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Transcriptional regulation of type I interferon gene expression by interferon regulatory factor-3 in Japanese flounder, *Paralichthys olivaceus*

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ABSTRACT

Type I interferon (IFN) induces the antiviral response in innate immunity. The type I IFN gene cloned from Japanese flounder (*Paralichthys olivaceus*) has a length of 1189 bp and consisting of 5 exons and 4 introns. In a phylogenetic tree of type I IFNs, Japanese flounder grouped with other Acanthopterygii. To gain insight into the transcriptional regulation of IFN gene, the 1.36 kb 5′-upstream region including numerous canonical motifs to bind transcription factors [for example, IFN regulatory factor (IRF)] was analyzed. In HINAE cells using a luciferase reporter assay, poly I:C-responsive transcriptional activity was found in the region from −634 to −179 bp. This region includes several IRF motifs. In the presence of poly I:C, overexpression of IRF3 and RLR strongly enhanced transcriptional activity. These results suggest that the transcriptional regulation of Japanese flounder type I IFN is regulated by IRF3 after triggering with dsRNA sensors.

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1. Introduction

Interferons (IFNs) are rapidly produced and released from – virus infected host cells or immune cells such as macrophages or dendritic cells, and trigger an antiviral response (Trinchieri, 2010). Three types of IFNs (type I, II and III) have been identified in vertebrates based on their structural features, receptor usage and biological activities (Kontsek et al., 2003; Kotenko et al., 2003; Pestka et al., 2004). The mammalian type I IFNs [IFN-α/β/ω/ε/κ/τ (caw)/δ (pig)] possess strong intrinsic antiviral activity, and induce a potent antiviral state in a wide variety of cells (Levy and Garcia-Sastre, 2001; Samuel, 2001). In most cell types, the expression of type I IFN is induced by pattern recognition receptors including Toll-like receptor (TLR)-3 and RIG-I-like receptors (RLRs) (Kawai and Akira, 2010). TLR-3 is expressed within intracellular vesicles and recognizes dsRNA such as viral nucleic acids and poly I:C, and then activates the signaling pathway leading to the activation of NFκB and IRF3/7, and to the induction of type I IFN (Kawai and Akira, 2011). RLRs recognize viral RNAs in intracellular space and transmit signals through the mitochondria-localized adaptor molecule IPS-1/MAVS/VISA/Cardif, causing kinases such as TBK1 and IKKε to activate IRF3/7 and induce the transcription of the type I IFN genes (Kawai and Akira, 2010). The secreted type I IFN binds with IFN-receptor (IFNR)-1 and IFNR-2, which signal through the JAK/STAT pathway. STAT associates with IRF9 to form the heterotrimeric complex IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to upstream IFN-stimulated response elements (ISRE) and activates the transcription of IFN-inducible genes (Honda et al., 2006).

Teleost type I IFN transcripts have been cloned from zebrafish (*Danio rerio*), carp (*Cyprinus carpio* L.) (Kitao et al., 2009), goldfish (*Carassius auratus*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), sea bass (*Dicentrarchus labrax* L.), gilthead seabream (*Sparus aurata*), green spotted puffer (*Tetraodon nigroviridis*), fugu (*Takifugu rubripes*), medaka (*Oryzias latipes*) and stickleback (*Gasterosteus aculeatu*) (Zou and Secombes, 2011). These genes can be subdivided into

Abbreviations: ATF2, activating transcription factor 2; BAC, bacterial artificial clone; CpG-ODN, cytosine-phosphate-guanosine oligodeoxynucleotides; dsRNA, double-strand RNA; IFN, interferon; IKKε, IκB kinase epsilon; IPS-1/MAVS/VISA/Cardif, IFN-β promoter stimulator-1/mitochondrial antiviral signaling protein/virus-induced signaling adaptor/CARD adaptor inducing IFNβ; ISGF3, IFN-stimulated gene factor 3; ISRE, interferon-stimulated response element; IRF, interferon regulatory factor; JAK/STAT, Janus kinase/signal transducer and activator of transcription; LGP2, laboratory of genetics and physiology 2; MDA5, melanoma differentiation-associated gene 5; NFκB, nuclear factor-kappa B; poly I:C, polyriboinosinic:polyribocytidylic acid; RIG-I, retinoic acid-inducible gene-I; RLR, RIG-I-like receptor; STAT, signal transducer and activator of transcription; TBK1, TANK-binding kinase1; TLR, Toll-like receptor.

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two-cysteine (2C)-containing forms and four-cystein (4C)-containing forms. Recent studies demonstrated that the 2C IFNs are widely expressed in various tissues and cell types after viral infection or stimulation with TLR ligands including poly I:C, imidazoquinoline S-27609 and CpG-ODN (cytosine-phosphate-guanosine) (Altmann et al., 2003; Casani et al., 2009; Kileng et al., 2008; Long et al., 2004; Strandskog et al., 2008; Sun et al., 2009). In contrast to the expression of 2C IFNs, the expression of 4C IFNs appears limited to certain specified cell types and signaling pathways (Zou et al., 2007). In order to understand how to produce type I IFN, it is very important to analyze the transcriptional mechanism of IFN gene. To date, nucleotide sequences of type I IFN promoters in zebrafish (IFN1-4), grass carp, Atlantic salmon (IFNa-c), and rainbow trout (IFN5) were identified (Sun et al., 2009, 2011; Chang et al., 2009; Hu et al., 2011). These IFN promoters respond to poly I:C stimulation, virus infection and the overexpression of transcriptional factors IRF3 and IRF7 (Sun et al., 2010, 2011; Aggad et al., 2009; Bergan et al., 2010).

