Cloning and characterization of interferon stimulated genes Viperin and ISG15, and their promoters from snakehead *Channa argus* *

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Abstract By suppression subtractive hybridization, rapid amplification of cDNA ends and gene walking methods, interferon stimulated genes (ISGs), Viperin and ISG15, and their promoters have been cloned and characterized from snakehead *Channa argus*. The Viperin cDNA was found to be 1474 nt and contain an open reading frame (ORF) of 1059 nt that translates into a putative peptide of 352 amino acid (aa). The putative peptide of Viperin shows high identity to that in teleosts and mammals except for the N-terminal 70 aa. The ISG15 cDNA was found to be 758 nt and contain an ORF of 468 nt that translates into a putative peptide of 155 aa. The putative peptide of ISG15 is composed of two tandem repeats of ubiquitin-like (UBL) domains, and a canonical conjugation motif (LRGG) at C-terminal. Viperin and ISG15 promoter regions were characterized by the presence of interferon stimulating response elements (ISRE) and γ-IFN activation sites (GAS). ISRE is a feature of IFN-induced gene promoter and partially overlaps interferon regulatory factor (IRF) 1 and IRF2 recognition sites. GAS is responsible for the γ-IFN mediated transcription. One conserved site for NF-κB was found in the promoter region of Viperin. This is the first report of conservative binding motif for NF-κB in accordance with the consensus sequence (GGGRNNYYCC) among teleost ISG promoters. Moreover, there were also TATA, CAAT and Sp1 transcription factor sites in Viperin and ISG15 promoters. In 5′ untranslated region (UTR), snakehead ISG15 gene contains a single intron, which differs from Viperin gene. The transcripts of Vipeirn and ISG15 mRNA were mainly expressed in head kidney, posterior kidney, spleen and gill. The expression levels in liver were found to increase obviously in response to induction by IFN-inducer poly I:C.

Keywords: **interferon, interferon stimulated gene (ISG), Viperin, ISG15, snakehead (*Channa argus*).**

The interferons (IFNs) are a family of cytokines that share the ability to produce an antiviral state in cells by inducing expression of interferon stimulated genes (ISGs)[1,2]. The earliest antiviral response of the host is nonspecific. Thus, IFN-mediated antiviral defense is able to respond during the early stages of a viral infection, and this response provides some degree of protection until the specific immune defense is able to establish. At present, IFN-like activity has been detected in cells and organs of rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), sea bass (*Lateolabrax japonicus*) and several salmonids after viral infection or treatment with double-stranded RNA (dsRNA)[3—5]. IFN molecules have been cloned and characterized from zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*) and channel catfish (*Ictalurus punctatus*) since 2003[6—8]. Significant progress has been made in isolating virally induced genes[9,10]. Several ISGs, such as *Mx1*, *Mx2* and *Mx3*[11—13], *Vig-1*[14] and *Vig2*[15], have also been identified in fish.

Viperin was identified recently as a group of antiviral proteins which can be induced by virus and interferon[14,16,17]. The Viperin homologues have been found in crucian carp (*Carassius auratus*), rainbow trout and mandarin fish (*Siniperca chuatsi*)[10,14,18]. The expression of rainbow trout *Vig-1* could be induced by viral haemorrhagic septicemia virus (VHSV)[14]. The stable expression of Viperin in fibroblast could reduce 90% of human cytomegalovirus (HCMV) production, and suppress the expression of some viral proteins[16]. Similar to teleost and human Viperin homologues, mouse mvig could be induced by vesicular stomatitis virus (VSV) and pseudorabies virus (PRV)[17].

ISG15 is among the most highly expressed pro-

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teins in mammalian cells after viral infection and treatment with IFN-β[19—21]. The strong and rapid induction of ISG15 during infection indicates an important role for this protein in innate immunity. ISG15 is composed of two tandem repeats of ubiquitin-like (UBL) domains, and can conjugate to cellular proteins through its conserved motif (LRGG). Extracellular ISG15 displays cytokine-like functions by inducing IFN-γ in T-cells and stimulating natural killer cell proliferation[22]. Several ISG15 orthologues have been reported in goldfish (*Carassius auratus*), pufferfish (*Takifugu rubripes*), zebrafish and channel catfish[9,23,24]. The ISG promoters have also been characterized from rainbow trout *Vig2* and *Mx1*[15,25], pufferfish *Mx*[13], mandarin fish Viperin[18], goldfish and Atlantic cod (*Gadus morhua*) ISG15[23,24].

The snakehead (*Channa argus*) is a species with a relatively high economic value in China, but the diseases caused severe economic losses[26]. Little is known about the immune defense mechanism in snakehead. The present study was performed to identify snakehead Viperin and ISG15 genes, and to compare the characteristics of these genes and their promoters with other known ISGs. These results will provide new insights into ISGs regulation and their antiviral mechanisms.

1 Materials and methods

1.1 Fish

Snakehead weighing about 250 g were purchased from a local fish market in Wuhan, Hubei Province of China, and maintained in aquarium (25 ℃) with aerated water for one week before sampling. The tissues were carefully removed and immediately stored in liquid nitrogen.

