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**Title:** Cloning and characterization of two genes encoding rainbow trout homologues of the IFITM protein family\*.

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\* The sequences reported in this paper have been deposited in the EMBL/GenBank database under accession numbers **AJ291989**, **AJ313031**, **AJ320156** and **AJ320157**.

## 1   **Abstract**

2   To identify genes connected with leukocyte function in rainbow trout a cDNA library derived from head  
 3   kidney leucocytes exposed to phytohemagglutinin (PHA) was screened. A clone with homology to the  
 4   previously described human interferon inducible transmembrane proteins (IFITM) was isolated. This clone  
 5   was used to screen a trout genomic library identifying two trout IFITM genes. Both genes are composed of  
 6   two exons, in common with human IFITM genes. The promoter regions of both genes were examined and  
 7   found to contain interferon, immune and stress-related transcription factor binding sites. Transcripts for both  
 8   genes were found in most tissues examined. Trout IFITM1 mRNA levels were highest in head kidney, but  
 9   present in all tissues studied. IFITM2 mRNA levels were highest in gill, heart and liver, and absent in head  
 10   kidney and blood. In response to conditions mimicking viral infection, transcription of the trout IFITM genes  
 11   was elevated in a trout spleen macrophage cell line (RTS11) over the examined timecourse (72 h). This  
 12   study suggests that the organization and transcription of the IFITM genes is well conserved between human  
 13   and rainbow trout.

14

15   **Key words:** *Oncorhynchus mykiss*, interferon-inducible, transmembrane, promoters, teleost.

16

17   Among the battery of interferon (IFN)-responsive genes that collectively mediate IFNs pleiotropic effects are  
 18   a family of small transmembrane (14-17kDa) proteins, IFITMs. The original member of the family was an  
 19   IFN responsive gene called human 9-27/leu13 protein, or IFITM1 (Lewin et al., 1991; Deblandre et al.,  
 20   1995). Human IFITM1 is expressed in leucocytes and endothelial cells and is often associated in leukocyte  
 21   cell membranes with CD81 (Chen et al., 1984). It is thought that this IFITM/CD81 complex may also interact  
 22   with lineage-specific components, such as CD19 and CD21 for cell-specific signaling activities (Tedder et al.,  
 23   1994; Levy et al., 1998). Induction of IFN can lead to IFITM1-triggered induction of a homotypic adhesion  
 24   pathway in leucocytes, leading to inhibition of cell growth in a subset of cell lineages; contributing to L-  
 25   selectin shedding necessary for movement of lymphocytes into lymphoid tissues, and allowing transient  
 26   maintenance of lymphocytes in lymphoid tissues during the generation of an active immune response  
 27   (Deblandre et al., 1995; Frey et al., 1997; Evans et al., 1993).

28         While there is some understanding of the functions of human IFITM1, the functions of other IFITMs  
 29   are largely unknown. More recently, expression of a number of IFITMs in hematopoietic stem cells and germ

cells suggests this IFITM family may be involved in promotion and maintenance of the pluripotent state of many progenitor cells through induction of an antiproliferative state, indicating a role for IFN as a signaling molecule in this process (Tersikh et al., 2001; Baird et al., 2001). The interferon-responsive nature of this gene family in mammals has been tied down to an IFN-stimulable response element (ISRE) found in the promoter-enhancer regions of many IFN-inducible genes (Reid et al., 1989; Pru et al., 2001).

IFITM proteins contain two transmembrane regions and in general there is a conservation of amino acid identity throughout the protein sequence with the exception of both termini where there is a high degree of variance. Members of the IFITM family have been isolated in a number of mammalian species. To date, a 14 KDD transmembrane protein in *Torpedo marmorata* (cartilaginous fish) (Morel et al., 1991) remains the only homologous sequence identified in other vertebrates. In the current study, we report the characterization of two rainbow trout IFITM genes.

A cDNA library derived from 4 h PHA-stimulated head kidney leucocytes was screened by differential plaque hybridization.  $\alpha^{32}\text{P}$ -dCTP-labelled cDNA probes were prepared from mRNA isolated from (a) head kidney and (b) a mixed pool of mRNA isolated from five tissues - brain, gill, heart, liver and muscle. One clone was isolated after three rounds of screening and identified as a homologue for the IFITM gene family by sequencing (MWG, Milton Keynes, UK).

A random-primed  $^{32}\text{P}$ -labeled, IFITM cDNA PCR product was used to identify trout IFITM genes from a rainbow trout lambda DASH II genomic library using nylon membranes (Amersham, Bucks, UK). The lambda DNA was extracted following the protocol of the lambda DNA Midi prep kit (Qiagen, Crawley, UK). The clones were then digested with several restriction endonucleases and the resulting fragments were subcloned into pBluescript II KS (-) (Stratagene, La Jolla, CA). Sequence data was evaluated, overlapping contigs generated and complete sequence for IFITM genes and immediate flanking regions was determined.

