1. Introduction

Interferon regulatory factors (IRFs) are a family of transcription mediators involved in the transcriptional regulation of type I interferon (IFN) and IFN-stimulated genes (ISGs) (Mamane et al., 1999). So far, 10 IRF members, IRF-1 to -10, have been identified in vertebrates, but only IRF-10 seems not to be present in mammals (Tamura et al., 2008). All IRFs share extensive homology in the DNA binding domain (DBD) covering the first about 120 amino acids of the amino terminus, in which a five tryptophan repeat cluster responsible for the binding to the promoters of target genes was located (Escalante et al., 1998, 2007; Honda et al., 2006). In addition, IRFs, except IRF-1 and -2, possess an IRF association domain (IAD) at the carboxyl terminus which interacts with other proteins to form transcriptional complexes (Eroshkin and Mushegian, 1999). In addition to regulating immune responses, IRFs also function in cell growth control, lymphocyte development, apoptosis and onco-gensis (Nguyen et al., 1997; Hisashi et al., 1998; Barnes et al., 2002; Honda et al., 2006).

IRF-3 is one of the best characterized members of the IRF family for its important roles in initiation and development of the IFN antiviral response in mammals (Hiscott, 2007). In most of nucleated cells, viral nucleic acids are recognized by the toll-like receptor (TLR) 3 and RNA helicases RIG-1 and MDA5 (Takeuchi and Akira, 2007). This event leads to the activation of IRF-3 and NF-κB. They, in turn, translocate to the nucleus, bind to IRF regulatory element (IRF-E) consensus in the promoter of IFN-β gene and initiate transcription. The early IFN produced then activate the classic JAK–STAT pathway and interferon-stimulated gene factor (ISGF)-3 which binds to interferon stimulating response element (ISRE) motifs found in ISGs including IRF-7. Subsequent viral activation of IRF-7 drives the induction of the later IFN-α/β cascade in a positive feedback way. However, the type I IFN response in plasmacytoid dendritic cells (pDCs) is under the control of IRF-7 and independent of IRF-3 (Honda et al., 2005). Unlike IRF-7 which is expressed predominantly in immune tissues and induced by IFNs and viruses, IRF-3 is constitutively expressed in most cells without induction by neither IFN stimulation nor virus infection (Honda et al., 2006; Hiscott, 2007; Paun and Pitha, 2007).
Fish are a group of vertebrates possessing an IFN system similar to that of mammals, but much less studied (Zhang et al., 2007; Robertsen, 2008; Chaves-Pozo et al., 2010). At present, fish IRF-1 to -8 have been identified and IRF-3 reported only for rainbow trout and Atlantic salmon (Yabu et al., 1998; Richardson et al., 2001; Collet et al., 2003; Zhang et al., 2003; Bena et al., 2005; Ordas et al., 2006; Sun et al., 2006, 2007; Jia and Guo, 2008; Holland et al., 2008, 2010; Shi et al., 2008, 2010; Kileng et al., 2009; Bergan et al., 2010; Xu et al., 2010). In zebrafish and spotted green puffer-fish genomes, IRF-3 sequences have also been predicted through database searches. Japanese flounder, Paralichthys olivaceus, is one of the major cultured marine fishes in China. Over the past decade, flounder farming industry of China has been suffered a growing damage from various viral epidemics (Zan et al., 2007). The aim of this study was to enrich the knowledge about the IFN system of flounder by studies of an IRF-3 gene, which will benefit the control of viral diseases of this commercially important species. Herein, we reported sequencing, tissue distribution and inducible expression of flounder IRF-3 and effect of its over-expression on fish type I IFN promoter. The flounder IRF-3 displays the highest sequence homology to the known fish IRF-3s and a capacity of activating grass carp type I IFN promoter. Further, our data demonstrate that both its spatial expression pattern and transcriptional modulation fashion are different from those of mammalian orthologs.