1.2 Obtaining of homologous cDNA fragments

Total RNA was extracted from head kidney of snakehead with Trizol (Invitrogen) following the manufacturer's instruction. PolyA^{+} RNA was prepared using PolyATract mRNA Isolation System (Promega). The isolated mRNA from head kidney in snakehead stimulated with poly I:C was used as the tester, and the unstimulated control as the driver. A subtractive cDNA library was constructed with the PCR-select cDNA subtraction kit (Clontech). Through random screening and sequencing colonies from the subtractive library, several cDNA fragments, which were compiled to about 1251 bp and 570 bp in length, were found to be homologous to those known Viperin and ISG15 genes.

1.3 Cloning of Viperin and ISG15 cDNA sequences by rapid amplification of cDNA ends (RACE)-PCR

5′ RACE, started with the full-length cDNA synthesis using the BD SMART^{TM} RACE cDNA Amplification Kit (Clontech). All primers used are listed in Table 1. The first round PCR was performed using the 5′ UPM and Viperin-R1 or ISG15-R1 primers. The cycling protocol utilized a touchdown method, with a hot start at 94 ℃ for 2 min, followed by 10 cycles of 94 ℃ 30 sec, 65 ℃ 30 sec, and 72 ℃ for 1 min, with the annealing temperature lowering down 1 ℃ for each cycle; and 25 cycles of 94 ℃ 30 sec, 55 ℃ 30 sec, and 72 ℃ for 1 min, followed by 72 ℃ for 6 min. The second round PCR was carried out with the 5′ nested primer and Viperin-R2 or ISG15-R2, and 1 μL of the first round PCR mixture, with a cycling protocol of 94 ℃ for 2 min, followed by 30 cycles of 94 ℃ 30 sec, 57 ℃ 30 sec, 72 ℃ 1 min, and an extension of 72 ℃ for 6 min. 3′ RACE, started with reverse transcription of PolyA^{+} RNA with Adapter-dT$_{17}$ primer, and the PCR conditions were the same as for 5′-RACE using the 3′ adapter primer and Viperin-F or ISG15-F.

Table 1. Primers used for cloning and expression studies

Primer	Sequence (5′-3′)	Application
Viperin-R1	CCAGAGTCTTCGCAGTGTCCA	Viperin 5′ RACE (first round PCR)
Viperin-R2	GCAGAGTGTCGAGACAGAGCT	Viperin 5′ RACE (second round PCR)
Viperin-F	GCAGAACTCAGGGCAGGAAG	Viperin 3′ RACE
ISG15-R1	CTGGCCGTTTACAAAGACCAG	ISG15 5′ RACE (first round PCR)
ISG15-R2	AGTTTGCCCACTGTATCATCTG	ISG15 5′ RACE (second round PCR)
ISG15-F	CGACAGAGGGAGGACAAACG	ISG15 3′ RACE and expression study

(To be continued)

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(Continued)

Primer	Sequence (5′-3′)	Application
Viperin-P1	CGATGCAGAGTGTCGAGACAGAGCTG	Vipeirn genomic walking (first round PCR)
Viperin-P2	GCTTCGGAGACGAGTGCTCATTGGA	Viperin genomic walking (second round PCR)
ISG15-P1	TTCAGTTTGCCCACTGTATCATCTGG	ISG15 genomic walking (first round PCR)
ISG15-P2	GCGTATGGACTGTGCCATTCAGCAT	ISG15 genomic walking (second round PCR)
Viperin-TF	ACTCTGGGCAACGGAAGGAT	RT-PCR primer used in expression study
Viperin-TR	GGAACGAGGCAGGAAACGCT	RT-PCR primer used in expression study
ISG15-TR	CGTTCATCATCTCCCGACCA	RT-PCR primer used in expression study
β-actin-F	CACTGTGCCCATCTACGAG	RT-PCR control used in expression
β-actin-R	CCATCTCCTGCTCGAAGTC	RT-PCR control used in expression
5′ UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTA-TCAACGCAGAGTCTAATACGACTCACTATAGGGC	5′ RACE PCR
5′ Nested primer	AACGCAGAGTACGCGGG	5′ RACE PCR
3′ Adapter	GGCCACGCGTCGACTAGTAC	3′ RACE PCR adaptor
Oligo dT adapter	GGCCACGCGTCGACTAGTACT$_{17}$	Genomic walking adaptor primer 1
AP1	GTAATACGACTCACTATAGGGC	First strand cDNA synthesis
AP2	ACTATAGGGCACGCGTGGT	Genomic walking adaptor primer 2

