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Genomic structure and transcriptional regulation of grass carp calmodulin gene

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ABSTRACT

A fish calmodulin (CaM) gene was characterized for the first time in grass carp. The CaM gene is about 12-Kb in size with identical intron/exon organization as that of mammalian CaM genes. When compared to mammalian counterparts, the 5′-promoter region of grass carp CaM gene contains a TATA box and has a much lower GC content and CpG dinucleotide frequency. Interestingly, the 5′-promoter of carp CaM gene is AT-rich with multiple IRS elements and putative binding sites for Pit-1, Sp1/Sp3 and AP1. Using luciferase reporter assay, a potent silencer region was identified in the distal region of grass carp CaM promoter. Besides, the CaM promoter activity could be upregulated by IGF but suppressed by PACAP, forskolin and over-expression of Sp1 and Sp3. These findings, taken together, indicate that grass carp CaM gene does not exhibit the typical features of housekeeping genes and its expression is under the control of hormone factors, presumably by coupling with the appropriate signaling pathways/transcription factors.

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Introduction

Calmodulin (CaM) is a ubiquitous Ca^{2+}-binding protein involved in many cellular functions including cell proliferation, survival and death [1–3]. Multiple CaM genes have been identified in vertebrates, e.g. three CaM genes in mammals [4–7], two in chicken [8,9] and frog [10,11]. All the genes from different vertebrate species encode the same CaM molecule with identical amino acid sequences, suggesting its high conservation during vertebrate evolution. Although all three CaM genes are ubiquitously expressed and coordinately regulated in mammals [12], the differential expression of these CaM genes during development [13,14] and in response to extracellular signals [13–16] has been reported. The mechanism about the differential regulation of these CaM genes has not been clearly elucidated. Several studies in mammals, however, suggest the difference of 5′-UTR and/or 5′-flanking promoter regions among three CaM genes may be responsible for their differential regulation [7]. In lower vertebrates, however, not much is known about the three variants of CaM gene and their regulation at the transcriptional level. At present, two CaM cDNAs have been reported in frog [10], but no functional study on gene regulation has been attempted. Similarly, multiple CaM cDNAs have been isolated in fish [17,18], but the full gene of fish CaM has not been cloned and the corresponding promoter sequence has yet to be characterized. We previously cloned a grass carp CaM cDNA [19]. Here, we further extended our study by PCR cloning of the full gene of grass carp CaM and functionally characterized the newly cloned CaM promoter by transfection studies. The data obtained clearly indicate that (i) the carp CaM gene does not exhibit the typical features of housekeeping genes as reported in mammals and (ii) its expression is under the modulation of neuroendocrine factors.

Materials and methods

Animals, reagents and plasmids. One-year-old Chinese grass carp (*Ctenopharyngodon idellus*) purchased from local markets were kept and treated as previously described [19]. Forskolin, H89, IGF-I and IGF-II were purchased from Sigma (Sigma, MD, USA). Pituitary adenylate cyclase activating-polypeptide 38 (PACAP38) was obtained from Peninsula Laboratories Inc. (Belmont, CA, USA). pGL3 plasmid was obtained from Promega (Promega Corporation, Madison, WI). gcPit-I-pcDNA3.1 was constructed in our lab. pCMV-Sp1 and pCMV-Sp3 were gifts from Professor Will W.M. Lee (The University of Hong Kong).

Genomic PCR. According to the cDNA of grass carp [19], specific primers (as listed in Supplemental Table 1) were designed for genomic PCR to amplify the full-length of CaM gene. Briefly, primers PU1 and PD1 were used to amplify the partial CaM gene containing intron II, III, IV and V by pfu DNA polymerase at the PCR condition: 94 °C 3 min followed by 30 cycles of 94 °C 1 min, 55 °C 1 min and 72 °C 5 min and then a final extension at 72 °C for 10 min. Similar PCR strategy was used to amplify the intron I of the fish CaM gene using nest PCR with primers PI-Ua and PI-Da, PI-Ub and PI-Db. The 5′-flanking region and 3′-flanking region

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of the grass carp CaM gene was amplified from a genome-walker library constructed according to the manufacturer recommended procedures (Universal GenomeWalker™ Kit, Clontech).

