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Promoter analysis and transcriptional regulation of a *Gig2* gene in grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

Grass carp reovirus (GCRV)-induced gene 2 (*Gig2*) is recognized as a new antiviral factor involved in response to viral infection. However, little is known about the mechanisms behind the transcriptional regulation of *Gig2* when infected by virus. In this study, the upstream promoter region of grass carp (*Ctenopharyngodon idella*) *Gig2* gene (*CiGig2*) was identified by homology cloning strategy. *CiGig2* promoter sequence was found to be 859 bp in length and contained three scattered IFN-stimulated response elements (ISRE). In addition, some grass carp *IRFs* (*CiIRF1*, *CiIRF2* and *CiIRF3*) ORF sequences were subcloned into the expression plasmids pET-32a and expressed in *Escherichia coli* BL21, then the expressed proteins were purified by affinity chromatography with the Ni-NTA His-Bind Resin. Gel mobility shift assay was employed to screen the transcriptional regulatory factor for *CiGig2*. The results revealed that the recombinant polypeptides of CiIRF1, CiIRF2 and CiIRF3 bound to *CiGig2* promoter with high affinity; indicating that IRF1, IRF2 and IRF3 could be the potential transcriptional regulatory factors for *Gig2*. Subsequently, *CiGig2* promoter sequence was cloned into pGL3-Basic vector and the ORFs of *CiIRF1*, *CiIRF2* and *CiIRF3* were cloned into the expression plasmids pcDNA3.1 (+). Then, pGL3-*CiGig2* promoter sequence and pcDNA3.1-CiIRFs were co-transfected into *C. idella* kidney (CIK) cells. The *in vivo* effects of CiIRFs on *CiGig2* promoter were measured by dual-luciferase assays in the transfected CIK cells. Our results showed that the roles of CiIRFs were diversified in regulating *CiGig2* transcription, e.g., CiIRF3 played a positive role in during this process; on the contrary CiIRF1 worked as a suppressor; however the effect of CiIRF2 on *CiGig2* transcription was not obvious. For further study the roles of the three ISREs in *CiGig2* transcription, we cloned three mutant *CiGig2* promoters called ISRE1mut-luc (deleted ISRE1), ISRE2mut-luc (deleted ISRE2) and ISRE3mut-luc (deleted ISRE3), respectively. *In vitro*, gel mobility shift assays showed that all three mutant promoters also were combined with CiIRFs. CIK cells were co-transfected with *CiGig2* promoter mutants (ISRE1mut-luc, ISRE2mut-luc or ISRE3mut-luc, respectively) and pcDNA3.1-IRFs. The results suggested that different ISRE played the diverse roles. ISRE2 is more important than ISRE1 and ISRE3 to the transcription of *CiGig2* induced by CiIRF1. ISRE1 and ISRE3 are important to the transcription of *CiGig2* induced by CiIRF2 and CiIRF3.

1. Introduction

The cytokines are secreted or membrane-bound proteins and involved in virtually every aspect of immunity and inflammation, including innate immunity, antigen presentation, bone marrow differentiation, cellular recruitment and activation, and adhesion molecule expression [1]. The field of cytokine has undergone a quiet but vigorous expansion in the last decade [2]. Cytokines cover a wide range of branching, including tumor necrosis factor (TNF), interferon (IFN), interleukin (IL), colony stimulating factor (CSF), transforming growth factor-β family (TGF-β family), growth factor (GF) [3]. For the past few years many new cytokines have been continuously reported in different animal species.

In recent years, significant progress in fish innate immune system has been made and many cytokines have been discovered [4–6]. In general, besides a series of cytokines similar to the counterparts of mammals, fish also has some unique innate

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immune factors, or anti-virus mechanism due to its aquatic environment [7]. *Gig2* had been identified from UV-inactivated GCHV-treated *Carassius auratus* blastulae embryonic (CAB) cells by suppressive substractive hybridization [8] and identified as a novel gene specific to non-amniote vertebrates [9]. *Gig2* transcript is highly up-regulated under the stimulation of GCHV or IRF7, indicating Gig2 plays a pivotal antiviral role [9,10].

In order to further understand the transcriptional regulatory mechanism of *Gig2* in response to virus infection in fish cells, we cloned *Gig2* promoter (KC196275) from grass carp (*Ctenopharyngodon idella*). The full-length of *CiGig2* promoter sequence is 859 bp containing three putative ISRE. IRF1, IRF2 and IRF3 could be the potential transcriptional factors for *CiGig2* by Non-radioactive EMSA. Co-transfected experiment indicated that IRF1 and IRF3 really regulate the transcription of *CiGig2*. CiIRF1 evidently decreased the transcriptional level of *CiGig2*; CiIRF3 is just the opposite. CiIRF2 could not regulate its transcription. The experimental results from ISRE mutations in *CiGig2* promoter showed that ISRE play a different role in the transcription of *CiGig2*. ISRE2 is more important to the transcription of *CiGig2* than ISRE1 and ISRE3 when induced by CiIRF1. ISRE1 and ISRE3 are important to the transcription of *CiGig2* by CiIRF2 and CiIRF3.