The rainbow trout spleen macrophage cell line, RTS11 was maintained as previously described (Ganassin & Bols, 1998). For stimulation studies, cells were plated at a density of >70% confluency in six well dishes and 48 h later were stimulated with the addition of double-stranded RNA, poly(cytidylic-inosinic) (Poly I:C) (Sigma-Aldrich, Dublin, Ireland) at a final concentration of 100  $\mu\text{g/ml}$ . Controls without Poly I:C were set up in parallel. After exposure the cells were harvested by direct lysis, and total RNA isolated using TRIzol reagent (Invitrogen, Carlsbad, CA).

Total RNA was extracted from healthy fish from head kidney, whole blood, brain, gill, gonad, heart, liver, muscle and spleen tissue samples using TRIzol. DNase-treated total RNA (1 µg) from tissues and cell culture was reverse transcribed with MMLV-RTase (Promega, Madison, WI) and IFITM-specific RT-PCR products were amplified using the following intron-spanning primers, IFITM1F (5'-TCGTTTGCACTAGAAGCTTT-3'), IFITM1R (5'-TGTGTGTATTAAAGAGATGAGCT-3'), IFITM2F (5'-TCGTTTGCCCGAGAAGCTTT-3') and IFITM2R (5'-GACAAAAGCAAAGACTGGAAGTACACTA-3'). To confirm cDNA integrity, a 260 bp intron-spanning  $\beta$ -actin fragment was amplified using gene-specific primers (5'-ATGGAAGATGAAATCGCCGC-3') and (5'-CGACATGGAGAAGATCTGGCA-3') based on [AJ438158](#).

By differential screening of a cDNA library generated from PHA stimulated head kidney leucocytes (Davidson et al., 1999) we identified a rainbow trout homologue of the IFITM protein family. The cDNA obtained was 0.7 kb in length, coding for a protein of 127 aa with a predicted molecular mass of 14.4 KDD. This clone was termed IFITM1 following the isolation of a second gene IFITM2 while screening the genomic library, as detailed below. The IFITM2 gene codes for a slightly larger protein of 128 aa.

The rainbow trout IFITMs share 85% aa identity and 87% aa similarity. Outside of this, the most closely related protein sequence is mouse IFITM3 with 32% identity and 50% similarity to trout IFITM1. Other IFITM sequences (mammalian and *Torpedo*) share between 26-31% identity and 39-48% similarity to trout IFITM1. Sequence conservation is highest for the central segment of the proteins with little homology at either end of the proteins as can be seen in the alignment in Fig. 1. ProfileScan (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>) identified one protein kinase C phosphorylation (PKC) site in trout IFITM1 and IFITM2 in the highly conserved region (74-SIK-76). Based on experimental results IFITM proteins have been shown to possess two transmembrane regions (Lewin *et al.*, 1991; Morel *et al.*, 1991). SOSUI ([http://sosui.proteome.bio.tuat.ac.jp/sosui\\_submit.html](http://sosui.proteome.bio.tuat.ac.jp/sosui_submit.html)) and TopPred2 ([http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)) transmembrane prediction programs were used to examine the trout sequences. Based on high hydrophobicity values, both proteins are predicted to contain two transmembrane regions at amino acid residues 52-72 and 99-121 (Fig. 1).

Two IFITM genes were isolated from a rainbow trout genomic DNA library screened with the full-length trout IFITM1 cDNA. 2.3 kb of IFITM1 nucleotide sequence that included 0.8 kb of promoter region, 1.1 kb of coding gene and 0.4 kb of 3' flanking sequence, together with 6.1 kb of IFITM2 sequence including 4 kb of promoter region, 1.1 kb of coding gene and 1 kb of 3' flanking sequence was determined

(**AJ320156** **AJ313031**). The positions of the exons and introns were defined by comparison with the rainbow trout IFITM1 cDNA. The genes contain two exons and one intron. While exon 1 is the smaller of the two exons, it contains the larger part of the coding sequence (77 aa). The human IFITM genes (**J04164** **X57351** **X57352**) are similar in size and are also composed of two exons. The relative sizes and location of intron/exon splice sites with respect to encoded amino acids is maintained between the two species.