Primer extension. To determine the transcriptional start sites of the fish CaM gene, primer extension assay was performed using purified fish mRNA and primer extension system from Promega (Cat# E3030) according to the manufacturer recommended procedure. The extension products were separated on 8% polyacrylamide sequencing gel with 8 M urea and then exposed to X-ray film for 3 weeks.

Construction of promoter–reporter plasmids. To construct plasmids containing serial deletions at the 5′-end of grass carp CaM promoter and luciferase reporter gene i.e. gcpCaM-Luc, the increasing deletion of 5′-flanking region of fish CaM gene was amplified by the primer PGW2 together with the downstream primer AP2, PDA1, PDA2, PDA3, PDA4, PDA5, PDA6 and PDA7, respectively (Supplemental Table 1). The PCR fragments obtained were then cloned into the upstream of the luciferase gene of pGL3. Similar PCR strategy was used to get the promoter constructs with 3′-deletion using primer PEA1 combined with primer AP2, PDA1, PDA2, PDA3, PDA4, PDA5, PDA6 and PDA7, respectively (Supplemental Table 1).

Transient transfection. Mouse αT3-1 cells seeded onto 24-well culture plate were transfected by lipofectamine with plasmids including 0.1 μg/ml of each promoter construct, 0.02 μg/ml pEGFP-N1 (internal control) and 0.38 μg/ml carrier DNA or 0.28 μg/ml carrier DNA plus 0.1 μg/ml of gcPit-I-pcDNA or 0.36 μg/ml carrier DNA plus 0.02 μg/ml pCMV-Sp1/pCMV-Sp3. For drug treatment with PACAP38, forskolin, H89 and cAMP analog, the transfected cells were treated with drug at appropriate concentration for 12 h before cell harvesting. The luciferase activity was determined using Promega Luciferase Assay System. To study the effects of IGF-I and IGF-II on the grass carp CaM promoter activity, the transfected cells were firstly serum starved for 6 h and then treated with IGF-I/II in serum-free medium for 6 h before cell harvesting. All the experiments were carried out at least three times in quadruplicate.

3. Results

3.1. Genomic organization of the grass carp CaM gene

Through genomic PCR, the full-length gene of grass carp CaM with 12 Kb in size was obtained (GenBank ID: AY656698, Supplemental Fig. 1). The fish CaM gene contains 6 exons and 5 introns, and the sizes of exons I, II, III, IV, V and VI are 102, 31, 144, 107 136 and 1019 bp, respectively, and their corresponding introns are 7413 bp (intron I), 204 bp (intron II), 2306 bp (intron III), 399 bp (intron IV) and 246 bp (intron V), respectively. The pattern of intron insertion in the carp CaM gene was found to occur at identical positions as those observed in the human and rat CaM I genes (Supplemental Table 2). However, the sizes of introns are notably different in the CaM genes between grass carp and mammals (Fig. 1). Similar to other vertebrate CaM genes, the intron I interrupts the coding sequence immediately after the ATG translation start codon. The C-terminal amino acid codons and the entire 3′-UTR are encoded in the last exon of the carp CaM gene, and this is also the largest exon in the fish CaM gene. The last canonical poly(A) signal (AATAAA) is located 16 nt upstream of the cleavage site at the end of 3′-UTR, which is consistent with the situation reported in most mammalian mRNAs [20].

3.2. Multiple transcriptional start sites at the fish CaM gene

To identify the transcriptional start site (TSS), primer extension was performed using fish mRNA and primer PGW2 complementary