1.4 Cloning of Viperin and ISG15 promoters

Genomic DNA was purified from the muscle by the phenol chloroform method[27]. The 5′ flanking region was obtained using a genome walking approach by constructing genomic libraries with a Universal Genome Walker™ Kit (Clontech). Each of the 2.5 μg genomic DNA was completely digested with *Dra*I, *Eco*RV, *Pvu*II or *Stu*I in a total volume of 100 μL, and four pools of adaptor-ligated DNA fragments were constructed. A pair of primers, Vipein-P1/Viperin-P2 or ISG15-P1/ISG15-P2 designed from the sequences at the 5′ end of Viperin or ISG15 cDNA, and the adaptor primers AP1 and AP2 were used for priming upstream amplification through two rounds of PCR. The cycling protocol included a two-step method for long-distance PCR. The primary PCR was performed with a hot start at 94 ℃ for 2 min; 6 cycles of 94 ℃ for 30 sec, 72 ℃ for 3 min with the temperature lowing down 1 ℃ for each cycle; and 30 cycles of 94 ℃ 30 sec, 67 ℃ 3 min, followed by 67 ℃ for 10 min. The secondary PCR was carried out with 1 μL of the first round PCR mixture under the conditions of 20 cycles of 94 ℃ for 30 sec and 67 ℃ for 3 min, followed by 67 ℃ for 10 min.

1.5 T-cloning, sequencing and database analysis

The PCR products separated on 1.2% agarose gels were cut out, purified (Omega), ligated into the T-vector (Takara), and used to transform competent DH-5α cells. Positive colonies were screened by the method of PCR. All sequences generated were used to search for similarities using BLAST at web servers of the National Center of Biotechnology Information. The amino acid sequences were translated through servers of ExPASy. Signal peptide prediction was finished through SignalP 3.0 software. Multiple sequence alignments and unrooted phylogenetic trees were constructed using the ClustalW 1.8 program and MEGA version 3.1. The sequences flanking Vipeirn and ISG15 5′ regions were analyzed by TRANSFAC software for potential transcriptional factor binding sites.

1.6 Expression analysis by RT-PCR

Three snakehead fish weighing about 250 g were cultured in aquarium with aerated water for one week. Total RNA was extracted from the head kidney, posterior kidney, spleen, intestine, liver, gill, heart, brain, skin and muscle, then treated with DNase I, and reverse transcribed to cDNA. The cDNA templates from three fish were mixed together, and 2 μL of which were used for PCR reaction. β-actin was used as an internal control. PCR conditions were as follows: 94 ℃ for 2 min; then 28 cycles of 94 ℃ 30 sec, 57 ℃ 30 sec, 72 ℃ 1 min (for Viperin) or 30 sec (for ISG15 and β-actin), followed by an extension at 72 ℃ for 6 min. In addition, we performed a comparative study on 6 snakehead fish. Three were injected intraperitoneally with 0.4 mL poly I:C (Sigma, 5 mg/mL), and the other three injected with phosphate buffer saline (pH 6.8 as the control). Be-

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cause poly I:C was found to be able to enhance the ISGs expression in other organs except for lymphoid tissue[18, 24], we selected the liver as the test tissue to examine the effects of Viperin and ISG15 on the ISGs expression.

2 Results

2.1 Isolation of Viperin and ISG15 cDNA

By screening of subtractive cDNA library, we identified many expressed sequence tags (EST) which are homologous to the known mammalian genes critical for innate immune function, including antiviral genes Viperin and ISG15, and the genes encoding for signal transducer and activator of transcription (STAT), IFN regulatory factors (IRFs), Rhamnose-binding lectin (UBL), major histocompatibility class (MHC) and other immune molecules. With the obtained 5′ RACE and 3′ RACE cDNA fragments and the analysis of overlapping sequences, the compilation of a full-length cDNA from the CAP site to the polyA tail was achieved, which produced a 1474 bp Viperin cDNA and a 758 bp ISG15 cDNA fragments (GenBank accession Nos. AY898793 and EF067846). Viperin cDNA contains an open reading frame (ORF) of 1059 bp that translates a putative peptide of 352 aa, with a 179 nt 5′ UTR and a 236 nt 3′ UTR. There is a polyadenylation signal (AATAAA) presented 14 nt upstream of the polyA stretch, and two ATTTA sequences in 3′ UTR, which are characteristic motifs possibly involved in rapid message degradation. ISG15 cDNA contains an ORF of 468 bp that translates a putative peptide of 155 aa. The 5′ UTR is 63 nt in length, and 3′ UTR of the transcript is of 227 nt containing three mRNA instability motifs and a polyadenylation signal at position of 23 nt upstream the polyA tail.