2. Materials and methods

2.1. Cells, vectors

C. idellus kidney (CIK) cells were kindly provided by Professor Pin Nie, Institute of Hydrobiology, Chinese Academy of Sciences and maintained at 28 °C in medium 199 supplemented with 10% fetal calf serum (FCS). Cells were seeded in 25 cm^2 flasks. pGL3-basic, *Escherichia coli* strains DH5α and BL21 (DE3) pLys were bought from Promega. The eukaryotic expression vector pcDNA3.1 (+) and the prokaryotic expression vector pET-32a (+) were purchased from Invitrogen and Novagen respectively.

During the process of cell culture, cells were washed with PBS and digested by 1 ml 0.25% trypsin for 3–5 min, afterwards, the trypsin digestion liquid was discarded and the cells were separated and suspended in 5 ml culture medium. After cells were re-suspended, 2–3 ml of medium was transferred into a new culture flask and the medium was supplemented to a total volume of 5 ml.

2.2. Cloning of CiGig2 promoter and its mutants

Homologous primers Gig2-Pro-F and Gig2-Pro-R, designed according to the known *C. auratus* genomic DNA of *CaGig2* (GQ181131) sequence, were used to clone grass carp *Gig2* promoter sequences. Genomic DNA was purified from whole blood of obviously healthy grass carp using a Universal Genomic DNA Extraction Kit (TaKaRa). PCR cycling conditions were: 1 cycle of 94 °C/5 min; 30 cycles of 94 °C/30 s, 61 °C/30 s, 72 °C/30 s; and 1 cycle of 72 °C/10 min.

Overlapping PCR was used to clone ISRE1mut, ISRE2mut and ISRE3mut sequences (Fig. 5A). PCR cycling conditions were the same as above. The primers used in clone were listed in Table 1.

2.3. Protein expression and purification

Grass carp *IRF1, IRF2* and *IRF3* coding sequences and their corresponding DBD-deleted sequences were digested with *EcoR* I/*Xho* I and subcloned into the *EcoR* I/*Xho* I site of pET32a (+) expression vector. Recombinant plasmid pET32a/CiIRF1-ORF, pET32a/CiIRF2-ORF, pET32a/CiIRF3-ORF and pET32a/CiIRF1-nDBD, pET32a/CiIRF2-nDBD, pET32a/CiIRF3-nDBD were sequenced and transformed into competent *E. coli* BL21 cells respectively. Cells were cultured in incubator shaker (ZHWY-200H) at 37 °C until the OD_{600}

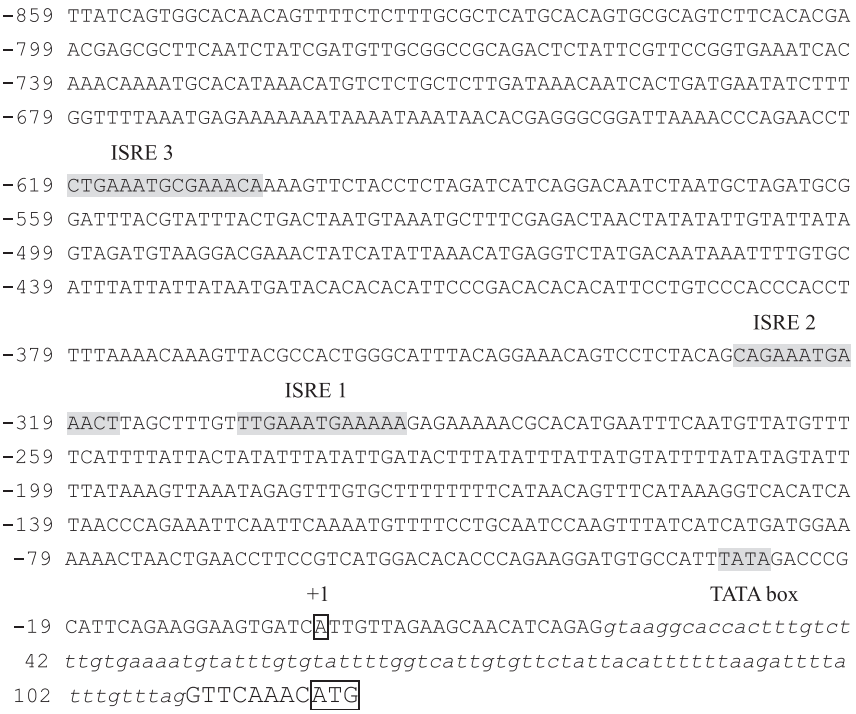

Fig. 1. Promoter sequence of *CiGig2* and their potential functional components. The promoter region and 5′ UTR are shown in capital letters. The 5′ UTR intron region is indicated with lower case italics. The three ISRE sites, TATA box are shaded in grey. The transcription start site of Gig2 gene is designated as position +1. The start code of the ORF and the putative transcription start site are indicated with boxes.