (qPCR) was performed as previously described (Long et al. 2013) to determine the expression level of the *Mractb1* transcripts. Primers for qPCR were designed using the Primer Premier 6.0 software. First-strand cDNA was synthesized using total RNA extracted from different tissues by the RevertAid First Strand cDNA Synthesis Kit (Thermo scientific) and used as template for qPCR analysis. *Mr18S-rRNA* was used as the internal reference for gene expression normalization.

Bioinformatic analyses

CpG islands in the upstream region were predicted by the Methprimer software using default parameters (http://www.urogene.org). Core promoter elements were predicted by the YAPP Eukaryotic Core Promoter Predictor (http://www.bioinformatics.org/yapp/cgi-bin/yapp.cgi).

Construction of plasmids

To dissect transcriptional activity of the 5′ flanking sequence of the *Mractb1* gene, the cloned upstream sequences were divided into 7 fragments according to location of the predicted GC islands and the TSSs (transcriptional start sites) revealed by 5′ RACE. The position of the first nucleotide of the initiation codon was regarded as +1. The length and relative location of fragments 1 to 7 are 558 bp (-558 to -1), 523 bp (-1080 to -558), 240 bp (-1317 to -1078), 249 bp (-1568 to -1320), 195 bp (-1763 to -1569), 357 bp (-2120 to -1764) and 501 bp (-2620 to -2120), respectively. These fragments were amplified and cloned into the SacI/NheI site of the pGL3-Basic vector (Promega) separately or in combination. Deletion of fragment 3 in the 5′ flanking

152 sequences was conducted via the PCR-driven overlap extension method previously described
153 (Heckman & Pease 2007) with primers Mractb1P42-F and Mractb1P24-R. The constructs were
154 named according to the inserted fragments, for example, pGL-Mba7654321 includes the whole
155 sequence, pGL-Mba1 contains only fragment 1 and pGL-Mba21 consists of both fragment 1 and
156 fragment 2. To compare the promoter activity of *Mractb1* gene with that of carp *actb1* gene, the
157 promoter of carp *actb1* gene was amplified by the primer pair Carpactb1-F1/Carpactb1-R1 and
158 inserted into the HindIII/NcoI site of the pGL3-Basic vector.
159

160 **Luciferase activity assay**

161 The EPC (endothelial progenitor cells) cells (ATCC® CRL-2872™) were maintained at 28°C
162 with 5% CO_2 in M199 medium (Hyclone) supplemented with 10 % fetal bovine serum,
163 100 μg/mL streptomycin, 100 U/mL penicillin and 2 μg/mL amphotericin B. One day before
164 transfection, cells were seeded into 24-well plates at a density of 2×10^5 cells/well. Transfection
165 was performed using the X-tremeGENE HP Transfection Reagent (Roche). The pRL-TK
166 plasmid from Promega was used as internal control for luciferase activity assays and co-
167 transfected with the promoter-luciferase constructs. The total amount of plasmid used for
168 transfection was 500 ng/well and the ratio between the promoter-luciferase vector and pRL-TK
169 was 5:1. Twenty-four hours after transfection, cells were lysed and subjected to luciferase
170 activity assays using the Dual-Luciferase® Reporter Assay System from Promega.
171

172 **Western blot**

EPC cells cultured in 6-well plates were transfected with 2 μg plasmid. Twenty-four hours after transfection, cells were lysed by the RIPA (radio immunoprecipitation assay) lysis buffer from beyotime. Then western blot was performed as previously described (Mo et al. 2010) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as loading control. The primary antibodies used for western blot were rabbit anti-Firefly Luciferase (Abcam, #ab185923, 1:5000) and mouse anti-GAPDH (Boster, #BM3876, 1:100). The secondary antibodies were goat anti-rabbit IgG (immunoglobulin) (Boster, #BM3894, 1:5000) and goat anti-mouse IgG (Boster, #BM3895, 1:5000), respectively. Intensity of the bands in the western blot image was analyzed by the ImageJ software (http://rsb.info.nih.gov/ij/).

Statistical Analysis

The results of dual luciferase assays and qPCR are presented as mean ± standard deviation. Independent samples T tests were performed using SPSS (statistical product and service solutions) 15.0 to analyze the significant difference between groups.

Results

Cloning of the 5′ flanking sequence for the Mractb1 gene

To clone the 5′ flanking sequence for the *Mractb1* gene, a 1293 bp genome sequence was firstly amplified using the primers Mractb1-F/ Mractb1-R designed according to the reported cDNA sequence (Zhu et al. 2005). Three rounds of genome walking experiments were subsequently

194 performed to clone the upstream sequence of the *Mractb1* gene. As shown in (Fig. 1), the length
195 of the DNA fragments obtained by the three genome walking experiments were 2094, 531 and
196 713 bp, respectively. Furthermore, PCR using the forward primer Mractb1-F and the reverse
197 primer Exon3-R (matches to the 3′ cDNA end) generated a 1593 bp sequence. Assembling of
198 these fragments resulted in a 4306-bp long contig, which contains both the 5′ flanking and full
199 sequence for the *Mractb1* gene. The obtained upstream sequence counted from the first
200 nucleotide of the initiation codon is 2620 bp in length, which has been submitted to the GenBank
201 database under the accession number KY038927.
202
203 **Identification of two transcripts for the Mractb1 gene**
204 Before characterizing the promoter of the *Mractb1* gene, 5′-RACE was performed to identify the
205 transcriptional start site (TSS). A clear DNA band in the range of 100 to 250 bp was obtained
206 (Fig. 2). The DNA fragments were purified and cloned into the pMD18-T vector. Subsequent
207 DNA sequencing revealed two 5′ cDNA end sequences for the *Mractb1* gene. One is 106 bp (10
208 clones sequenced) and the other is 145 bp (6 clones sequenced) in length (Fig. 2). Further
209 analysis revealed that the *Mractb1* gene possesses 2 transcript variants (designated Mractb1_tv1
210 and Mractb1_tv2, respectively), which only differ in the 5′ UTR (untranslated region sequence)
211 (Fig. 3A). PCR using different forward primers (Exon1-F and Exon1′-F) and the same reverse
212 primer (Exon3-R) amplified the full-length sequence for the 2 transcripts (Fig. 3A, B), which are
213 1186 and 1225 bp in length, respectively. Since the transcripts contain different exon 1 (Fig. 3A),
214 we speculate that they are driven by alternative promoters (promoter-1 and promoter-2).