2.2 Analysis of Viperin and ISG15 protein sequences

The amino acid sequence of snakehead Viperin shared approximately 71% identity to Viperin of goldfish, 72% to zebrafish, 75% to rainbow trout, 69% to mouse and human (Fig. 1). A divergent region of 70 amino acids was found at N-terminal. Sequence analysis of the putative peptide presented a short hydrophobic N-terminal region which constitutes a signal peptide. Three putative N-glycosylation sites (at positions 112, 148 and 197) suggest that snakehead Viperin is a glycoprotein. Similar to *Vig-1* in rainbow trout, snakehead Viperin also has an iron-sulfur motif CNXXCXXC at the position of 70-182. The amino acid sequence of snakehead ISG15 has the homology of 50%, 47%, 25%, 23% with rainbow trout, goldfish, mouse and human ISG15 respectively (Fig. 2). Snakehead ISG15 in the C-terminal retains Leu-Arg-Gly-Gly (LRGG) amino acids that have been shown to be critical for the first step in the conjugation of ubiquitin to intracellular proteins. Some amino acids have been substituted in conserved motif. European sea bass (*Dicentrarchus labrax*), killifish (*Misgurnus anguillicaudatus*) and oriental weatherfish (*Lucania parva*) have substituted the C-terminal Gly with Asp or Glu[24]. In addition, snakehead ISG15 retains Arg_{128} which corresponds to Lys_{129} of the human ISG15. The residue is critical for the polymerization of ubiquitin monomers after covalent ligation to intracellular proteins.

Though the result of two phylogenetic trees (Fig. 3) reveals the considerable sequence divergence among teleosts and mammals, teleost Viperin and ISG15 are clustered together and they originated from the same ancestor. Snakehead and mandarin fish Viperin are first clustered together, and snakehead Viperin and ISG15 are closely related to those of rainbow trout, but far away from those of crucian carp and zebrafish.

2.3 Promoter region of snakehead Viperin and ISG15

The sequences of Viperin and ISG15 gene promoters were deposited into GenBank (accession Nos. EF384270 and EF384271, respectively). The transcription initiation sites were determined by the full-length capped cDNAs of Viperin and ISG15. About 3 kb of the snakehead Viperin promoter and its 5′ flanking region were sequenced through genome walking (Fig. 4, partial sequence). A TATA box is located from −52 to −55 which may be bound by TATA binding peptide (TBP). A CAAT box is located from −186 to −189 which is believed to determine the efficiency of transcription. Two of the putative interferon stimulated response element (ISRE) sites are located between −131 and 141, −150 and −161. Three γ-IFN activation sites were found between −170 and −178, −322 and −330, −1158 and −1166. One binding site for the NF-κB transcription factor was found between −300 and −309, and one NF-κB like site was between −326 and −336.

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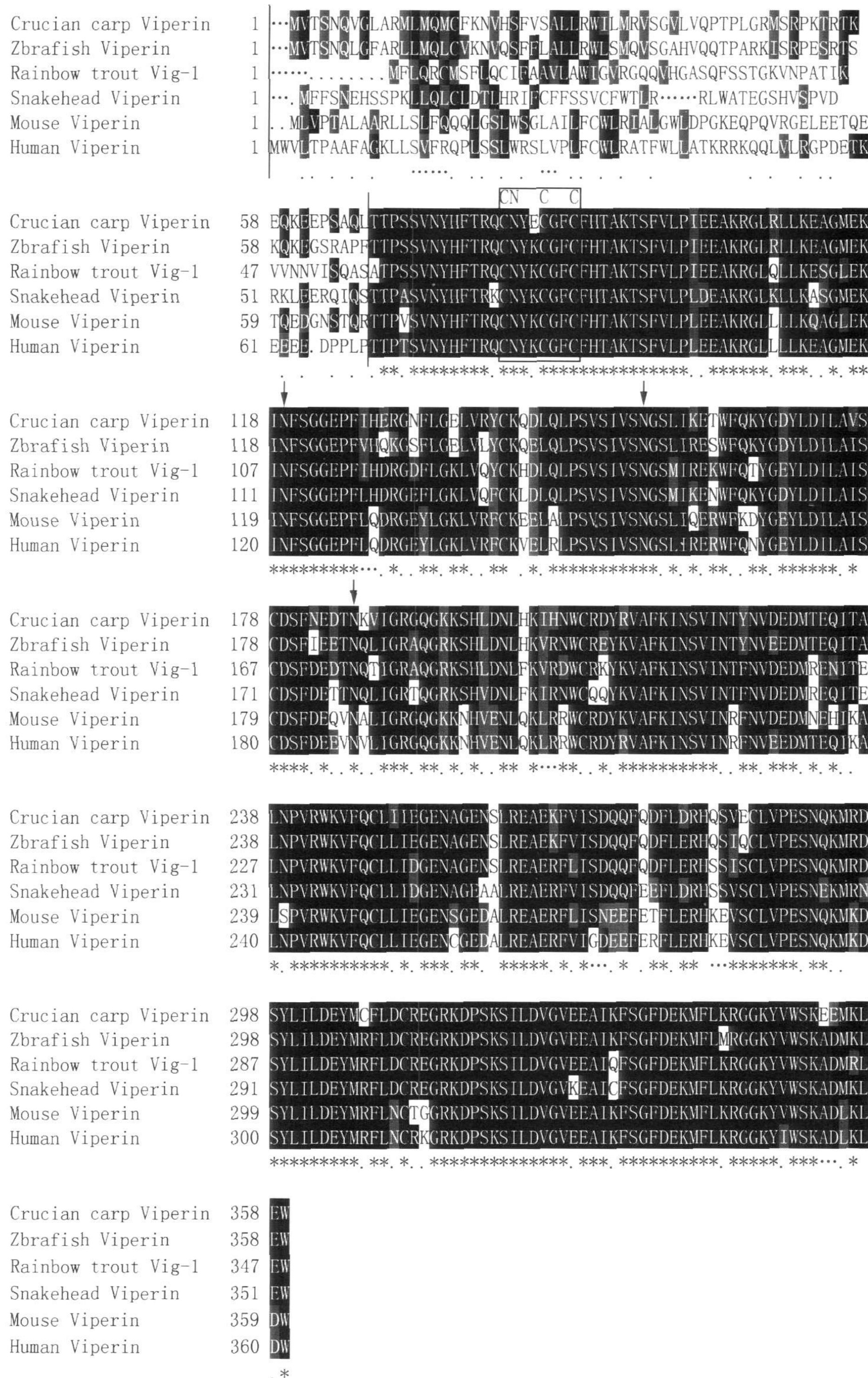