Recently, RLRs including RIG-I, MDA5 and LGP2 genes were identified in teleosts, and these antiviral functions were also shown in zebrafish, rainbow trout and Japanese flounder (Biacchesi et al., 2009; Chang et al., 2011; Ohtani et al., 2010, 2011). However, the RLR signaling pathway through cytosolic dsRNA recognition to induce IFN production is unknown in teleosts.

In this report, the full-length type I IFN gene and the 5′-upstream control region were identified from Japanese flounder. To clarify how type I IFN transcription is regulated, the 5′-upstream region was analyzed using a luciferase reporter assay in HINAE cells stimulated by poly I:C or co-transfected with various transcription factors. We also examined how recognition of dsRNA by RLR affected type I IFN gene expression.

2. Materials and methods

2.1. Cells

The hirame natural embryo (HINAE) cell line, which was derived from a Japanese flounder embryo (Kasai and Yoshimizu, 2001), was maintained in Leibovitz's L-15 (Life Technologies, Carlsbad, CA, USA) medium containing 10% FBS (Life Technologies), 100 units/ml of penicillin, 100 μg/ml of streptomycin and 250 ng/ml of amphotericin B at 20 °C.

2.2. Probe preparation and genomic BAC library screening

A Japanese flounder genomic BAC (bacterial artificial clone) library (Katagiri et al., 2000) was screened for IFN gene with specific PCR-derived probes including the open-reading frame (ORF) region of Japanese flounder type I IFN gene. High-density replica filters spotting 49,152 BAC clones with an average insert size of 165 kb, were hybridized as reported previously (Katagiri et al., 2000). Japanese flounder IFN genomic BAC clones were screened and used to determine the IFN gene organization and 5′-upstream region.

2.3. Southern blot analysis

Southern blot analysis was conducted with 10 μg of Japanese flounder genomic DNA extracted from peripheral blood, which was digested to completion using restriction enzymes, *Bam*H I (Fermentas, Burlington, ON, Canada), *Eco*R I (Fermentas) and *Hin*d III (Fermentas) as described in a previous study (Hikima et al., 2001). DNA fragments encoding Japanese flounder type I IFN gene (approximately 1.2 kb) amplified by PCR using specific primers (JF-IFN-F2 and JF-IFN-R2 in Table 1) were used as a probe for the hybridization.

2.4. Sequence analysis of Japanese flounder IFN gene and promoter

The genomic sequences for Japanese flounder IFN gene and promoter were obtained from BAC clone number 35-12-P using the primer pair (Table 1). The sequence was amplified by expand high fidelity PCR system (Roche, Basal, Switzerland) and cloned into pJET1.2/blunt vector (Fermentas). The deduced amino acid sequence of Japanese flounder type I IFN gene was aligned using ClustalX software, and phylogenetic analysis generated from MEGA 4.0 program (http://www.megasoftware.net/) (Kumar et al., 2008). To find the transcription factor binding sites in Japanese flounder type I IFN promoter, MatInspector (http://www.genomatix.de/en/index.html) software was used with zebrafish provided database.

2.5. Reporter constructs

Four constructs of the promoter regions were amplified by PCR from BAC clone 35-1-P and cloned into pGL3 vector (Promega, Madison, WI, USA) using the primers specified in Table 1. The PCR products containing promoter region were digested with *Mlu* I (Fermentas) and *Hin*d III, and then ligated with pGL3 vector [named pGL3-IFNp(-1267), pGL3-IFNp(-634), pGL3-IFNp(-458) and pGL3-IFNp(-179)]. All of constructs were purified with cesium chloride gradient ultracentrifugation and dialyzed to distilled water.

2.6. Effecter constructs

The ORF regions of Japanese flounder IRF1 (GenBank ID: AB005883), IRF3 (GenBank ID: GU017417) and IRF7 (GenBank ID: GU017419) were amplified using specified primer sets (shown in Table 1) and cloned into pJET1.2/blunt vector to confirm the sequence. After sequencing, correct clones were digested with restriction enzymes, and ligated into pcDNA4/HisMax (Life technologies) expression vector (named pcDNA4-IRF1, pcDNA4-IRF3 and pcDNA4-IRF7). The construction procedure of the expression vectors for Japanese flounder LGP2 and MDA5 (i.e. pcDNA4-LGP2 and pcDNA4-MDA5) were described in previous studies (Ohtani et al., 2010, 2011).

2.7. Quantitative real-time PCR analysis

One microgram of total RNA extracted from healthy Japanese flounder tissues with TRIzol reagents (Life technologies) was treated with DNase I (Fermentas) and the cDNAs were synthesized with reverse-transcribed with High capacity cDNA reverse transcription kits (Life technologies). Quantitative real-time PCR (Q-PCR) was performed using FastStart Universal SYBR Green master (ROX) (Roche) with StepOne Plus Real-time PCR system (Life technologies). Primers used for Q-PCR were designed with the software Primer Express (Life technologies). The amplification efficiencies of these primer sets were examined according to the StepOne Plus instructions. These primer sequences and efficiency values (%) are listed in Table 1. The specificity of the PCR amplification for all primer sets was verified from the dissociation curves. The relative expression levels for Japanese flounder IFN were determined using the Japanese flounder β-actin gene as an internal reference using comparative Ct ($2^{-\Delta\Delta Ct}$) methods (Livak and Schenittgen, 2001). The cDNA samples of three individual experiments were examined, and the statistical *p*-values were calculated by Student's *t*-test.

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