215

216 **Expression of the Mractb1 transcripts in different sexes and tissues**

217 To shed light on the activities of the promoters in different sexes and tissues, the expression
218 levels of the 2 transcript variants were measured by qPCR assays. As shown in (Fig. 4), the
219 *Mractb1* gene demonstrate quite different expression levels among tissues and between sexes.
220 The highest overall expression level of the *Mractb1* gene was found in the gonad and gill,
221 followed by the intestine, muscle and liver. Sexual dimorphism could be found for the expression
222 level of the *Mractb1* gene in all the tissues (Fig. 4). The expression levels of both Mractb1_tv1
223 and Mractb1_tv2 in the female gonad (ovary) and gill is significantly higher than those in the
224 corresponding male tissues, while muscle and intestine of the males demonstrated higher
225 expression than those of the females (Fig. 4). Difference between the expression of the two
226 transcripts in same tissue was identified in the male gill and the female gonad and muscle.
227 Mractb1_tv2 showed higher expression in the male gill, but lower expression in the female
228 muscle and gonad when compared to Mractb1_tv1 (Fig. 4). These findings indicate that
229 expression of the *Mractb1* gene is regulated by tissue and sex specific factors.

230

231 **Molecular dissection of the Mractb1 gene promoter**

232 Three CpG islands were predicted in the 5′ flanking sequence of the *Mractb1* gene. These CpG
233 islands are located at -1762 to -1569 (i1, 194 bp), -1437 to -1320 (i2, 118 bp) and -747 to -542
234 (i3, 206 bp), respectively (Fig. 5A). The 2620 bp flanking sequence was divided into 7 segments
235 (from Mba 7 to Mba1) according to location of the CpG islands and the TSSs (Fig. 5A).

Transcriptional activities of the upstream fragments were investigated by dual luciferase assays. When characterized separately, Mba7, Mba6, Mba3 and Mba1 demonstrated no or minimal transcriptional activity, while high promoter activity was detected for Mba5, Mba4 and Mba2, each of them contains a predicted CpG island (Fig. 5B), suggesting importance of the CpG islands in transcription. Based on structure of the *Mractb1* transcripts and transcriptional activities of the upstream fragments, we concluded that the two alternative promoters are in Mba54 (promoter 1) and Mba21 (promoter 2), respectively. Activity of the two promoters and regulations by their corresponding upstream regions were further characterized. For the promoter-1, combination of Mba5 and Mba4 only slightly increased the promoter activity, Mba6 displayed no effect on Mba54, while fuse Mba7 to Mba654 significantly enhanced the promoter activity (Fig. 5C). As for the promoter-2, significant synergistic effect was found between Mba2 and Mba1; however, Mba3 demonstrated negative effect on the activity of Mba21 (Fig. 5D). Serial deletion experiments indicated that Mba654321 had the highest transcriptional activity, followed by Mba7654321 and Mba54321, while Mba4321, Mba321 and Mba21 only displayed basal promoter activity (Fig. 5E). Furthermore, interaction between the two alternative promoters was observed. Mba54 (promoter-1) and Mba21 (promoter-2) demonstrated significant synergistic effects when combined (Mba5421). Both Mba3 and Mba6 can enhance activity of Mba5421 and the interaction between Mba3 and Mba6 is necessary for the full activity of the *Mractb1* gene promoter (Fig 5F). Taken together, the upstream fragments demonstrate different effects under different circumstances. For example, Mba7 enhances activity of the promoter-1 but inhibits activity of the whole promoter (Fig. 5C, E). On the contrary, Mba3 inhibits activity

257 of the promoter-2 but is indispensable for the full activity of the whole promoter (Fig. 5D, F).
258 These findings suggest complex interactions among genetic elements in regulating expression of
259 the *Mractb1* gene.
260
261 **Genetic elements in the Mractb1 gene promoter**
262 As shown in (Fig. 5A, B), the CpG islands in the promoter of the *Mractb1* gene are intimately
263 associated with the promoter activity. It's interesting that despite no TSS was identified in the
264 first CpG island (i1, Mba5), it still directs high level of transcription (Fig. 5B). Further
265 investigation indicated that this region contains a CCAAT box, a CArG box and a GC box (Fig.
266 6). Both CCAAT box and CArG box were reported to be important for the constitutive
267 expression of carp and human *beta-actin* gene (Liu et al. 1990; Quitschke et al. 1989). The
268 second CpG island (i2) encompasses the first TSS and an initiator element (InR) (Fig. 6). The
269 third CpG island (i3) contains the second TSS, a GC box and an InR (Fig. 6). Since no TATA
270 box was identified in the core promoters for the *Mractb1* gene, the InRs may play important
271 roles in transcription initiation.
272
273 ***Mractb1* promoter demonstrates comparative activity with the carp *actb1* promoter**
274 To justify whether the *Mractb1* promoter cloned in this study has the potential to be used as a
275 genetic tool in gene function and transgenesis studies, the activity of which was compared with
276 that of carp *beta-actin* (*actb1*) gene promoter, a promoter which is commonly used in transgenic
277 fish. The fragment with the highest promoter activity (Mba654321) was designated as Mbap.

The promoter of the carp *actb1* gene (designated as Cbap) was cloned into the pGL3- basic vector as well. Dual luciferase assays in EPC cells indicated that the Mbap demonstrated comparative activity with the Cbap (Fig. 7A). Western blot using antibody against the firefly luciferase detected similar expression level of firefly luciferase in the EPC cells transfected with pGL-Mbap and pGL-Cbap (Fig. 7B, C); however, the empty vector pGL-basic demonstrated no luciferase expression at all. These results indicate that the *Mractb1* promoter is as active as the carp *actb1* promoter and which can be used for transgenesis and gene function studies for prawn and related species in the future.

Discussion

As a house keeping gene, the promoter of *actb1* is cloned from a wide range of organisms and extensively used for gene function and transgenesis studies (Barman et al. 2015; Cho et al. 2011; Hwang et al. 2003; Kong et al. 2014; Liu et al. 1991; Noh et al. 2003). Although the CDS (coding sequence) and 3′ UTR sequence of the *Mractb1* gene have been cloned previously (Zhu et al. 2005), the promoter remains to be characterized. In this study, we cloned the upstream sequence of the *Mractb1* gene, identified the TSSs, investigated expression of the transcripts in different tissues of both sexes and characterized transcriptional activity of the *Mractb1* gene promoter. Both luciferase assays and western blot indicated that the *Mractb1* promoter demonstrated comparable transcriptional activity to that of the carp *actb1* promoter in the EPC cells, suggesting that it is a valuable tool for gene function and transgenesis studies for

299 *Macrobrachium rosenbergii*.