Fig. 1. Multiple sequence alignments of snakehead Vipeirn with other species Viperin. The identical and similar residues are shown with asterisk (*) and dot (.) respectively, a divergent region at N-terminal is shown, and three *N*-glycosylation sites are indicated by arrows. GenBank accession Nos.: Human AF442151, Mouse NM_021384, Snakehead AY898793, Rainbow trout AF076620, Zebrafish NM_001025556, Crucian carp AY303809.

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Fig. 2. Multiple sequence alignments of snakehead ISG15 with other species ISG15. The identical and similar residues are shown with asterisk (*) and dot (.) respectively, the C-terminal LRGG amino acids are in the box. GenBank accession Nos.: Human BC009507, Mouse NM_015783, Snakehead EF067846, Goldfish AF206323, Rainbow trout AF510711.

Fig. 3. Phylogenetic relationships of Viperin and ISG15 protein sequences. The bootstrap confidence values shown at the nodes of the tree are based on 1000 bootstrap replications. (a) Viperin phylogenetic tree. Accession Nos.: Chimpanzee XM_515283, Cow NM_001045941, Red jungle fowl XM_426208, Orangutan CR858150, Norway rat NM_138881, Pig NM_213817, Dog XM_846183, Chinese perch AY395718. (b) ISG15 phylogenetic tree. Accession Nos.: Water buffalo DQ118136, Sheep NM_001009735, Chimpanzee XM_520842, Norway rat XM_216605, Cow NM_174366, Dog XM_536714, Zebrafish XM_682249, Crucian carp AY303810 and AY303811.

In the whole snakehead ISG15 promoter and its 5′ flanking region which covers about 1.7 kb, the putative binding sites for TATA, CAAT, NF-κB like and ISRE were identified (Fig. 5). The TATA and CAAT boxes are located between −45 and −48, −284 and −287 respectively; one of the putative ISRE site is between −97 and −108; three γ-IFN activation sites are between −357 and −365, −506 and −514, −1563 and −1571, and two NF-κB like sites are between −587 and −596, −1142 and −1151. In the 5′ UTR, snakehead ISG15 gene contains a single intron, which differs from Viperin gene.