2. Materials and methods

2.1. Fish, cells and virus

Flounders (P. olivaceus) (158–170 g, n = 36) were purchased from a local fish farm. Fish were acclimatized in aerated seawater tanks at 18 °C for 1 week before use. FC95307 cells, a flounder gill epithelial cell line, were maintained at 24 °C in MEM medium (Gibico) supplemented with 10% fetal calf serum (FCS) (HyClone), 100 U/ml penicillin and 100 μg/ml streptomycin. Lymphocystis disease virus (LCDV) China strain LCDV-cn was isolated from tumour-carrying flounders as previously described (Hu et al., 2004). The viral titers were measured by a 50% tissue culture infective dose (TCID50) assay (5 × 106 TCID50/ml H9252 with polyI:C (Sigma) (10 mg/ml, 1000 U/ml). Two group of flounders were intraperitoneally (i.p.) injected with polyI:C (Sigma) (10 mg/ml, 100 μl per fish) and LCDV-cn (5 × 108 TCID50/ml, 200 μl per fish), respectively. Control fish were injected with the same volume of phosphate-buffered saline (PBS). The head kidney and gill were collected for real time PCR assays at 0, 1, 2, 3, 4, 5, 6 and 7 day after injection.

2.2. Challenge of flounders with polyI:C and LCDV

Two group of flounders were intraperitoneally (i.p.) injected with polyI:C (Sigma) (10 mg/ml, 100 μl per fish) and LCDV-cn (5 × 108 TCID50/ml, 200 μl per fish), respectively. Control fish were injected with the same volume of phosphate-buffered saline (PBS). The head kidney and gill were collected for real time PCR assays at 0, 1, 2, 3, 4, 5, 6 and 7 day after injection.

2.3. RNA extraction

Total RNA was extracted from fish tissues using Isogen (Nippon Gene). RNA sample were treated with DNase I to remove genomic DNA contamination using the Turbo DNA-free kit (Ambion). The RNA concentration was determined by measuring absorbance at 260 nm, and its quality was monitored by A260 nm/A280 nm ratios > 1.8.

2.4. cDNA cloning

From 1 μg of total RNA extracted from head kidney of a flounder 4 days post injection with LCDV-cn, a double strand cDNA pool was produced using a SMART cDNA Library Construction Kit (Clontech). Based on the conserved sequences of known fish IRF-3s, degenerate primers were designed. Partial cDNA was amplified with a combination of first-round and nested PCR using degenerate primer pairs IRF3-hF1/IRF3-hR1 and IRF3-hF2/IRF3-hR2, respectively. 3'-rapid amplification of cDNA ends (RACE) was performed by a first round PCR using primers IRF3-3F1 and CDS III/3′, followed by a nested PCR using IRF3-3F2 for sense primer and CDS III/3′ for antisense primer. 5′-RACE was performed by a first round PCR using 5′ UPM for sense primer and IRF3-5R1 for antisense primers followed by a nested PCR using 5′ NUP for sense primer and IRF3-5R2 for antisense primers. The full coding region of flounder IRF-3 was amplified using primer pair IRF3-3F-IRF3-fr with Advantage 2 Polymerase Mix (Clontech). The amplicon was cloned into the EcoRI and HindIII sites of pcDNA3.1/myc-His(−) A vector (Invitrogen). The integrity of the recombinant plasmid was confirmed by sequencing. Primers used in the present study are specified in Table 1.

2.5. Sequence analysis

Sequence result of flounder IRF-3 was compared with the Genbank/EMBL database by using the BLASTX and BLASTP search programs (http://blast.genome.ad.jp). The nucleotide sequence was translated to protein sequence using Translate Tool (http://www.expasy.org/tools/dna.html). The multiple alignment of protein sequences was produced by the Clustal W program (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). The phylogenetic tree was created using the neighbor-joining algorithm within MEGA version 3.1 (Kumar et al., 2004). Bootstrap values were calculated with 1000 replications to estimate the robustness of internal branches. The sequence of grass carp type I IFN promoter (accession no. GU139255) was analyzed by TRANSFAC software for potential transcriptional factor binding sites (Wingender et al., 2000).