300

301 Identification of two TSSs and the results of promoter activity assays indicated that the *Mractb1*
302 gene possesses two alternative promoters which initiate transcription from different TSSs and
303 lead to the generation of two transcript variants. These two transcript isoforms contain different
304 initial exons but share the same open reading frame (ORF) and 3′ UTR, indicating that they only
305 differ in the 5′ UTR. Existence of alternative promoters is common for mammalian genes. It was
306 reported that 18% of human genes have evidence of alternative promoter usage (Landry et al.
307 2003). Alternative promoters may differ in tissue and developmental stage specificity and
308 transcriptional activity (Landry et al. 2003). The usage of alternative promoters may account for
309 distinct mRNA levels of the *Mractb1* gene transcripts in different tissues and sexes, and different
310 5′ UTR of the two transcript variants may affect their translation efficiency. Moreover, molecular
311 dissection for the activities of different upstream regions revealed significant synergistic effects
312 between the two alternative promoters. Although the *actb1* gene promoters of human and carp
313 were well characterized, it is unknown whether they possess alternative promoters as well.

314

315 Three CpG islands were identified in the 5′ flanking sequence of the *Mractb1* gene. These CpG
316 islands are closely related to the activity of the *Mractb1* gene promoter according to luciferase
317 assays. All of them demonstrated transcriptional activity and both of the identified TSSs are
318 located in the two proximal CpG islands, consistent to the discovery that most CpG islands
319 function as sites of transcription initiation by destabilizing nucleosomes and attracting proteins

that create a transcriptionally permissive chromatin state (Deaton & Bird 2011). CpG islands are prevalent in the promoter of housing keeping (HK) genes and more than three quarters (78.7%) of human HK (housing keeping) genes predominantly have a CpG+/TATA- core promoter (Zhu et al. 2008). CpG-islands usually contain multiple GC-boxes, and GC-boxes coupled with InR elements are essential to initiate transcription in the absence of TATA-box (Butler & Kadonaga 2002). Consistent with this notion, both GC-box and InR, but no TATA-box were found in the core promoters of the *Mractb1* gene.

Genetic elements including the CCAAT-box and CArG-box were reported to be important for the constitutive expression of human and carp *actb1* gene (Liu et al. 1990; Quitschke et al. 1989). These elements were also identified in the *Mractb1* promoter. Fuse the DNA fragment containing these elements (Mba5) to the proximal promoter sequence (Mba4321) dramatically increased the promoter activity (Fig. 5A, E), suggesting the function of these elements in regulating the *Mractb1* gene. Like in the carp *actb1* gene promoter, a distal upstream region (Mba7) was found to negatively regulates the *Mractb1* promoter activity. Moreover, the upstream fragment Mba6 and 5′ end of the first intron Mba3, and the interaction between these two regions are necessary for the constitutive transcriptional activity of the *Mractb1* gene promoter. These results indicate that in addition to the conservative elements, other unknown elements and factors are involved in regulating the *Mractb1* gene.

Conclusions

The *beta-actin* (*Mractb1*) gene promoter of *M. rosenbergii* was cloned and characterized. Two alternative promoters were identified for the *Mractb1* gene, which direct the generation of two transcripts with different 5′ UTR. Genetic dissection of the upstream sequence of the *Mractb1* gene revealed one distal negative element and two proximal positive elements regulating the activity of the *Mractb1* gene promoter. Finally, the Mractb1 promoter demonstrated comparative activity to the carp (Cyprinus carpio) *actb1* promoter. Our investigations provide a valuable genetic tool for gene function studies and shed light on the regulation of the *Mractb1* gene.

Acknowledgements

The authors would like to thank Dr. Bolan Zhou from the Hunan university of arts and science for technical assistance.

References

Alam MS, Lavender FL, Iyengar A, Rahman MA, Ayad HH, Lathe R, Morley SD, and Maclean N. 1996. Comparison of the activity of carp and rat β-actin gene regulatory sequences in tilapia and rainbow trout embryos. *Molecular Reproduction & Development* 45:117.

Arenal A, Pimentel R, Guimarais M, Rodríguez A, Martínez R, and Abad Z. 2000. Gene Transfer in Shrimp (*Litopenaeus schmitti*) by Electroporation of Single-cell Embryos and Injection of Naked DNA into Adult Muscle. *Biotecnologia Aplicada* 17:247-250.

Banu R, and Christianus A. 2016. Giant Freshwater Prawn *Macrobrachium rosenbergii* Farming: A Review on its Current Status and Prospective in Malaysia. *Journal of Aquaculture Research & Development* 7:423.

Barman HK, Mohanta R, Patra SK, Chakrapani V, Panda RP, Nayak S, Jena S, Jayasankar P, and Nandanpawar P. 2015. The β-actin gene promoter of rohu carp (*Labeo rohita*) drives reporter gene expressions in transgenic rohu and various cell lines, including spermatogonial stem cells. *Cellular & Molecular Biology Letters* 20:237-247.

Bennett DJ, and Jennings RC. 2013. Successful Agricultural Innovation in Emerging Economies: Transgenic marine algae for aquaculture: a coupled solution for protein sufficiency. Cambridge University Press, 233-246.

Bonami JR, and Widada JS. 2011. Viral diseases of the giant fresh water prawn *Macrobrachium rosenbergii*: A

review. *Journal of Invertebrate Pathology* 106:131-142.

Butler JEF, and Kadonaga JT. 2002. The RNA polymerase II core promoter: a key component in the regulation of gene expression. *Genes & Development* 16:2583-2592.

Cho YS, Sang YL, Kim YK, Dong SK, and Nam YK. 2011. Functional ability of cytoskeletal β-actin regulator to drive constitutive and ubiquitous expression of a fluorescent reporter throughout the life cycle of transgenic marine medaka *Oryzias dancena*. *Transgenic Research* 20:1333-1355.

Deaton AM, and Bird A. 2011. CpG islands and the regulation of transcription. *Genes & Development* 25:1010-1022.

Feng J, Li G, Liu X, Wang J, and Wang YQ. 2014. Functional analysis of the promoter region of amphioxus *beta-actin* gene: a useful tool for driving gene expression in vivo. *Molecular Biology Reports* 41:6817-6826.

Gunning P, Leavitt J, Muscat G, Ng SY, and Kedes L. 1987. A Human *Beta-actin* Expression Vector System Directs High-Level Accumulation of Antisense Transcripts. *Proceedings of the National Academy of Sciences of the United States of America* 84:4831-4835.

Hanley S, Smith TJ, Muller F, Maclean N, Uzbekova S, Prunet P, and Breton B. 1998. Isolation and functional analysis of the histone H3 promoter from atlantic salmon (*Salmo salar L.*). *Molecular Marine Biology & Biotechnology* 7:165.