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-1700 AAGGCCCTGCGGATGAGGGGTGGTTCCTGATCAAAGCGGGCCCTCC

Sp1

-1650 AGGGGGACAGGCACTGGGGAGGGGATCGCAGTAGAACGGCCCCTAAAGTATCCCGCAGAGACTCTGTAAGAGTGG

Sp1

-1575 TCTTACCTGTGATGGGGAAAAGCAGTGGGAGGAACAGTTACCCCTCTGAGGAGACCTAAGAGACACAAAGACTGG

-1500 CCAATTTATTTTATTTTTTATTTTTTATTTTTTTATAGCGTTAAAGGTAGAATCTCCTTTTCACTTTTAAATGTC

-1425 ATGATTTATGGATGTAAGTCTTCCATCCTACCGACATGGATGAGGTTTGCAGCGTTTTAAATGAGCAGTGAACCA

-1350 TTTTCACAGTGATGTAACTAATGTGAGGTCATGCCACGTGATATTACATTAGGCAAAGCTCACTGGCAGATAATG

C-jun

-1275 ACTTTAGCAGAGAGCAACCAGCCATCACTTTAGTTATGTATCTATACATCACAACTGATCATATATGTAATAAGA

C/EBP alpha

-1200 TAGTGACATTAACCTGTGGCATCCAGGCCCTCTA**TTACAATAA**CCGGGAAGTCTGGTTTTCTTCTAGATTCCACG

GAS

-1125 CAGCTGGGTAGCAGCTCCAGTACAGATGTTGCCTCTGGGATGATGTCACCACACTGCAGAACAGACAAAATCAGT

-1050 TTGCTGCTGTGAGAGAAGACAGGGGCGAGTCGTAGCCGGTGACGTGCAGAACACGCACTACTACGAGCCACGGGG

-975 AACAACAATAGAGGAAAGTCTCAAGTGCTTTGGGCCATTTACAAGCCGCTGCTATGCCATATTCTACCACATGAG

-900 GGCGCAGGTCTGCAGAATCTTTCATATTTTTGCACCATTGTAAGTAAAACTATGTAACTAAACATGACTGTGCAC

-825 CTCATAATCAGATTATAGGAACCTTTTTCAGGACTTCGTACGCCTCCTCAAACCTGTAGAAAACATCAGAGTTGA

-750 CGATGTTCAGAGTAGATGGGTGATACTGTGGCGCTGCTGAGGGCACCACATAGTACTTCCTTGGCATTTCTGTCC

-675 CGGTGCGTGGCCACAGATACTGAGTCCAAAAGTTTTCACGTTGTAAGTACGAGCACACAAGCACTGGGTCGTGTT

-600 GAATTAAACCTCGCAGGAGTCGCTCTGTTAGGGAGGCGCTCTCAGAATTGGCCTTTAAAAAATAGCCCTTGATGA

-525 GAGAATCTTTGGTGTGTGGCAGGAAACAGGACATGGGACATAAAACACACTCGGGATGCAAATCTCTCGACAATT

Oct-1

-450 TGTCTTTGAGCTCCGCGTAAAACTTGGCTCCTCTGACCCGGTCGCTGTTGCACACAAGGAGGGAATAACAGGTGC

-375 CGTCAAGTGCATCTCTGAACAGCCGTGGCACTTCTCCACGGGGCTTTTCCTGAATTCCAAAATAAAGGGGCGCTC

NF-κB like/GAS NF-κB(consensus)

-300 CGTCAAGCTCCACTGAAAAAACACGAGAGGACCATTGGGGAAGAAGGGACATGGTGCGTCGTGCCATAGTCCAAG

-225 CGTTGTGATCAGTTTCTAGTCTGGACAGATGATACCCAATGGAAAGTA**TTACATAAA**CTGAAGGGGAAACGAAAG

CAAT box GAS ISRE

-150 TAAAAAGCCGAAACGAAACTCAACCCGTAATAAAAGTCAAAGTCTTGCTGGCGATTCTCTGTGACCTACTTCTGT

ISRE

-75 CTGGTGCTCACCTGCCGTAATATATGGCACCTGTTGTGAGAACGACGCATTTACGCATAGCCTGCTGTAAATACA

TATA box

+1 GTGAGTCATTGTAACGCC

Fig. 4. The sequence of snakehead Viperin promoter and its 5′ flangking region. GAAA element and its complement are in grey, the TATA and CAAT are boxed, the putative binding sites of ISRE and NF-κB are double underlined, GAS sites are in bold, and the putative binding sites for C-jun, Sp1, Oct-1 and C/EBP alpha are underlined.

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−1725 AGGTACCAGGGGTTGCAACATGGCAACCAGCACCAGAGGACCAACTTCCTATGGTAGTACCCTCTTGACCTTCAG