2.6. Tissue distribution

Reverse transcription polymerase chain reaction (RT-PCR) was used to examine tissue distribution of flounder IRF-3 mRNA in healthy fish. Total RNA at 0.2 μg from various tissues was reverse-transcribed separately in a 20 μl reaction system with Superscript First Strand Synthesis System (Invitrogen). PCR was performed in a 25 μl reaction system with 1 μl reverse-transcribed product. Primer pair IRF3-3F-IRF3-fr (Table 1) was used for the amplification of flounder IRF-3. For an internal control, the flounder β-actin transcript (accession no. EU090804) was amplified with primers β-actin F/β-actin R (Table 1). Amplified products were electrophoresed on 1.2% agarose gels containing ethidium bromide (EtBr) and visualized under ultraviolet light.

2.7. Real time PCR assay

A quantitative real time PCR assay was employed to study inducible expression profile of flounder IRF-3, type I IFN (accession no. AB511962) and Mx (accession no. AB110446) genes upon challenge with polyI:C and LCDV. 1.0 μg of total RNA from each target tissue (3 individuals each) was reverse-transcribed into cDNA by random primers using Superscript First Strand Synthesis System (Invitrogen). Real time PCR was performed on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA). The PCR mixture in 20 μl volume contained 1 × SYBR Green Real time PCR Mast Mix (TOYOBO), 0.2 μM each of specific forward and reverse primers (Table 1) and 1.0 μl diluted cDNA (50 ng/μl). PCR conditions were 94 °C for 4 min, followed by 40 cycles of 94 °C for 20s, 60 °C for 30s, 72 °C for 30s. Flounder 18s rRNA (accession no. EF126037) was used as endogenous control. All the samples were amplified in triplicates. Fluorescent detection was performed after each extension step. A dissociation protocol was performed after thermocycling to verify that a single ampli-
Table 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5′ → 3′)</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRF3-hF1</td>
<td>ATCCCNCTCGAGACGCCGNYT</td>
<td>First round homology PCR</td>
</tr>
<tr>
<td>IRF3-hR1</td>
<td>AGGGTCGGTCGCTTATATCCTG</td>
<td>First round homology PCR</td>
</tr>
<tr>
<td>IRF3-hF2</td>
<td>GTCTTTGGTCTCTCTTCTCTG</td>
<td>Nested homology PCR</td>
</tr>
<tr>
<td>IRF3-hR2</td>
<td>TGTTTGTCGCTTATATCCTG</td>
<td>Nested 5′-RACE PCR</td>
</tr>
<tr>
<td>IRF3-3F1</td>
<td>GAAGAGGTGGTGATTGGTGGATA</td>
<td>First round 3′-RACE PCR and tissue PCR</td>
</tr>
<tr>
<td>IRF3-3F2</td>
<td>CGGGCAACAGCAGTATCCAGTAA</td>
<td>Nested 3′-RACE PCR</td>
</tr>
<tr>
<td>IRF3-5R1</td>
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<td>First round 5′-RACE PCR</td>
</tr>
<tr>
<td>IRF3-5R2</td>
<td>GTTGCTCTTATATCCTG</td>
<td>Nested 5′-RACE PCR</td>
</tr>
<tr>
<td>IRF3-Ff</td>
<td>GGGAATTCCTCCTGCACTT</td>
<td>Expression plasmid construction</td>
</tr>
<tr>
<td>IRF3-fR</td>
<td>GCAAGCTTCTGCTTATAT</td>
<td>Expression plasmid construction</td>
</tr>
<tr>
<td>IRF3-tR</td>
<td>GGCTTGAGTTTGATCCGACATG</td>
<td>Tissue PCR</td>
</tr>
<tr>
<td>/H9252-actinF</td>
<td>GAAATGGGAACCGCTGCCTC</td>
<td>Tissue PCR</td>
</tr>
<tr>
<td>/H9252-actinR</td>
<td>GTTGGCGTACAGGTCCTAC</td>
<td>Real time PCR</td>
</tr>
<tr>
<td>IFN-pF</td>
<td>CGCTCGACGCTTGAATAGGAAGTTGTGGTGGATA</td>
<td>First round 3′-RACE PCR and tissue PCR</td>
</tr>
<tr>
<td>IFN-pR</td>
<td>GGAAGCTTCTGCTTATAT</td>
<td>Nested 3′-RACE PCR</td>
</tr>
</tbody>
</table>

Note: (1) N represents all four nucleotides; H, A, C or T; R, G or A; Y, C or T. (2) Restriction sites are underlined.