Heckman KL, and Pease LR. 2007. Gene splicing and mutagenesis by PCR-driven overlap extension. *Nature Protocols* 2:924.

Hong JK, Suh EJ, Kwon SJ, Lee SB, Jin AK, Lee SI, and Lee YH. 2016. Promoter of chrysanthemum actin confers high-level constitutive gene expression in Arabidopsis and chrysanthemum. *Scientia Horticulturae* 211:8-18.

Hwang G-L, Rahman MA, Razak SA, Sohm Fdr, Farahmand H, Smith A, Brooks C, and Maclean N. 2003. Isolation and characterisation of tilapia β-actin promoter and comparison of its activity with carp β-actin promoter. *Biochimica et Biophysica Acta* 1625:11-18.

Kong HJ, Kim JL, Kim WJ, Kim HS, Yeo SY, Park JY, and An CM. 2014. Genomic cloning and promoter analysis of the *beta-actin* gene from Korean rose bitterling (*Rhodeus uyekii*). *Genes & Genomics* 36:861-869.

Kosuga M, Enosawa S, Li XK, Suzuki S, Matsuo N, Yamada M, Roychowdhury J, Koiwai O, and Okuyama T. 2000. Strong, long-term transgene expression in rat liver using chicken *beta-actin* promoter associated with cytomegalovirus immediate-early enhancer (CAG promoter). *Cell Transplantation* 9:675-680.

Landry JR, Mager DL, and Wilhelm BT. 2003. Complex controls: the role of alternative promoters in mammalian genomes. *Trends in Genetics* 19:640-648.

Leary S, Underwood W, Anthony R, Cartner S, Corey D, Grandin T, Greenacre CB, Gwaltney-Bran S, McCrackin MA, and Meyer R. 2013. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. *University of Alaska Anchorage*.

Ling SW. 1969. The general biology and development of the *macrobrachium rosenbergii* (de man). *FAO Fisheries Report* 57:589-606.

Liu Z, Xiang J, Zhou G, and Gong Z. 2001. Foreign gene transfer into Chinese shrimps (*Penaeus chinensis*) with gene gun. *Chinese Science Bulletin* 46:766-769.

Liu ZJ, Moav B, Faras AJ, Guise KS, Kapuscinski AR, and Hackett PB. 1990. Functional-Analysis of Elements Affecting Expression of the *Beta-actin* Gene of Carp. *Molecular and Cellular Biology* 10:3432-3440.

Liu ZJ, Zhu ZY, Roberg K, Faras A, Guise K, Kapuscinski AR, and Hackett PB. 1991. Isolation and characterization of *beta-actin* gene of carp (*Cyprinus carpio*). *Mitochondrial DNA* 1:125-136.

Long Y, Song G, Yan J, He X, Li Q, and Cui Z. 2013. Transcriptomic characterization of cold acclimation in larval zebrafish. *BMC gonomics* 14:612.

Mo S, Wang L, Li Q, Li J, Li Y, Thannickal VJ, and Cui Z. 2010. Caveolin-1 regulates dorsoventral patterning through direct interaction with β-catenin in zebrafish. *Developmental Biology* 344:210-223.

New MB. 2005. Freshwater prawn farming: global status, recent research and a glance at the future. *Aquaculture Research* 36:210-230.

New MB, and Nair CM. 2012. Global scale of freshwater prawn farming. *Aquaculture Research* 43:960–969.

Noh JK, Cho KN, Han EH, Kim AR, Lee JS, Dong SK, and Kim CG. 2003. Genomic Cloning of Mud Loach *Misgurnus mizolepis* (*Cypriniformes*, *Cobitidae*) β-Actin Gene and Usefulness of Its Promoter Region for Fish Transgenesis. *Marine Biotechnology* 5:244-252.

Preston NP, Baule VJ, Leopold R, Henderling J, Atkinson PW, and Whyard S. 2000. Delivery of DNA to early embryos of the Kuruma prawn, *Penaeus japonicus*. *Aquaculture* 181:225-234.

Quitschke WW, Lin ZY, Deponti-Zilli L, and Paterson BM. 1989. The beta actin promoter. High levels of transcription depend upon a CCAAT binding factor. *Journal of Biological Chemistry* 264:9539-9546.

Rasmussen RS, and Morrissey MT. 2007. Biotechnology in Aquaculture: Transgenics and Polyploidy. *Comprehensive Reviews in Food Science and Food Safety* 6:2-16.

Saurabh S, and Sahoo PK. 2008. Major diseases and the defence mechanism in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). *Proceedings of the National Academy of Sciences, India - Section B: Biological Sciences* 78:103-121.

Shi Y, Soderlund M, Xiang J, and Lu Y. 2015. Function and regulation domains of a newly isolated putative β-actin promoter from pacific white shrimp. *Plos One* 10:517-528.

Shi Y, Xiang J, Zhou G, Ron TB, Tong H-I, Kang W, Sun S, and Lu Y. 2016. The Pacific White Shrimp β-actin Promoter: Functional Properties and the Potential Application for Transduction System Using Recombinant Baculovirus. *Marine Biotechnology* 18:349-358.

Sin FYT. 1997. Transgenic fish. *Reviews in Fish Biology and Fisheries* 7:417-441.

Zhou B, Long Y, Song G, Li Q, and Cui Z. 2015. Molecular characterization of the lgals1 gene in large scale loach *Paramisgurnus dabryanus*. *Gene* 577:65-74.

Zhu J, He FH, Hu SN, and Yu J. 2008. On the nature of human housekeeping genes. *Trends in Genetics* 24:481-484.

Zhu XJ, Dai ZM, Liu J, and Yang WJ. 2005. Actin gene in prawn, *Macrobrachium rosenbergii*: characteristics and differential tissue expression during embryonic development. *Comparative Biochemistry & Physiology Part B Biochemistry & Molecular Biology* 140:599-605.