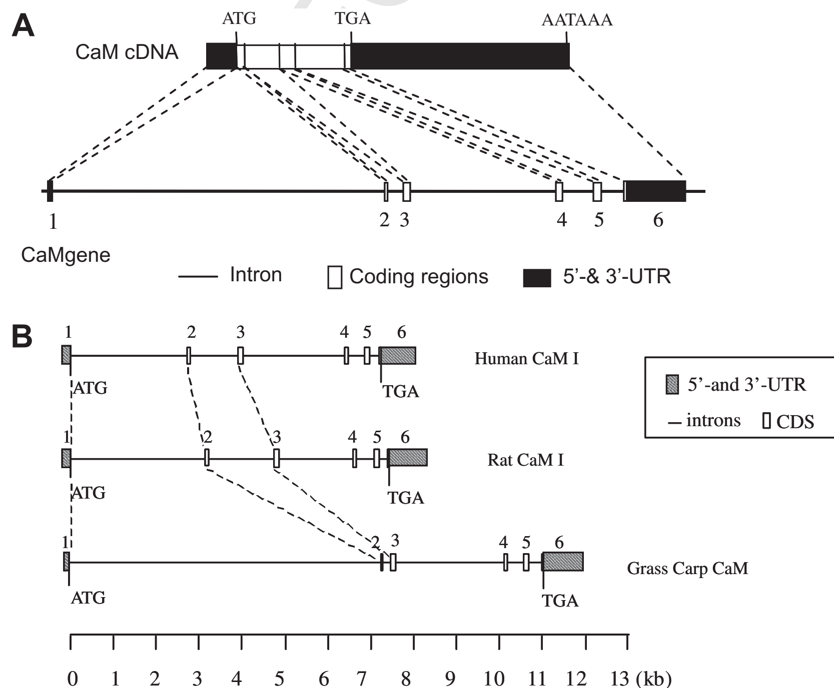

Fig. 1. Genomic organization of fish CaM gene. (A) Schematic illustration of the genomic organization of the grass carp CaM gene. Coding region from ATG to TGA is shown by open box. 5′- and 3′-UTR are indicated by hatched box. ATA indicates the AATAAA consensus polyadenylation signal site. The number 1–6 represents exons 1–6 of the fish CaM gene. (B) Comparison of the structures of vertebrate CaM I genes. The grass carp CaM gene was aligned with human/rat CaM I genes with respect to the ATG at the 3′-end of exon 1. Boxes and lines represent exons (numbered from 1 to 6) and introns, respectively. Coding regions are shown as white boxes, and non-coding regions as grid boxes. The CaM I genes of human and rat are from the studies of [5,23]. Grass carp CaM gene is from the present study. The scale bar at the bottom represents the distance in kb with respect to ATG.

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to the sequence at −72 to −50 bp upstream of the ATG, and five extension products were obtained with the sizes of 49(a), 95(b), 97(c), 111(d) and 161(e) nucleotides, respectively (Supplemental Fig. 2). The position of the product (a) with 49 nucleotides in the carp CaM gene is consistent with the predicated TSS using the program PROSCAN (http://bimas.dcrt.nih.gov/molbio/proscan) and therefore, this position was designated as the "+1" in this study (Supplemental Fig. 2). The other products were thus designated as position −46, −48, −62 and −102 corresponding to the extension products with sizes of 95(b), 97(c), 111(d) and 161(e) nucleotides, respectively.

3.3. Features of the 5′-flanking promoter region

Analysis of the 1.5 Kb of 5′-flanking region of the carp CaM gene has revealed that a typical TATA box (TATATA) is located 24 bp upstream of the first TSS (Fig. 2). Another putative TATA box is located 143 bp upstream of the TSS. There are a total of 10 putative CAAT boxes in the 5′-flanking sequence. However, there is no CAAT box around the position −80 as reported in other calcium binding protein, e.g. the chicken ovalbumin gene [21]. Unlike other vertebrate CaM promoters with high GC content (>50%), the grass carp CaM promoter region is AT-rich (67%). Regions with high GC content are only found in two regions i.e. −1 to −100 bp and −1400 to −1600 bp upstream of the ATG start codon in the 5′-flanking sequence of the carp CaM gene (Fig. 2 and Supplemental Fig. 3A). As a result, the CpG dinucleotide frequency averaged over 100 nucleotides are much lower than that of mammalian CaM promoters (Supplemental Fig. 3B). In addition, a couple of AT-rich insulin response sequences (IRS) and other putative regulatory elements including the binding sites for Pit-1, Sp1 and Ap1 can be identified in the 5′-promoter region of grass carp CaM gene by computer-assisted sequence analysis using TESS site search (http://www.cbil.upenn.edu/tess) (Fig. 2).