−1650 ATATTACTTTGAAAACAATCCCTTAACCTTGTTTTAAACAGTTGTCCAGTGACCTTGTGTACACTGTAACAGGCA

−1575 TTGCTTGCTGTAATTCCACATTGTGATATTGACCATTCAAATATCCATTTAAATATCCTGCTATTGTGCTTCCAA

GAS/C/EBPalpha Pit-1

−1500 TGTAAGTGAGGGTTGGGGAAAATTACAGAACCCTTGTTGAGCTGAAGCTGAGAAACGGTGACACAAAGGACAGCA

−1425 TGACATAATCACCATCACGTCTAGAAAACTAAGTGACCTAGATTAGTGAAGACCAAAGGTCAGACATGATGATGC

−1350 ATGAATCATAATGATATTTGTTGTCTTAAGTTATTCATAATGACTTCATTCAGTGGATACATTATTTACAGCTAA

−1275 AGTAATTGTTAACACACACACACAACTTCTTACCACTGACTCTCATTTTACAGGCTGCCCAAATACCTGCTGACC

−1200 TAGTGGACAAACTTGAGCGATTGGCACTGGTTGATTTCCGCACCCAGCAGGGACTGACCTGTTTGGAGAAAGCTA

NF-κB like

−1125 TCCAATTTGCAGATCAGCTTCATGTTGTTGACATATCAGGAGTTGAACCAATGGATTCAGTTGTAGAGGACAGGT

Sp1

−1050 GTGGTACACGCAGTGACATATCAGTTGAATTTAAAATCCTACAAAAAATAAGCAACAACTGCAGCACAATTTTAA

−975 ACTCTTCTGAAACCTGCTGTATGACTGTGGGAATGTTTGATCCGCTCAGGGCATTATACCTGAGAGACGACTCTG

−900 TGATGGAAGGGGACTGTGCTGAAGAACTGCTGCAGTTCTCCAAAAACACAGTTGAAGAATATTTTGTAGCACCAC

−825 CAGGTAATAAAAGTTTTTTGTTTGTTTTTTTTAAATCTTGTTTTTTTTTTTATGGTAAAGATCTTTGACTATTTC

−750 TTATTTTTCACCACAGGAAATATTCCTCTACCAAAGAGGGAGGAGAGGGCTGCCATCCTCAAACACTCCGAGCT

−675 CTGATGTTTATGGATTTGTTTTTTGTTTGTATTTTTTTGTTTGTATTTAATGTATTTATTTGTTTGCATCAGGAG

−600 TGTTGGCCGAGGCCAAATGCTTATATAAATGGAAAAACACATCACTTTCATTTGACAGCCATGGTTAAAATATAT

NF-κB like

−525 GCCTGTATCATTTTCTCAAAACAGAGAATTAGGTGTGTCCACACAGAGCAGTTGCTCATGATTGGAATTTAGTTA

GAS

−450 TCTTGGCAAGTCCAGTTTTTTTTTCTAATACCTAATATAAATAAAGTTTTATCTTTTCATCTTTTGTGCAAACT

−375 ATTTGAAATGTTGCTTAAAATTATAACGTGTTCATCAAGTAATCAAGTGGTTGATAAAAGGATCGAGCCTTTTTT

GAS

−300 TATTGCAACTACACAATCCTGAAAGTCCCCTGCAAGTGTATTAATTCTAGTAATTAACATATACAGATGTGTATT

CAAT box

−225 CTCTGATTAGAACTGGGATATTTGGTGGAAGAATTATAAACATGCTTTAGATTTGTGTCATGATCAAAACCAAAA

−150 CATAACAAGTCCTCATTGTTGCAGAGTCACGTTTGGAGAATAGAAAGCGAAAGTTATAAAGGATGAGAAGGGAAA

ISRE

−75 ACGAATCCTACGCAGAGCGTGTATGTGTATATAAACAGTGGTGAGCTCAAACCTCAGGCACAGCTGCTTCTCACA

TATA box

+1 ACATTTCGTTACCGATCGAGTTTCCTGAAGGACCTTGCTGTTTCTGAACAGgtaggagtctttaacagcaattta

+76 gttctacgaggttgaaaagtgttttgactttttagattttaaaattactgactttgtctttcagACCATTT

Fig. 5. The sequence of snakehead ISG15 promoter and its 5′ flangking region. GAAA elements and its complement are in grey. The TATA and CAAT are boxed, the putative binding sites of ISRE and NF-κB are double underlined, GAS sites are in bold, and the putative binding sites for Sp1, Pit-1 and C/EBP alpha are underlined. Intron is indicated in lowercase with consistent gt/at mode.

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2.4 Expression of Viperin and ISG15 genes in snakehead fish

The tissue expression patterns indicated that snakehead Viperin and ISG15 were transcribed mainly in the head kidney, posterior kidney, spleen, gill, less in liver, and little in other tissues (Fig. 6 (a)). After intraperitoneal injection with poly I∶C, the expressions of snakehead Viperin and ISG15 in liver were found increased approximately 7- and 4.5-fold respectively when compared with the control fish (Fig. 6(b)).

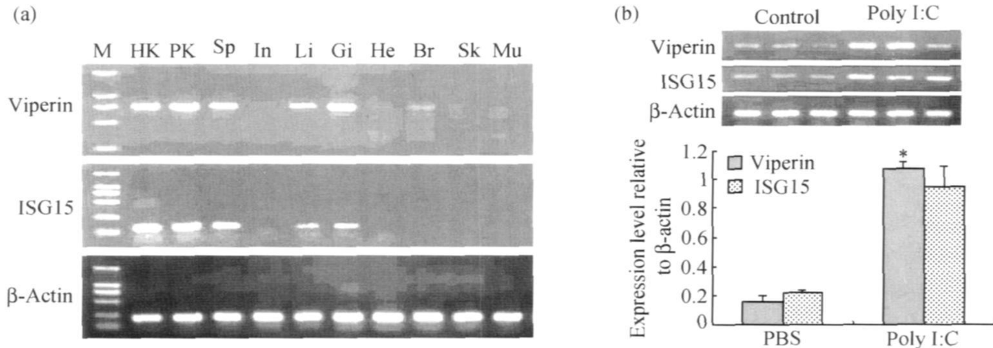

Fig. 6. Expression profile of Vipeirn and ISG15 in various tissues (a), and poly I∶C induced expression in liver (after 24 hours, $n=3$) (b). (a) HK, head kidney; PK, posterior kidney; Sp, spleen; In, Intestine; Li, liver; Gi, Gill; He, heart; Br, brain; Sk, skin; Mu, muscle. (b) the expression level is calculated relative to the β-actin. * indicates statistical significance between induced and controlled fish.