Table 1(on next page)

Sequences for primers

1 **Table 1. Sequences of primers**

Primer name	Sequence（5'-3'）	Note
Mractb1-F	TTCCCATCCATTGTCGGCAG	cDNA amplification
Mractb1-R	GCATTCTGTCAGCGATTCCTGG	cDNA amplification
GSP1	TGACCCATACCAACCATCACAC	5'-RACE
GSP2	ACCGGAGCCATTGTCTACAACCAAC	5'-RACE
Exon1-F	ACTCGCTCTTCGACATC	cDNA cloning
Exon1'-F	AGTCTGTCACTTGCTCC	cDNA cloning
Exon3-R	TGTCTGTAGAAATGAATTTATTC	cDNA cloning
Mractb1-SP1	GTACTGATATGAAGGCGTGTTCAG	Genome walking
Mractb1-SP2	GATAATGATGGTAAGGCAAACATTG	Genome walking
Mractb1-SP3	TACACCTGGAGTGTCTAAGCAG	Genome walking
Mractb1-SP4	CTACTCCTGAAGATGTCGAAGAGCGAGTG	Genome walking
Mractb1-SP5	TAACATCTGAAATGAAAGCGGACGAAACTG	Genome walking
Mractb1-SP6	CAAACGTCTTGCCTTATATGGACATGGAG	Genome walking
Mractb1-SP7	GTAGAAAGACCGGGATTTCTTTCGGT	Genome walking
Mractb1-SP8	ACTGGGCGTAACTACTATGCCTCTAA	Genome walking
Mractb1-SP9	GTTGAAGGGAAATGTACTGAGAACA	Genome walking
QMractb1-F1	TCAGGAGTAGCACGTACAC	qPCR
QMractb1-F1'	ATCACTGGTGCTCGTTG	qPCR
QMractb1-R0	ACAATGGATGGGAACAC	qPCR
QMr18S-rRNA-F	TAGTTGGAGGTCAGTTCC	qPCR
QMr18S-rRNA-R	ATTCCAGAGTAGCCTGC	qPCR
Mractb1P1-F	ACTGAGCTCTCCCGAAGTGATCACTG	Promoter analysis
Mractb1P1-R	AGACTGCTAGCTTTGTATTAGCTGCAAGAGAAAG	Promoter analysis
Mractb1P2-F	ACTGAGCTCTCATTTAGTAAGTAGGAGAG	Promoter analysis
Mractb1P2-R	AGACTGCTAGCAGCAAGTGACAGACTGAAC	Promoter analysis
Mractb1P3-F	ACTGAGCTCAGGTAAGTACACGTTGGC	Promoter analysis
Mractb1P3-R	AGACTGCTAGCTGAGATAAATTATGGAAC	Promoter analysis
Mractb1P4-F	ACTGAGCTCTTTGCCCTTCCGCGAAATTTAC	Promoter analysis
Mractb1P4-R	AGACTGCTAGCGGTGAGAGTGTACGTGCTAC	Promoter analysis
Mractb1P5-F	ACTGAGCTCACATTATGGAAACATTTC	Promoter analysis
Mractb1P5-R	AGACTGCTAGCATTCTTTGCATGTGACGAG	Promoter analysis
Mractb1P6-F	ACTGAGCTCATGATGAGGGTCACGCGTTAAG	Promoter analysis
Mractb1P6-R	AGACTGCTAGCTTCGGCGAACTGGGCGT	Promoter analysis
Mractb1P7-F	ACTGAGCTCTGCGCTGCTTTTACCAAATAC	Promoter analysis
Mractb1P7-R	AGACTGCTAGCTCATCATCTTTACCATC	Promoter analysis
Mractb1P42-F	TACACTCTCACCTCATTTAGTAAG	Promoter analysis
Mractb1P24-R	CTTACTAAATGAGGTGAGAGTGTA	Promoter analysis
Carpactb1-F1	ACGAAGCTTTCAAACTGTGGCACCATC	Promoter analysis

Carpactb1-R1	ACGCCATGGCTGAACTGTAAATGAATG	Promoter analysis

Figure 1

Cloning of the 5’-flanking sequence of the *Mractb1* gene

The 5′-flanking sequence of *Mractb1* was cloned by three rounds of genome walking based on asymmetric interlaced PCR. The dashed line represents the assembled 5′ flanking sequence. The rectangular blocks and the dotted arrows indicate the cloned sequences and the primers used for each round of genome walking respectively. The electrophoretograms were displayed accordingly. The numbers above the electrophoretograms indicate different round of PCR for each walking step.