3.4. Functional analysis of the grass carp CaM promoter

To test the promoter activity of the grass carp CaM gene, a series of promoter–reporter constructs were prepared and their promoter activity was investigated in αT3-1 cells. The 1.37 Kb upstream of the fish CaM gene, as well as other constructs with 5′-end deleted sequence acted as strong promoter in alpha-T3-1 cells. The 1.5 Kb of full-length 5′-flanking region, however, showed very low basal promoter activity when compared to the promoterless pGL3, indicating that there is a strong silencer between −1509 and −1369 in the carp CaM promoter. There should be another weak silencer between −1157 and −909 because this region deletion resulted in a 2-fold increase of luciferase activity (Fig. 3A). To our surprise, the inhibitory effect of silencer located between −1509 and −1369 bp upstream of the fish CaM promoter was reversed by 3′-end deletion from +9 to +49 at the 5′-UTR (Fig. 3B). Indeed, the 3′-end deletion by removing 41 bp of 5′-UTR caused the carp promoter constructs with different 5′-flanking sequence to have similar promoter activity in terms of luciferase expression, implying that the 5′-UTR of the carp CaM gene plays an important role in coupling the mechanism of gene regulation with the promoter sequence.

3.5. Regulation of the grass carp CaM gene promoter activity

To test the hormone effects on carp CaM promoter activity, αT3-1 cells transfected with the fish CaM promoter constructs were treated with 100 nM IGF-I or IGF-II. Both IGF-I and II significantly increased the carp CaM promoter activity in all the 5′-deletion constructs (Fig. 4A, upper panel and Supplemental Fig. 4). In addition, IGF-I/II also dose-dependently increased the CaM promoter activity with the length of −132 bp (Fig. 4A, lower panel).

The functional role of the cAMP-dependent pathway in carp CaM promoter activity was investigated in αT3-1 cells by treatment with PACAP38 (Fig. 4B). PACAP38 is a member of the glucagon/secretin peptide family and is known to increase cAMP production in αT3-1 cells [22]. In this case, PACAP38 (1 μM) significantly reduced luciferase expression in αT3-1 cells transfected with all the promoter constructs. Besides, parallel treatment with forskolin (10 μM), an adenylate cyclase activator, also induced a drop in CaM promoter activities in αT3-1 cells (Fig. 4B). Moreover, the inhibitory action of forskolin was mimicked by 3 μM 8-Br-cAMP treatment (Fig. 4B). However, PKA inhibitor H89 (3 μM) had no effects on basal CaM promoter activity (Supplemental Fig. 5A). In accordance with these findings, H89 could not block the inhibitory effect of forskolin on the CaM promoter activity (Supplemental Fig. 5B).

To investigate whether the regulatory elements identified by TESS have any effect on fish CaM promoter activity, αT3-1 cells were cotransfected with CaM promoter-carrying constructs and the expression vectors for the transcription factors Pit-1, Sp1 or/and Sp3, respectively. Pit-1 over-expression had no effect on the fish CaM promoter activity (Supplemental Fig. 6), indicating that the Pit-1 sites present at the carp CaM gene promoter may not be functional. Sp1 and Sp3 over-expression, however, significantly reduced the fish CaM gene promoter activity. Apparently, over-expression of Sp3 was more effective than that of Sp1 in reducing the promoter activity (Fig. 4C). These data suggested that the Sp1 binding site located at −92 to −100 of the 5′-flanking of the carp CaM gene are functional, and probably the site can bind Sp3 more effectively than Sp1.

4. Discussion

We previously isolated a CaM cDNA from grass carp and found it is phylogenetically related to the CaM I gene of mammals [19]. Here, we characterized the structural organization of the carp CaM gene and found it has the same genomic organization as its human and rat counterparts (Supplemental Table 2). However, the intron sizes of the CaM I genes vary greatly among species, especially for introns I and II. The intron I with 7.413 Kb in fish CaM gene is much larger than those in human (2.9 Kb) [5] and rat (3.16 Kb) [23] CaM I genes. In contrast, the intron II in the fish CaM gene is much smaller (204 bp) than its corresponding region in mammalian models (1.16 and 1.5 Kb for human and rat CaM I, respectively) [5,23].