Fig. 1. Nucleotide and deduced amino acid sequences of flounder IRF-3 cDNAs. The start and stop codons and the deletion region in the shorter cDNA are indicated by boxes. The putative tryptophan (W) repeats are shaded in gray. The RNA instability motifs (attta) is underlined. The polyadenylation signals (aataaa) are in boldface.
Table 2

<table>
<thead>
<tr>
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<th>Accession number</th>
<th>Identity (%)</th>
<th>Similarity (%)</th>
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<td></td>
<td></td>
</tr>
<tr>
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<td>GU017417</td>
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<td>71.6</td>
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<tr>
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<td>57.7</td>
</tr>
<tr>
<td>Other subfamilies</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>AY647434</td>
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<td>40.5</td>
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<td>U20338</td>
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<td>U73036</td>
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<td>40.2</td>
</tr>
</tbody>
</table>

2.8. Luciferase assay

The ability of flounder IRF-3 to activate fish type I IFN promoter was studied using a luciferase reporter gene assay. A region of grass carp type I IFN promoter spanning from +17 to −704 nt relative to the transcription start site was amplified with the primer pair IFN-pIFN-pR (Table 1) and inserted into the Xhol and HindIII sites of the pGL3-basic vector (Promega) which contains a promoterless firefly luciferase gene downstream of the multiple cloning sites. The integrity of the recombinant plasmid was confirmed by sequencing. The pRL-SV40 vector (Promega) expressing Renilla luciferase was used as an internal control for transfection efficiency. FG9307 cells were seeded in 24-well plates (1.5 × 10^5 cells per well) overnight before transfection. Cells of each well were cotransfected with 200 ng of the IFN promoter-luciferase vector, 100 ng of pRL-SV40 (Promega) and 200 ng of either empty pcDNA3.1 or pcDNA3.1-IRF-3 vector for 24 h using Lipofectamine 2000 (Invitrogen) in 500 μl serum-free MEM. Basic control cells were cotransfected only with the IFN promoter-luciferase vector and pRL-SV40. Cells were subsequently incubated in fresh MEM with 2% FBS for a further 48 h and lysed with the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity of the lysates were measured in a Junior LB9509 Luminometer (Berthold Technologies, Bad Wildbad, Germany).

2.9. Statistical analysis

Statistical analysis was performed using SPSS13.0 software. Data were analyzed via one-way analysis of variance (ANOVA) when comparing the different treatments at one time point, followed by Duncan’s multiple comparison test. The value of P < 0.05 was considered to be significant.

3. Results

3.1. Cloning and identification of flounder IRF-3 cDNA

Using consecutive techniques of RT-PCR and RACE, two cDNAs differing in the length of 3′-UTR of an IFN-like peptide were isolated from head kidney of Japanese flounder (Fig. 1). The longer cDNA (accession no. GU017417) consisted of 2202 bp nucleotides containing a 1404-bp open reading frame (ORF) with 245 bp of 5′-UTR and 553 bp of 3′-UTR. In the 3′-UTR, one RNA instability motif (AUAAA) and two downstream polyadenylation signals (AATAAA) were found. The distal polyadenylation signal is 11 bp upstream of the poly(A) tail. The shorter cDNA (accession no. GU017418) was generated by an alternative polyadenylation at an adenine residue 226 bp upstream of the distal polyadenylation site. The proximal polyadenylation signal locates 19 bp upstream of this adenine residue. The predicted peptide consists of 467 amino acid residues. It exhibits the highest homology (50.7–57.6% identity) with fish IRF-3 sequences in a BLASTP analysis (Table 2) and, thus, was designated as flounder IRF-3. Additionally, it shares identity of 27.5–31.0% to IRF-3 from vertebrates other than fish and 24.1–27.2% to vertebrate IRF-7 (Table 2) which along with IRF-3 group constitutes the IRF-3 subfamily. A lower identity (14.7–25.0%) was observed between flounder IRF-3 and other IRF subfamily proteins. Like other known IRF-3 proteins, flounder IRF-3 possesses a DBD at the N-terminal shared by all IRF members and an IAD and a serine-rich domain (SRD) at the C-terminus (Fig. 2). The SRD of the IRF-3 group constitutes the IRF-3 subfamily. A lower identity (14.7–25.0%) was observed between flounder IRF-3 and other IRF subfamily proteins. Like other known IRF-3 proteins, flounder IRF-3 possesses a DBD at the N-terminal shared by all IRF family proteins. The IRF-3 family is classified into four subfamilies. The nodes of the phylogenetic tree matched the known taxonomic relationship among IRF members. Flounder IRF-3 was clustered into IRF-3 group which together with IRF-7 group forms the IRF-3 subfamily. In the tree, flounder IRF-3 shows the closest phylogenetic distance to fish IRF-3s and then to mammalian IRF-3s. These data support the authenticity of the nomenclature for flounder IRF-3.