Potential transcription factor binding sites in both genes were identified using MatInspector ([www.genomatix.de/cgi-bin/matinspector/matinspector.pl](http://www.genomatix.de/cgi-bin/matinspector/matinspector.pl)) and TESS (<http://www.cbil.upenn.edu/tess/>) and revealed the presence of interferon-inducible and other immune-related transcription factor binding sites (Fig. 2). The promoters are similar to other interferon-inducible promoters previously isolated from rainbow trout by possessing a TATA box as well as ISRE sites (Boudinot et al., 2001; Collet & Secombes, 2001). Trout IFITM2 possesses many of the same type of transcription factor binding sites as IFITM1 (Fig. 2B). An alignment with the three characterized human IFITM promoters (Reid et al., 1989; Lewin et al., 1991) showed no observable conservation, either in terms of sequence similarity or transcription factor binding motifs, between the two species, other than the presence of ISRE sequences, not shown. In contrast to the trout genes the human IFITMs lack TATA boxes– this divergence is characteristic of human and fish interferon responsive promoters isolated to date.

To ascertain the expression patterns for the trout IFITMs in healthy rested fish, total RNA was isolated from head kidney, whole blood, brain, gill, gonad, heart, liver, muscle and spleen samples and subjected to RT-PCR assay using gene-specific primers (Fig. 3A). The band corresponding to the IFITM1 transcript was most intense in head kidney, high in gill and liver and was visible in all other tissues. In contrast the IFITM2 transcript was not present in either head kidney or whole blood. IFITM2 levels were highest in gill, liver and heart, detectable levels were observed in the other tissues. The observed expression pattern implies that the genes are found at higher levels in some tissues involved with immune or haematopoietic function in teleost fish, such as head kidney, gill and liver (Iwama & Nakanishi, 1997).

To investigate whether the similarity of the trout IFITM genes to their interferon-responsive human homologues extends beyond sequence conservation and structural organization, their response to indirect IFN induction was studied. RTS11 cells was exposed to the synthetic dsRNA molecule (Poly I:C). Poly I:C has been found to stimulate IFN production in mammalian cell cultures. Eaton (1990) showed that four species of salmonids including rainbow trout, produced an antiviral IFN-like response following

intraperitoneal injections of Poly I:C. RTS11 cells were harvested after 24, 48 and 72 h exposure, total RNA extracted and RT-PCR amplification performed. Both IFITMs are more highly expressed in the exposed cells compared to the controls indicating a possible role for interferon-induced upregulation of both genes in teleosts (Fig. 3B).

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19

1 Fig. 1. Comparison of amino acid sequences of trout IFITMs with selected IFITM sequences, mouse (mm)  
2 IFITM3, human (hs) IFITM1, and *Torpedo* IFITM. The two transmembrane regions are underlined and the  
3 protein kinase C phosphorylation site is indicated with an arrow.

4  
5 Fig. 2. Nucleotide sequences of the 5' flanking regions of the trout IFITM genes. Nucleotide positions are  
6 counted from the initiation codon ATG that is designated as +1. Potential *cis*-acting elements are labelled  
7 with a single underline. (A) Rainbow trout IFITM1 sequence. (B) Rainbow trout IFITM2 sequence. Only 760  
8 bp of the obtained 4 kb presented in this figure.

9  
10 Fig. 3. Tissue expression of trout IFITM1 and IFITM2, determined by RT-PCR using intron spanning primers.  
11 (A) Levels of IFITM transcripts in different tissues. (B) IFITM expression levels in RTS11 cells exposed to  
12 dsRNA (Stim). Unstimulated RTS11 cells (Con) were assessed concurrently. IFITM1 (27 cycles of PCR  
13 amplification), IFITM2 (29 cycles) and  $\beta$ -actin (21 cycles).

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IFITM1 : -----MDQPPFYQPEFVPMKGNKYRLEDTHGAPKFQHTVVVLGQEPQPVVPHPRDHI : 51
IFITM2 : -----MDQSPSYQPEFVPMNGNKYRLEDPHGAPKFQHTVVVLGLEPQPVVPQPRDHI : 51
mmIFITM3 : MNHTSQAFITAASGGQPPNYERIKEEYEAEMGAPHGASASVRTTVINMPREVS--VPDHV : 58
hsIFITM1 : -----MHKEEHFAVLGAPPSTILPRSTVINIHSETS--VPDHV : 37
torpedo : -----MEYRTDQV-----PMSPRS-VQGAPGTLP--IRDHL : 28