Consistent with the phylogenetic relationship between fish CaM mRNA and mammalian CaM I mRNA [19], several features of the carp CaM gene also indicated its close relation with mammalian CaM I gene rather than with CaM II and CaM III genes. Firstly, five TSS are identified in the fish CaM gene, which is consistent to the reports that mammalian CaM I gene tends to have more TSS than CaM II and III genes [23] [5]. Secondly, both the carp CaM gene and mammalian CaM I gene have a typical TATA box with similar location at their 5′-flanking regions (i.e. 24, 32 and 28 bp upstream of the first TSS at carp, human and rat CaM I genes, respectively). In contrast, no typical TATA box was found in vertebrate CaM II and CaM III gene promoters [4,7,11,24,25]. In addition, a highly conserved H1 element [GCGGAGG(G)A] identified in mammalian CaM I gene promoters has also been found in the fish CaM gene promoter. Again, the H1

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actatagggcacgcgtggtcgacggcccgggctggtctggccaatcataaaagctgtgac -1450

atgctgagccaaaatctctccagcgtcaacaaagacagtggacttgatgcaataagtaat -1390
   Pit-1
attccgacgaaagaaactagtgccgtttagagaagtagtccctcaatgtaaagtgttctt -1330
                                        CSE
agaccgttaattaatgtaatgtgatcataataaattattttactagttgtcggcttgtc -1270
                                      IRS
cttattttttattcatagattcggtgattttaattaataagtaatttttaattcgtat -1210
   IRS
cctatattcctaaatgtattcaaaaacacgttaatttaacttggtgtgataaagaacaaa -1150

aataaacttaagacaatagttatttataatattatattgatataacttttaattatttc -1090
                                    Pit-1              Pit-1
atgtttatttattactcatcaatgaacatttcagtttaaaatttaactgttcacttgga -1030

attattttaattaataaattaattaagagaccaaaggaatgctttatgtaagacgacttt -970
   IRS          Pit-1
ctactgtgacgataaaaaaggtcttaattcacaaaaaaagctcattcttgaagttcattt -910

ttgaatgtgaatgttgactgtgtttactcttcttgctgtacatttgccacagtcttgtc -850
                    Pit-1
ttatgcactacacttaggcctattttttgtttatcacatttatagttttaatagtctat -790
                    IRS
caagcccctgcatttaaaggggccacaatattcctgtaggtgttatcctcctcattacaa -730

atgtaggctactggatgaatttactttagatttactttaaatttattcaggccaactt -670

ctgtgtttctacacaacttatagaacatattgataggtcattttgggaagatctgggtc -610
                              Pit-1
tgcttagagataaaaatgatcagcatgtgagaatgtatatgtatatatatatatattttt -550
                                                          IRS
tttaaataatcccatatttgtatctgtaaaatatctttgtaggcctacgttattcttaca -490
                IRS       IRS
acaatgaagtgaaaattatatagcctactgaatatgtgaacctttatgagtgagatgcat -430
                                                 AP1
attttgttcccacagttttcatattgtactacgttagtatgtttagtatctacaataggа -370
 IRS            Pit-1
ctaacaataacagtgtgtaaagtgatccaagaaataattacattacatcaaatacacatt -310

attgatctgtattgcgtgctcatatatttaaattattttataagaaaataataagtaata -250
                                      IRS
ttactattctaataaacctgctggtttacataaaaacatttaatagaatgtaaatatgct -190

gttttcttgcattagtgctgaaaactttttaaacaaaatgtgctatacactttttcttct -130

cgggactggagtggttatgataattagatgggcggggcttgtgactgcactatgcggaag -70
                                  Sp1
tgagtgatagggggtccgtacggcggaggaatacaccaaagtatatagtaccggatcacct -10
        1              H1
tcaatactcTGCGAGCGACAGTCTCACGGTGGAGCTTTGAACTGAGAGCGGAGCACCACC 51
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Fig. 2. Nucleotide sequence of the grass carp CaM gene 5′-flanking region. Nucleotide numbering starts with the first transcription start site (TSS). The other four TSSs from primer extension analysis (Supplemental Fig. 1) are indicated by bold italic letters. Putative cis-acting elements in the 5′-flanking region of the carp CaM gene are underlined. The possible TATA box is indicated by bold letters and the putative CAAT box shown with dot underline; CSE, Ca^{2+}-sensitive element; IRS, insulin response sequence; AP1, activating protein; Pit-1, pituitary-specific transcription factor; Sp1, stimulating protein 1.