3.2. Phylogenetic analysis

In order to determine the phylogenetic position of flounder IRF-3, overall amino acid sequences of 43 IRFs covering IRF-1 to −10 from various vertebrates were collected to construct a phylogenetic tree (Fig. 3). The vertebrate IRF family is classified into four subfamilies. The nodes of the phylogenetic tree matched the known taxonomic relationship among IRF members. Flounder IRF-3 was clustered into IRF-3 group which together with IRF-7 group forms the IRF-3 subfamily. In the tree, flounder IRF-3 shows the closest phylogenetic distance to fish IRF-3s and then to mammalian IRF-3s. These data support the authenticity of the nomenclature for flounder IRF-3.
Fig. 2. Multiple alignment of flounder IRF-3 amino acid sequence with other IRF-3 proteins. The putative DNA-binding domain (DBD) and IRF-association domain (IAD) are shaded in gray. The conserved tryptophan (W) residues composing a “tryptophan cluster” are boxed in the DBD. The residues identical in all sequences are shown with asterisks (*), whereas those with strong homologies and weak similarities are marked by colons (:) and dots (.), respectively. The accession number of frog (Xenopus laevis) is NM_0010086119 and those of other species are listed in Table 2.

### 3.3. Tissue distribution of flounder IRF-3 mRNA

The mRNA expression of flounder IRF-3 in various tissues was examined by RT-PCR (Fig. 4). The results showed that flounder IRF-3 was expressed constitutively in gill, intestine, heart, head kidney, kidney, liver and spleen of healthy flounders. The higher expression was detected in the head kidney, spleen and kidney, whilst other positive tissues have a low expression level. No expression was detected in brain, stomach, gonad, muscle and skin following up to 40 cycles of PCR amplification.
Fig. 3. Phylogenetic tree of the IRF family proteins in vertebrates. The tree was depicted on the overall sequences by neighbor-joining method. The numbers at nodes are bootstrap values (1000 replicates) in percent. The Japanese flounder IRF-3 is underlined. The accession numbers of the IRF-1 sequences are: Human, X14454; Mouse, M21065; Chicken, L39766; X. tropicalis, BC075398; Rainbow trout, AF332147. The accession numbers of the IRF-2 sequences are: Human, X15949; Sheep, AF228445; Chicken, X95478; X. tropicalis, BC080892. The accession numbers of the IRF-4 sequences are: Human, U52682; Mouse, AK089319; Chicken, AF320331. The accession numbers of the IRF-5 sequences are: Human, NM_002200; Cow, BT021607; X. laevis, BC082862; Atlantic salmon, BT044767. The accession numbers of the IRF-6 sequences are: Human, X73037; Pig, AF327368; Chicken, DQ250733; X. laevis, BC108593. The accession numbers of the IRF-7 sequences are: Human, M91196; Rat, NM_011008722; Duck, DQ266446; X. laevis, BC077289. The accession numbers of the IRF-8 sequences are: Human, M91196; X. laevis, BC070535. The accession numbers of other IRF sequences are listed in Table 2.