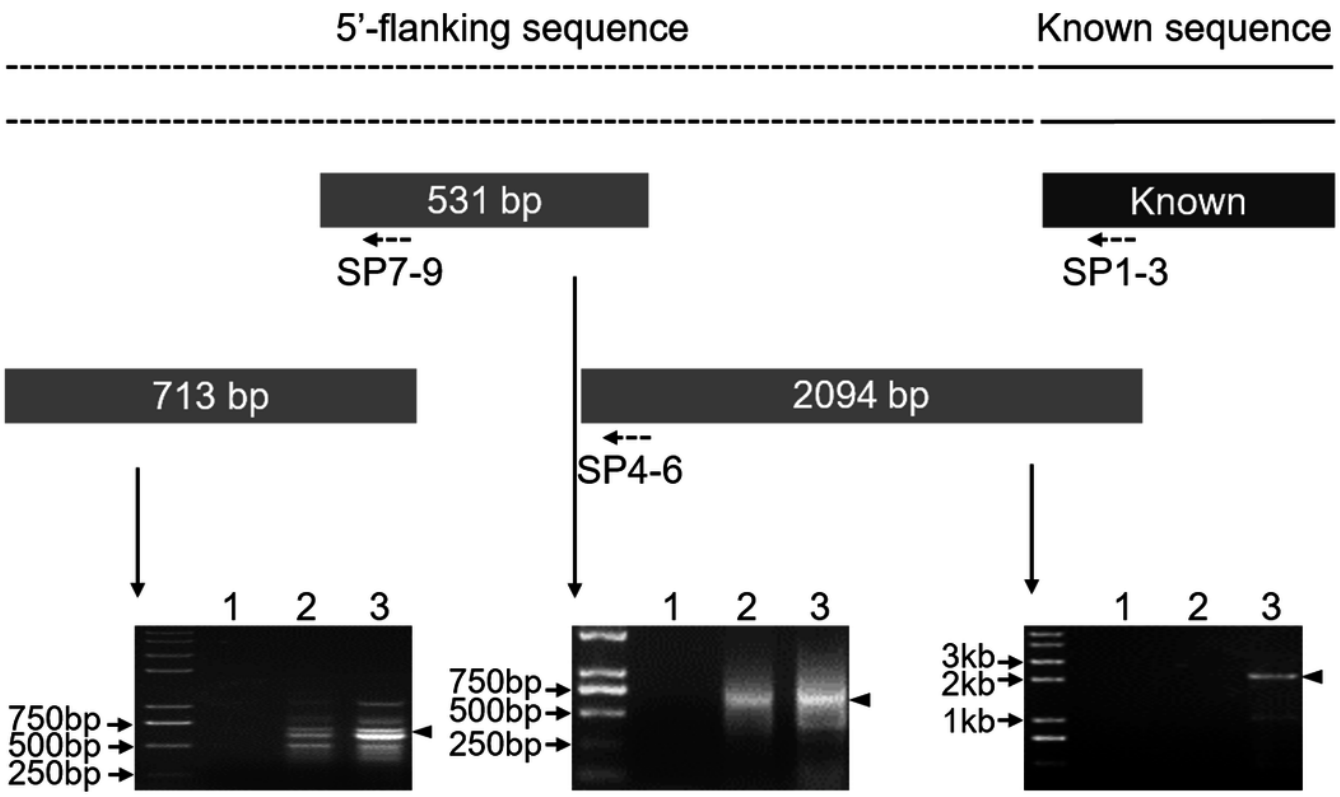

Figure 2

Determination of the transcription start sites of the *Mractb1* gene

The shaded rectangles represent the known cDNA sequence (black) and the 5′ cDNA end sequence obtained by 5′ RACE (grey), respectively. The electrophoretogram shows the 5′ RACE product (indicated by an arrow head). The base peak maps indicate the two 5′ cDNA end sequences identified by DNA sequencing. M, DNA marker; GSP1 and GSP2, gene specific primers; In, 5′ RACE inner primer.

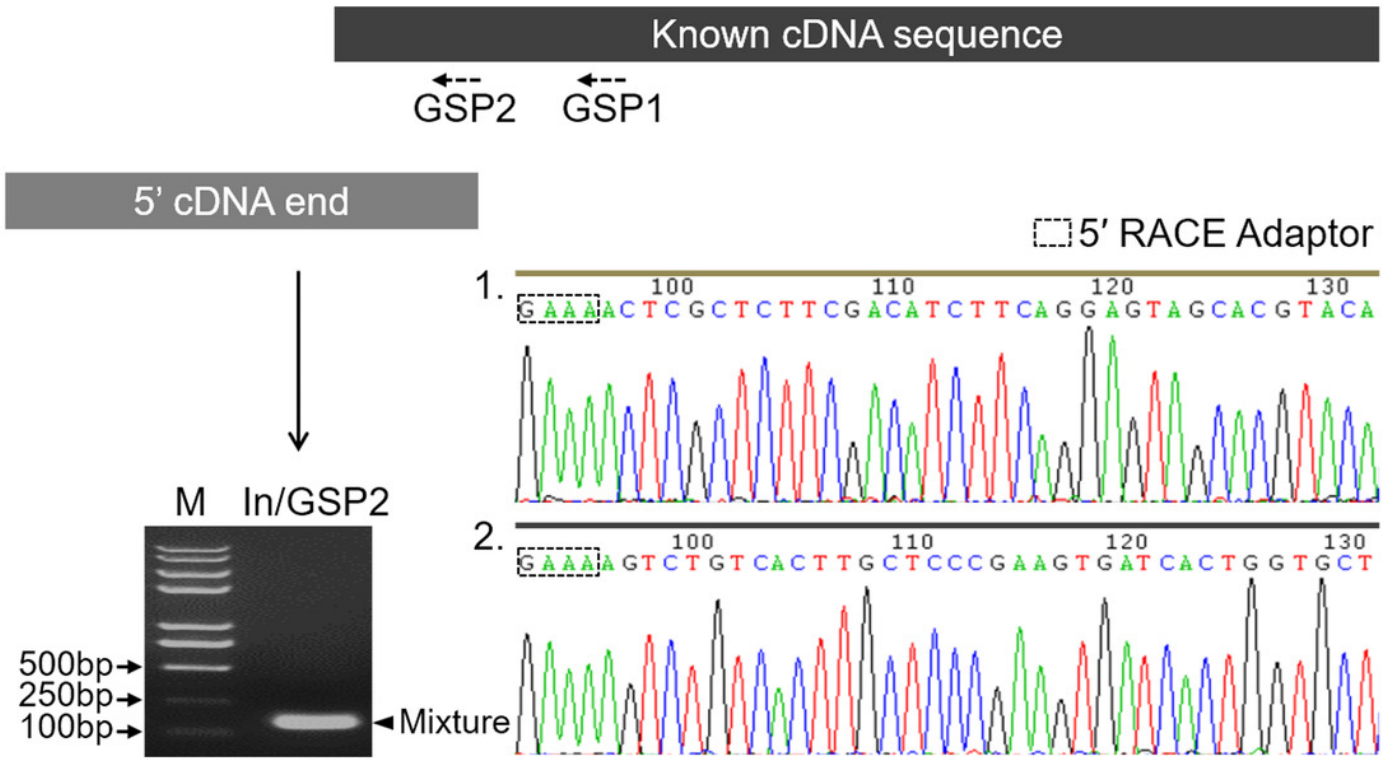

Figure 3

Transcripts of the *Mractb1* gene

(A) Structure of the *Mractb1* gene and its two transcript variants with different initial exon. The numbers and arrows indicate the relative position of the exons and coding sequence (CDS). The first base of the start codon was defined as position +1. The numbers in the rectangles indicate different exons of the Mractb1 gene. The alternative first exons are designated as exon 1 and exon 1'. **(B)** Electrophoretogram for the two transcripts. Primers used to amplify the two transcripts are Exon1-F, Exon1-F' and Exon3-R as displayed in (A). M: DNA marker.