3 Discussion

Compared with mammals[19,28], only a few IFN system genes of teleosts have been isolated and characterized[10,23]. In this study, the suppression subtractive hybridization technique was used for screening the differentially expressed genes in snakehead fish treated with poly I∶C. Sequencing revealed that Viperin and ISG15 were the most abundant ESTs in the library. Snakehead Viperin displayed very high sequence similarity with those known mammalian and teleost Viperin except for 70 aa at the N-terminal, which is highly diverse in all Viperin homologues. The short hydrophobic stretch at the N-terminal of Viperin does not allow the protein to enter the rough endoplasmic reticulum (ER)-Golgi pathway[14]. This suggests that Viperin seems to reside at the cytosolic face of the ER, and could be required for the antiviral effect through interference with transport of critical viral components, transmembrane glycoproteins, from the ER to the Golgi[16]. Existence of the N-glycosylation sites indicates that snakehead Viperin is a cellular glycoprotein. The residues 70—182 are highly homologous among Viperin, MoaA, NIRJ and PooIII protein families, especially the iron-sulfur motif CNXXCXXC[29], in which the cysteines were shown to be important to the biological function of the coordination of a Fe-S cluster. The conservation of C-terminal indicates that this region is a functional domain in the non-specific antiviral response[29].

Snakehead ISG15 contains ubiquitin like (UBL) domains. The conserved C-terminal "LRGG" is essential in ubiquitinylation for ISG15 and target proteins[30]. Some UBL proteins are expressed as inactive precursors with a few amino acids following the conserved motif. These UBL proteins are activated by proteases releasing their active conjugating tail[30]. Some teleost species, like channel catfish and bastard halibut (*Paralichthys olivaceus*) may need protease activity to expose their active conjugationg motifs[24]. The reported promoters of ISGs in teleosts include those for *Vig2* and *Mx1* in rainbow trout[15,25], *Mx* in pufferfish[13], ISG15 in goldfish and Atlantic cod[23,24], and Viperin gene in mandarin fish[18]. Snakehead Viperin and ISG15 promoters contain ISREs with the consensus sequence ($GAAAN_{1-2}$ GAAAS, S=C/G, N=A/T/G/C). However, only one base is different in ISRE between Viperin and ISG15 promoters. ISRE is an important characteristic of IFN-induced gene promoter, also partially overlaps the IRF1/2 recognition sites (AAAASYGAAASY, Y =T/C) (IRF-E). The presence of ISRE sites in the Viperin and ISG15 promoter regions implies that the expression of Viperin and ISG15 can be induced by virus and IFN signal through the activation of a cascade of reactions. Like rainbow trout *Vig2* and *Mx1* genes[15,25], snakehead ISG15 contains one ISRE, its

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Viperin gene contains two ISREs, whereas Atlantic cod ISG15 contains three ISREs[24]. These suggest that number of ISRE is different among the ISGs and even in the same ISG if varies from one species to the other.

Snakehead Viperin and ISG15 promoters also contain three conservative γ-IFN activation sites (GAS) with a consistent sequence motif (TTNCNNNNAA)[31], which is responsible for the γ-IFN-mediated transcription of the target genes. Moreover, we found that the element GAAANN and its complement are repeated in snakehead Viperin and ISG15 promoters, suggesting that these elements provide potential inducibility by IFN signal. In snakehead Viperin promoter region, a NF-κB site (GGGRNNYYCC, R=A/G) and a NF-κB like site were identified. The presence of binding motif for NF-κB is very interesting as it was indicated that the NF-κB transcription factor is involved in the stimulation of the type I IFN promoter[32]. Like other IFN-induced gene promoters, such as IRF1 and IRF2[33,34], the conservation of NF-κB site in snakehead Viperin promoter suggests that the activation of NF-κB also involves in induction of teleost ISGs. Snakehead ISG15 promoter lacks consensus NF-κB sites, which is in agreement with the reported ISGs[23,24], but it has two NF-κB like sites, whose function is unknown at present. In 5′ UTR, different from Viperin gene in which no intron was found, ISG15 gene contains an intron, suggesting that these two ISGs have different transcription patterns. Though the possibility of NF-κB sites presented in snakehead ISG15 promoter can not be excluded, from the comparisons of ISRE, NF-κB site, 5′ UTR structure, and protein sequences between Viperin and ISG15, we assume that these two genes have different regulatory mechanisms and different functions in IFN system.

Snakehead Viperin and ISG15 were transcribed mainly in immune organs, and a low expression level was also detected in other tissues. Our findings indicated that poly I:C can induce the expression of Viperin and ISG15 genes in snakehead liver, and that tissue distribution and induced expression of Viperin and ISG15 genes are the same with other reported teleost ISGs[18,24]. It is possible that IFN directly stimulates Viperin and ISG15 genes expression because poly I:C was considered as an IFN-inducer.

In conclusion, we have reported the molecular cloning and characterization of Viperin and ISG15 genes and their promoters from snakehead. These results not only provide the significant evidence for ISGs structure and function, but also help us to understand the teleost IFN system and ISGs regulatory mechanism. The challenge of future work is to conduct more functional studies of these genes and determine their regulatory mechanisms by which anti-viral defence is established.

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