3.4. Induction of flounder IRF-3, type I IFN and Mx transcripts by polyI:C and LCDV

In order to obtain some functional clues of flounder IRF-3 gene, its transcriptional modulation and expression kinetics in vivo upon polyI:C and LCDV challenges were investigated and compared with those of flounder type I IFN and Mx genes using real time PCR method. We selected head kidney and gill to perform the study owing to their immune importance. The three genes were all up-regulated by polyI:C and LCDV in the two organs with the highest inducibility being seen for Mx (Fig. 5). On the whole, the induction of flounder IRF-3 was most rapid but slightest among the three genes, meanwhile, LCDV had a weaker inductive action than polyI:C. In head kidney, the expressions of IRF-3, type I IFN and Mx showed approximate 13-, 47- and 53-fold increases in response to polyI:C-stimulation at their highest levels which appeared at day 2, 2 and 3.
Fig. 4. RT-PCR analysis of flounder IRF-3 expression in various tissues. Transcripts were amplified by RT-PCR with the PCR primer set presented in Table 1 and electrophoresed on 1.2% agarose gels containing EtBr. Japanese flounder β-actin was used as an internal control. This figure presents the result from one of four fish which had a similar tissue distribution profile. Abbreviation used: M, DL2000 marker; H kidney, head kidney.

post injection, respectively (Fig. 5(A)), whilst they showed approximate 6-, 15- and 26-fold increases in response to LCDV-infection at the highest levels which appeared at day 3, 4 and 4 post injection, respectively (Fig. 5(B)). In gill, IRF-3, type I IFN and Mx reached their highest expression levels at day 2, 3 and 4 post infection with about 8-, 30- and 99-fold increase in response to polyI:C-stimulation, respectively (Fig. 5(C)), whilst they reached the highest expression levels at day 3, 4 and 4 post-injection with about 4-, 10- and 18-fold increase in response to LCDV-infection, respectively (Fig. 5(D)). It is also noteworthy that the induction of IRF-3 is less persistent than that of the other two genes, particularly than that of the Mx, in all instances.

3.5. Activation of fish type I IFN promoter by flounder IRF-3

For examination of the ability of flounder IRF-3 to activate fish type I IFN promoter, a luciferase assay was performed. Because the promoter of flounder type I IFN gene has not yet been identified, we used grass carp type I IFN promoter in this study. Sequence analysis revealed four IRF-binding elements (IRF-E, -71, -78, -117 and -486 nt sites relative to the transcription start site), one NF-κB-binding site (−99) and two ATF/c-Jun elements (−434 and −530) in grass carp type I IFN promoter (Fig. 6(A)), which are characteristic motifs of known type I IFN promoters and essential to their activity (Sen, 2001; Bergan et al., 2006). A region (from +17 to −704) containing these elements was amplified from grass carp type I IFN promoter to construct a promoter–luciferase construct (Fig. 6(B)). The luciferase assay was conducted in FG-9307 cells. As shown in Fig. 6(C), cells of basic control and empty vector control had a similar luciferase level. In contrast, cells transfected with flounder IRF-3 construct showed a higher (9.7-fold increase) luciferase level than the empty vector control. This thus suggests an induction of the type I IFN promoter by over-expressed flounder IRF-3.