element has a similar location in the 5′-flanking region of the fish CaM gene as those of CaM I genes from rat and human (i.e. −41, −49 and −53 for carp, rat and human, respectively) [5,25]. However, the H3 element, which is only found in the promoter regions of vertebrate CaM II and III but not in CaM I genes [26,27], cannot be found in the 5′-flanking region of the carp CaM gene. Moreover, the Purkinje cell element (PCE1) mediating Purkinje cell specific expression and identified in the mammalian

CaM II promoter can be found neither in mammalian CaM I gene promoters [28] nor in fish CaM gene promoter.

Unlike mammalian CaM I gene promoter with a high level of GC-rich content, the 5′-promoter region of grass carp CaM gene has a much lower GC content (only 32.7%) and CpG dinucleotide frequency. High levels of GC content and CpG dinucleotide frequency are the typical features of housekeeping genes [29]. Apparently, the 5′-promoter sequence of the carp CaM gene does not exhibit the typical features of housekeeping genes. It is worth noting that a high AT-rich (about 85% A + T) rather than GC-rich content can also be observed in the CaM gene promoter of *Tetrahymena thermophila* [30]. It is still unclear if an increase of GC content in the CaM gene promoter can be correlated with the extent of progressive evolution of animals in terms of the Ca^{2+}-sensing ability via CaM signaling.

By TESS analysis, only one Ca^{2+}-sensitive motif (AGGGA) was found in the opposite strand of the fish CaM gene promoter. However, this motif is commonly found in mammalian CaM gene promoters [26,27]. In addition, nine insulin response sequences (IRS) with the core sequence of T(G/A)TTT(T/G)(G/T/A) can be found at the carp CaM promoter, while it is not so abundant in other vertebrate CaM gene promoters.

IGF-I and II were found to increase the basal promoter activity of carp CaM gene, and the responsive element for the hormone treatment seems to be located at the proximal promoter region because of the minimal promoter showing maximal luciferase activity (Fig. 4A and Supplemental Fig. 4). This is consistent with our previous study that IGF-I/II upregulate CaM gene expression at the transcriptional level, but not at the post-transcriptional level [19]. Unexpectedly, PACAP38 and forskolin treatment significantly decreased the carp CaM promoter activity (Fig. 4B), which is in contrast to that PACAP38 and forskolin increases CaM mRNA level in goldfish pituitary cells [18]. This discrepancy may be resulted from the use of different cell models, species-specific variation, or difference in the research methodology. Unlike multiple Sp1 sites in mammalian CaM gene promoters, only one canonical Sp1 binding site 5′-GGGCGGGGC-3′ was identified in the 5′-promoter region of fish CaM gene, and Sp1/Sp3 was found to negatively regulate the