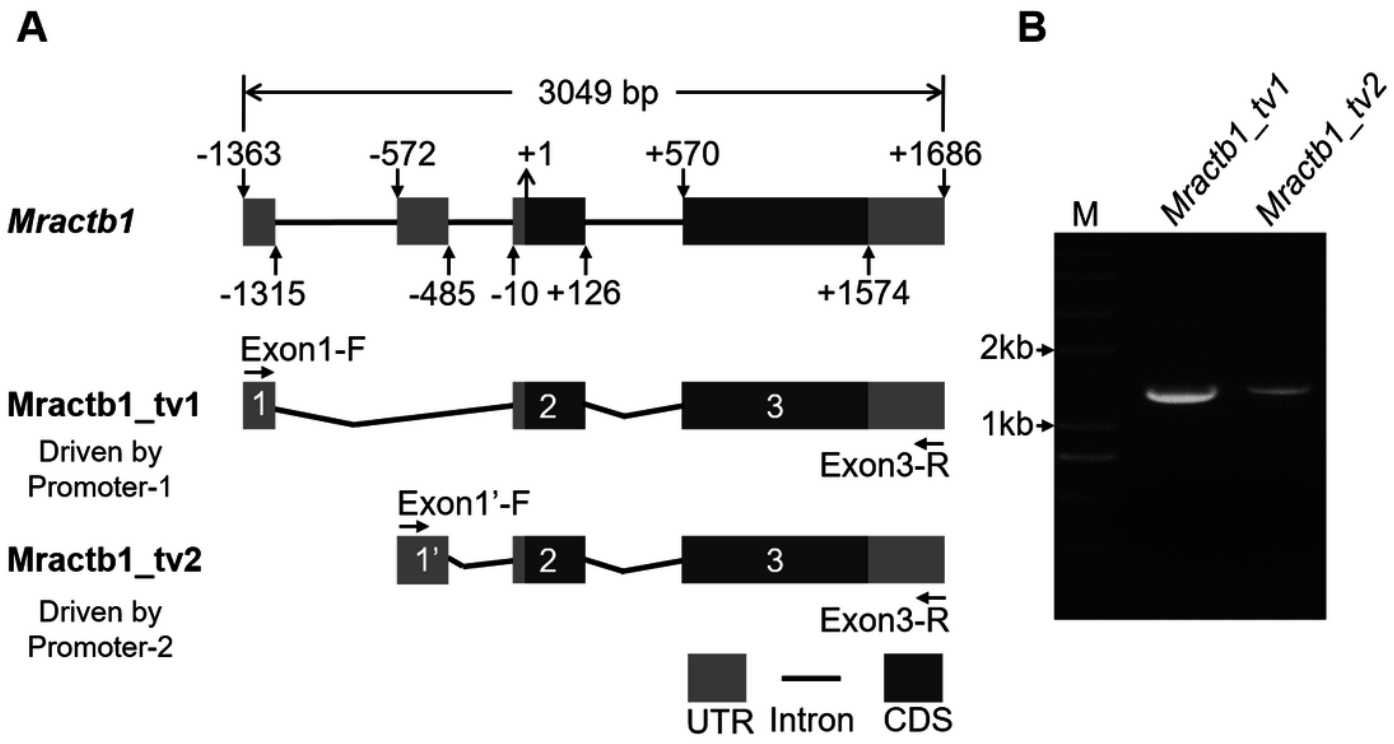

Figure 4

Expression of the two *Mractb1* transcripts in different sexes and tissues

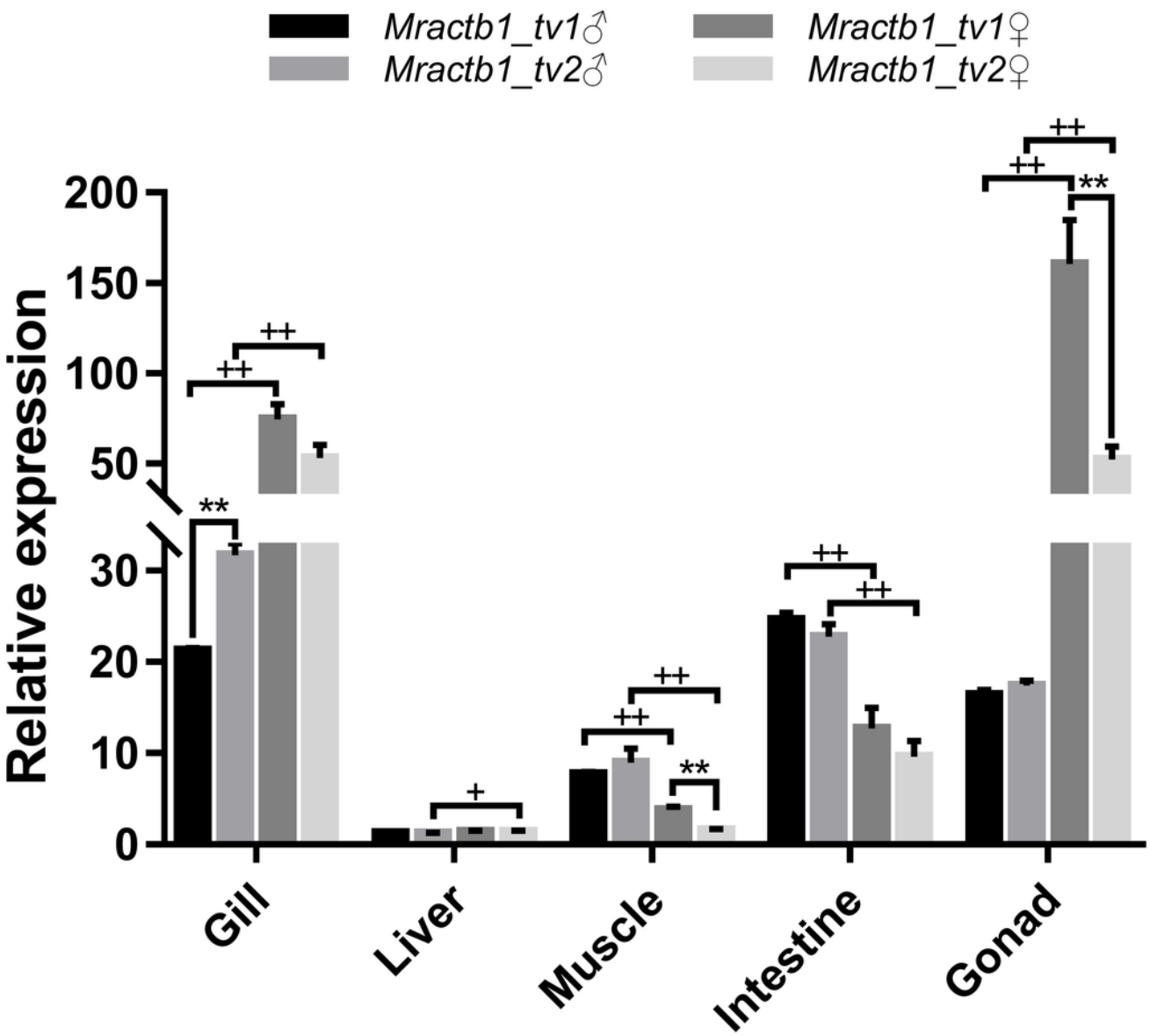

Figure 5

Molecular dissection of the *Mractb1* gene promoter.

(A) Schematic diagram of the DNA fragments subjected to promoter activity assays. The 2620 bp 5'-flanking sequence was divided into 7 fragments (rectangles shaded with different color) according to the location of the TSSs and CpG islands (i1: -1762/-1569, i2: -1437/-1320, i3: -747/-542) predicted by the Methprimer software (http://www.urogene.org/). The arrow heads indicate TSSs. The dashed rectangles represent first exons of the two transcripts. **(B)** Promoter activity of the flanking regions characterized individually. **(C)** Characterization of promoter-1. **(D)** Characterization of promoter-2. **(E)** Deletion analysis of the whole sequence. **(F)** Interaction between the two alternative promoters. The DNA fragments were cloned into the pGL3-basic vector to generate promoter activity analyzing plasmids. The constructs were transfected into EPC cells and the transcriptional activities were analyzed by dual luciferase assays. The relative luciferase activity represents the ratio between the firefly luciferase activity and the corresponding renilla luciferase activity. The bars indicate mean ± SD (n = 3).