4. Discussion

In this study, we cloned two cDNA variants of an IRF-like peptide from head kidney of Japanese flounder. They differ only in the 3′-end. Sequence analysis revealed that the two cDNAs were generated by alternative polyadenylation, which might represent a regulatory mechanism of the expression of this gene. We have observed that the longer variant is the predominant type in head kidney (data not shown). Whether this phenomenon is tissue-specific remains to be investigated. Based on the homology with fish IRF-3 orthologs, the inferred peptide was identified as flounder IRF-3. The phylogenetic analysis further confirmed the authenticity of known type I IFN promoters and essential to their activity (Sen, 2001; Bergan et al., 2006). A region (from +17 to −704) containing these elements was amplified from grass carp type I IFN promoter to construct a promoter–luciferase construct (Fig. 6(B)). The luciferase assay was conducted in FG-9307 cells. As shown in Fig. 6(C), cells of basic control and empty vector control had a similar luciferase level. In contrast, cells transfected with flounder IRF-3 construct showed a higher (9.7-fold increase) luciferase level than the empty vector control. This thus suggests an induction of the type I IFN promoter by over-expressed flounder IRF-3.
of the nomenclature by clustering it into the group of vertebrate IRF-3. The putative flounder IRF-3 protein possesses three conserved domains, an N-terminal DBD and C-terminal IAD and SRD, which are found in all known IRF-3s. The DBD is typical of all IRF members, characterized by a cluster of five well-spaced tryptophan residues. This region forms a helix–turn–helix motif that binds to the ISRE/IRF-E consensus in target promoters (Au et al., 1995; Escalante et al., 1998, 2007). Studies revealed that three of the five tryptophans are crucial to DNA–protein interactions, which orientate and stabilize amino acid contacts in the IRF molecule with GAAA core sequences of the ISRE/IRF-E consensus (Escalante et al., 1998, 2007). The IAD is another conserved domain in IRF family members except IRF-1 and -2, responsible for the formation of IRF homo/hetero-dimers and associations with other transcription factors (Eroshkin and Mushegian, 1999). Unlike the DBD and IAD, the SRD exists strictly in IRF-3, -5 and -7 (Holland et al., 2008; Xu et al., 2010). It is important for virus-induced phosphorylation and less conserved in IRF-3 than in IRF-7 across various species (Holland et al., 2008). Flounder IRF-3 SRD has a conserved SSL motif where the two serines are determined to be essential phosphorylation sites in mammalian IRF-3 (Lin et al., 1998; Yoneyama et al., 1998; Hiscott, 2007). Two other serine residues, Ser 453 and Ser459, of flounder IRF-3 SRD were also found conserved in fish species, which may be equivalent to the serine/threonine cluster between amino acids 396 and 405 of human IRF-3 SRD (Lin et al., 1998; Hiscott, 2007) and, thus, probably serve as subsidiary phosphorylation targets. Collectively, these structural characteristics of flounder IRF-3 reveal that it may have a similar activation mechanism and functions as mammalian IRF-3 in antiviral response.

In mammals, IRF-3 is constitutively expressed in most tissue and cell types and not transcriptionally induced by neither IFN stimulation nor virus infection, whereas IRF-7 is predominantly expressed in immune tissues, especially in pDCs and induced by viruses and type I IFNs (Honda et al., 2006; Hiscott, 2007; Paun and Pitha, 2007). To date, only two studies have been reported for IRF-3s in fish species, showing that they were expressed constitutively and ubiquitously in all examined organs (Holland et al., 2008; Bergan et al., 2010). In contrast to these literatures and reports for mammalian IRF-3 (Au et al., 1995; Honda et al., 2006), our study demonstrates that the constitutive expression of flounder IRF-3 was restricted to certain tissue types, especially to the immune tissues where the lymphoid and myeloid cells are abundant, and not detected in brain, gonad, stomach, muscle and skin. Unlike flounder IRF-3, fish IRF-7s were reported to be expressed constitutively in a wide variety of tissue types at high levels (Zhang et al., 2003; Sun et al., 2007; Holland et al., 2008; Jia and Guo, 2008). Other known fish IRFs such as IRF-1, -2, -4, -5, and -8 were also reported to be expressed ubiquitously in various tissues of healthy fish (Yabu et al., 1998; Richardson et al., 2001; Ordas et al., 2006; Sun et al., 2006; Shi et al., 2008, 2010; Holland et al., 2010; Xu et al., 2010). The higher expression of floun-
Flounder IRF-3 was found to be up-regulated upon challenge with polyI:C and LCDV, though the induction by LCDV is weak. The inductions of rainbow trout IRF-3 transcripts by treatment with polyI:C, type I and II IFNs, LPS, IL-1β and the TLR7/8 agonist, R848, have also been reported (Holland et al., 2008; Purcell et al., 2006). Likewise, Atlantic salmon IRF-3 was up-regulated by virus, polyI:C and type I IFN (Bergan et al., 2010). In contrast, mammalian IRF-3 is not transcriptionally modulated by stimuli. These results suggest that the expression of fish IRF-3 gene is differently regulated from that of mammalian IRF-3, which is probably explained by the presence of fish-specific binding sites for transcription factors in fish IRF-3 promoters. PolyI:C, a synthetic mimic of dsRNA, is a potent inducer of fish type I IFNs and ISGs (Bergan et al., 2006). In mammals, it stimulates production of IFNs mainly through the RIG-1 and TLR3 pathways, which implicates activation of the promoter by IRF-3, IRF-7 and NF-κB (Alexopoulou et al., 2001). The mechanism by which polyI:C up-regulates fish IRF-3, however, remains to be further studied. Unlike polyI:C, LCDV is a DNA virus not producing dsRNA molecule over its replication cycle. Thus, it may activate host’s antiviral response through a different signaling pathway. This might account for the weaker induction of flounder IRF-3 as well as type I IFN and Mx by LCDV. In pDCs of mammals, CpG-rich viral DNA triggers the IFN response via the TLR9-MD2 pathway (Honda and Taniguchi, 2006). Whether this pathway exists in fish is an interesting subject for future study. Our study demonstrated a more rapid but much weaker and less persistent induction of flounder IRF-3 than that of Mx by both polyI:C and LCDV in the head kidney and gill. A similar result was also seen between IRF-3 and type I IFN except in the head kidney of polyI:C-stimulated flounders where they had a similar transcription kinetics. The comparison between type I IFN and Mx showed that the former was less induced by both stimuli. Further, their expression kinetics were different when challenged with polyI:C but similar when with LCDV. In the former case, type I IFN transcripts reached peak levels earlier than those of Mx. This phenomenon can be explained by different signalling pathways the two stimuli triggered in the antiviral response. Collectively, it seems that the induction of flounder IRF-3 is an early event in the antiviral response, which occurs before the induction of type I IFN and Mx, and thus IRF-3 possibly participates regulation of the type I IFN pathway. On the other hand, as a proinflammatory cytokine, it is not surprising for flounder IRF-3 to have a lower abundance and stability than Mx which is considered as an effector protein of IFN response. Taken together, the distinct transcriptional modulation fashion and spatial expression pattern of flounder IRF-3 relative to mammal orthologs open an exciting field of functional research for fish IRF-3.