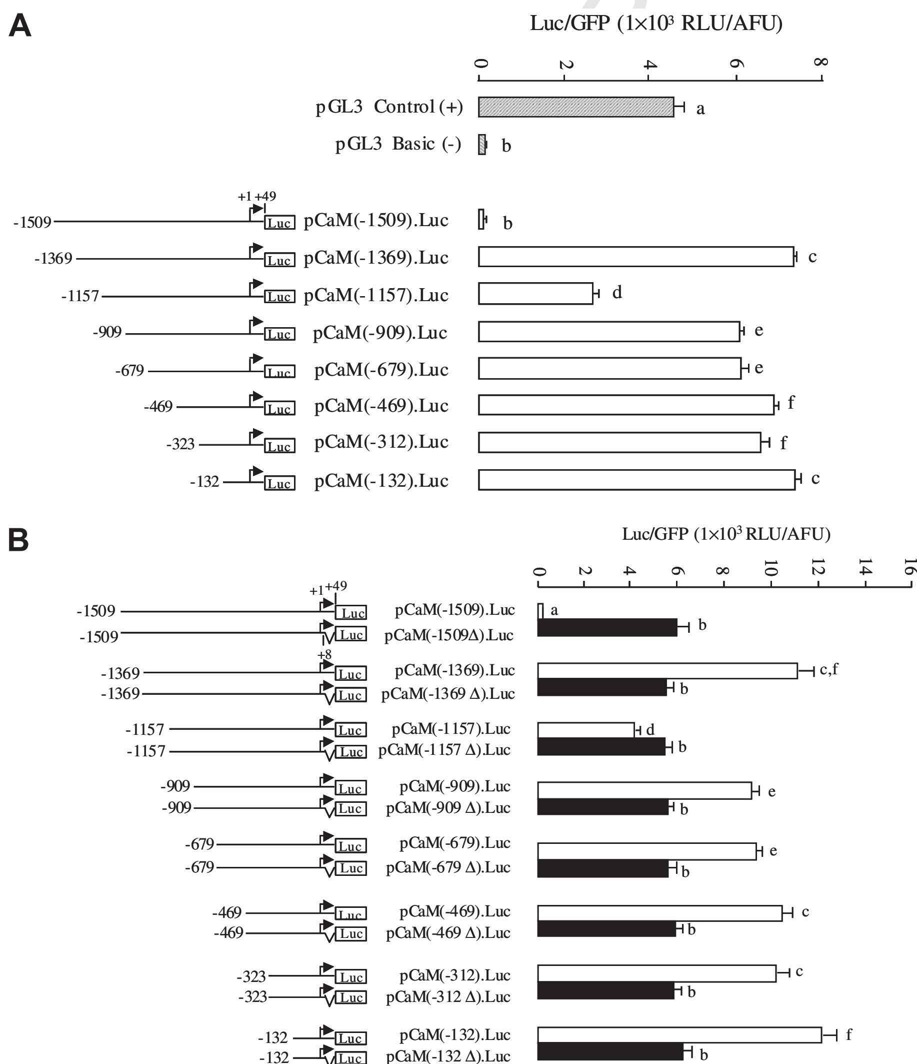

Fig. 3. Deletion analysis of grass carp CaM promoter activity. (A) 5′-deletion analysis of grass carp CaM promoter activity (pCaM.Luc) in αT3-1 cells. Constructs with increasing 5′-end deletion of grass carp CaM promoter are shown in the left. The first transcription start site is indicated as +1. The 5′-untranslated sequence and the promoter sequence are positively and negatively numbered, respectively. (B) Analysis of grass carp CaM gene promoter activity by 3′-end 41 bp-deletion of 5′-UTR in αT3-1 cells. Constructs with 5′-end deletion mutants of grass carp CaM promoter region are shown in the left. Corresponding constructs containing 41 bp 5′-UTR deletions are shown in parallel. Results are presented as the mean ± SEM ($N = 3$). Experiment groups with a similar magnitude luciferase activity ($P > 0.05$) are denoted with the same letter (ANOVA followed by Fisher LSD test).