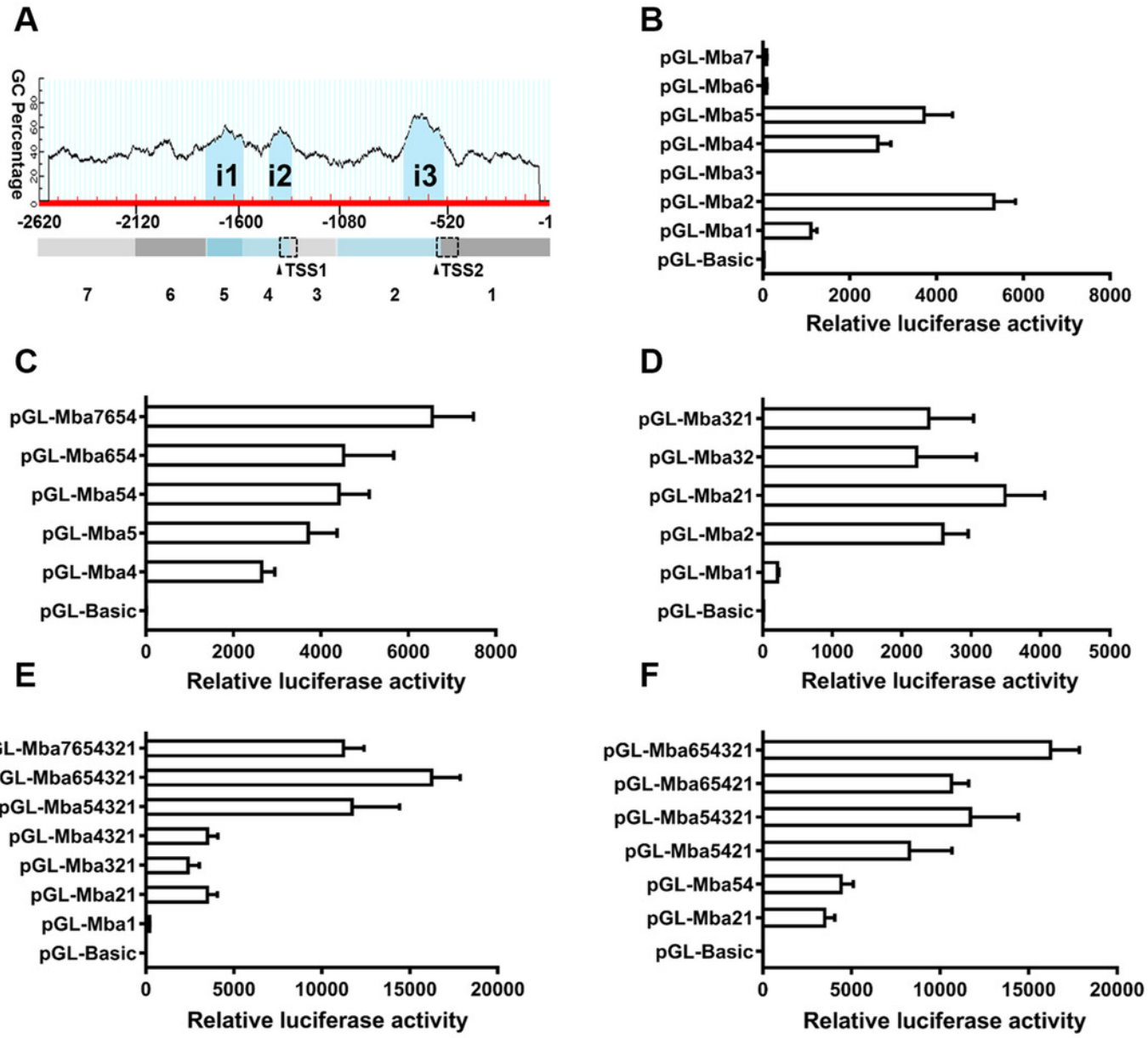
A
GC Percentage
i1
i2
i3
-2620
-2120
-1600
-1080
-520
-1
TSS1
TSS2
7
6
5
4
3
2
1
B
pGL-Mba7
pGL-Mba6
pGL-Mba5
pGL-Mba4
pGL-Mba3
pGL-Mba2
pGL-Mba1
pGL-Basic
0
2000
4000
6000
8000
Relative luciferase activity
C
pGL-Mba7654
pGL-Mba654
pGL-Mba54
pGL-Mba5
pGL-Mba4
pGL-Basic
0
2000
4000
6000
8000
Relative luciferase activity
D
pGL-Mba321
pGL-Mba32
pGL-Mba21
pGL-Mba2
pGL-Mba1
pGL-Basic
0
1000
2000
3000
4000
5000
Relative luciferase activity
E
pGL-Mba7654321
pGL-Mba654321
pGL-Mba54321
pGL-Mba4321
pGL-Mba321
pGL-Mba21
pGL-Mba1
pGL-Basic
0
5000
10000
15000
20000
Relative luciferase activity
F
pGL-Mba654321
pGL-Mba65421
pGL-Mba54321
pGL-Mba5421
pGL-Mba54
pGL-Mba21
pGL-Basic
0
5000
10000
15000
20000
Relative luciferase activity

Figure 6

The conserved genetic elements in the *Mractb1* 5’-flanking region.

The conserved genetic elements in the *Mractb1* 5’-flanking region. The CpG islands are shown in red. The exons are shown in uppercase and the introns are shown in lowercase. The numbers at the left represent relative position of the nucleotides and the first base of the start codon is regarded as +1. The donor/acceptor sites for splicing are shown in red italics. The arrows indicate transcription start sites. The CCAAT-box, CArG-box, GC-box and InR are underlined.


CCAAT-box
CArG-box
GC-box
InR
GC-box
InR

Figure 7

Mractb1 promoter demonstrates comparative activity with carp *actb1* gene promoter.

(A) Relative luciferase activity. **(B)** Western blot of firefly luciferase. **(C)** Relative levels of the firefly luciferase protein. The *Mractb1* promoter (Mbap) and carp *actb1* promoter (Cbap) were cloned into the pGL3-basic vector and the resulted constructs were transfected into EPC cells. Dual luciferase assays and western blot were performed to analyze the activities and protein levels of the luciferase reporter, respectively. GAPDH was used as loading control for western blot. The relative intensity represents ratio of the brightness of the firefly luciferase bands to those of GAPDH. The bars indicate mean ± SD (n = 3).