Mammalian IRF-3 is involved in the activation of type I IFN promoters through the binding to the GAAA core sequence of the IRF-E consensus (Escalante et al., 2007). In order to determine whether flounder IRF-3 has such an activity, we over-expressed it in FG9307 cells and analyzed its impact on grass carp type I IFN promoter using a luciferase assay. We observed that over-expressed flounder IRF-3 could exert an inductive action on grass carp type I IFN promoter in untreated FG9307 cells. This result is in line with previous studies of other IRF-3s from mammals and other fish (Juang et al., 1998; Dahlberg et al., 2006; Holland et al., 2008; Bergan et al., 2010), which are capable of inducing type I IFN and other gene promoters either in the presence or absence of virus or polyI:C. One of those studies also demonstrated an enhanced activity of over-expressed IRF-3 by challenge with virus (Juang et al., 1998). In our preliminary studies, the impact of both virus and polyI:C on the activity of flounder IRF-3 was inconsistent in FG9307 cells (data not shown). Thus, the action of flounder IRF-3 on luciferase activity was studied in their absence, which may reflect better any effect of over-expressed flounder IRF-3 in the case of the lack of virus or polyI:C interference. The increased luciferase level seen in flounder IRF-3 construct transfected cells provides an evidence supporting the induction of grass carp type I IFN promoter by flounder IRF-3. In the sequence of grass carp type I IFN promoter, essential transcription factor binding sites such as IRF-E, NF-κB and ATF/c-Jun elements and a number of IRF-core binding motifs GAAA/TTCGs are present. It is thus likely that the bindings of flounder IRF-3 as well as other transcription factors of host cells to them activated the transcription of downstream luciferase reporter gene.

In conclusion, this study demonstrates the present of an IRF-3 gene in Japanese flounder. Its expression profile and effect on fish type I IFN promoter were also described. The data indicate a limited tissue distribution and an inducible expression profile by polyI:C and LCDV for flounder IRF-3, two expression characteristics distinct from mammalian orthologs. This study provides important insights into the unique expression profile of flounder IRF-3, further studies of which may give a better understanding of the functions of fish IRF-3.

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