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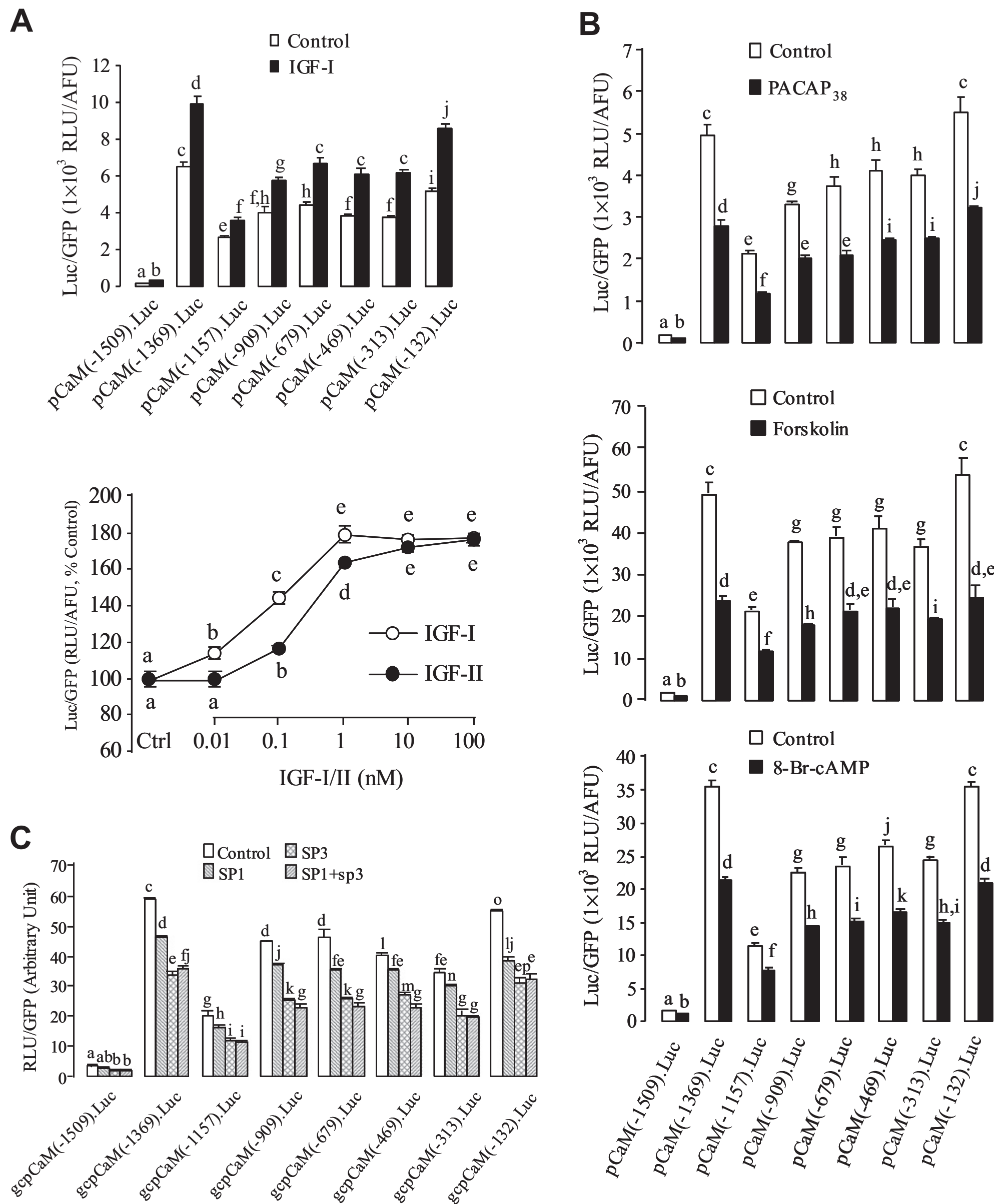

Fig. 4. Regulation of the fish CaM promoter activity by IGF, PACAP38, forskolin and Sp1/Sp3. (A) Up-regulation of fish CaM promoter activity by IGF-I and IGF-II. αT3-1 cells were cotransfected with sequential 5′-deletion constructs of grass carp CaM promoter and pEGFP-N1, and then treated with 100 nM IGF-I (upper panel) for 12 h. The luciferase activity from each sample was measured and normalized with respect to parallel expressed GFP, and the data were displayed as an arbitrary unit (Luc/GFP: RLU/AFU). Similar transfection was performed to investigate dose-dependent effects of IGF-I/II on activities of pCaM(−132).Luc in αT3-1 cells (lower panel). (B) Effects of PACAP, forskolin and cAMP analog on promoter activity of grass carp CaM gene in αT3-1 cells. Cells with transfected CaM promoter constructs were treated with 1 μM of PACAP38 (upper panel), 10 μM forskolin (middle panel) or 3 μM 8-Br-cAMP (lower panel) for 12 h followed by luciferase assay. (C) Effect of Sp1/Sp3 on the activities of grass carp CaM promoter in αT3-1 cells. pCaM(−1509 to −132).Luc constructs were cotransfected with Sp1 expression vector or/and Sp3 expression vector into αT3-1 cells and luciferase activity was measured after 24-h transfection. Results are presented as the mean ± SEM ($N = 3$). Experiment groups with luciferase activity of a similar magnitude ($P > 0.05$) are denoted with the same letter (ANOVA followed by Fisher LSD test).

fish CaM promoter activity in contrast to the positive regulation activity of Sp1 on rat CaM I gene promoter [31,32]. The cause of the discrepancy observed between these mammalian studies and our fish study is still unclear and remains to be determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.10.057.

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