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IFITM1 : IWSIDCSLVYCNPFCLGMLAVYFSSIKSRDRKMVGDLLEGARKHGKTACCFNTVTLTTLAILGL : 111
IFITM2 : IWSIDCSLVYGNPLCLGMLAVYFSSIKSRDRKMVGDLLEGARKHGKTARCFNVVTLTTLVILGL : 111
mmIFITM3 : VWSIFNTLFMNFCCLGFLAYAYSVKSRDRKMVGDTVGAQAYASTAKCLNISTLVLSILMV : 118
hsIFITM1 : VWSIFNTLFNLNWCCLGFLAFAYSVKSRDRKMVGDTVGAQAYASTAKCLNIWALILGILMT : 97
torpedo : PWSIFNLFYMNVCCLGLTAMIFSVKSRDRKVVGDVEGARHYGSTARSINIAATVLGILLI : 88

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IFITM1 : L-----FFFITYGIIYQVAH- : 127
IFITM2 : L-----FLFIIYGFFIYNISHL : 128
mmIFITM3 : V-----ITIVSVIIIVINAQNLHT : 137
hsIFITM1 : LGFILLLVFGSVTVYHIMLQIIQEKRGY- : 125
torpedo : L-----ILIGLAATGTIQALKYKG : 107

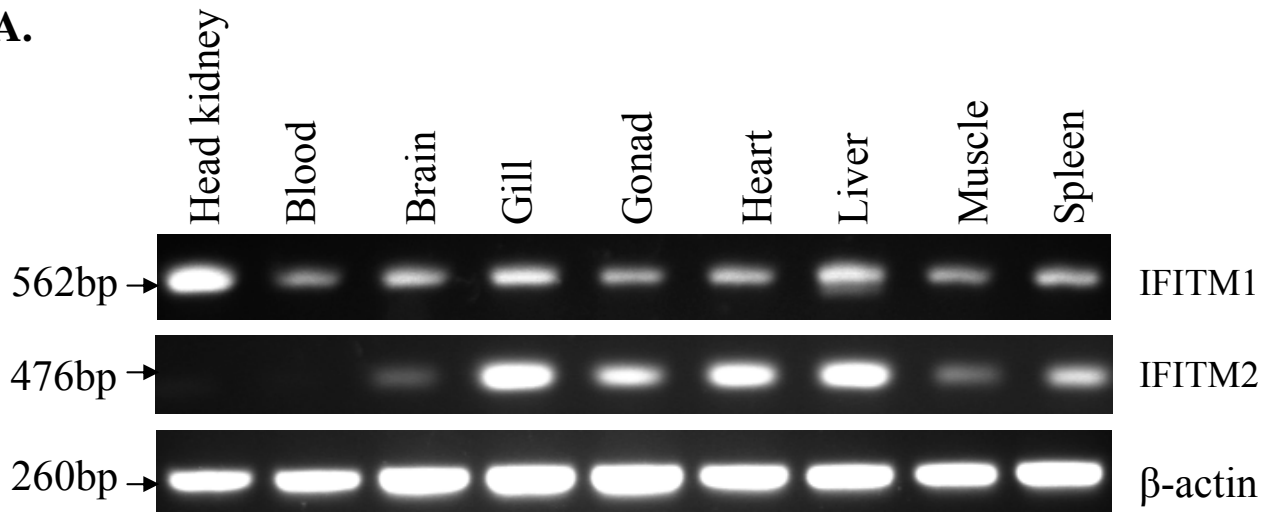
```

**A**

tgacccactgaagagatgagtccttcagtagagactt~~aaagg~~ttgagaccgagtttgcgtc -700  
 tctgacatgggtaggcagaccgttccat~~aaaaatggagctct~~ataggagaaagccctgcc -640  
 IRF-1/2  
 tccagctggttgcctt~~aaaaattctagggacaatt~~aagaaat~~aagaaatggaggaatgggc~~ -580  
 GAF PU.1  
 c~~aaaattc~~at~~aaagtgg~~tgatcctaactgacct~~aagacaggg~~aattgttact~~aggattaa~~ -520  
 TCF II ETS-1  
 atgccaggaattgtg~~aaaaactgagctcaaatgtat~~ttggct~~aagatgtgagaggaagt~~c -460  
 GATA-3 PU.1  
 tagtgtg~~agaagatggaact~~aggtg~~aaacttggc~~at~~acattctaacc~~agttagagggcag -400  
 ICSEB IRF-1/2  
 gatagacagactggtat~~accacagacagaagtgg~~t~~aacc~~cagacatgtcacaggggctat -340  
 aaagctg~~aagcatccgttcttaacgttatct~~catg~~accaa~~at~~atggc~~agtg~~acaggaagt~~ -280  
 ISGF3 GATA-1/3 IRF-1 ETS-1  
 ccagtc~~aaat~~at~~ctttgggtat~~acact~~ccccaaa~~agcagc~~acaaa~~acgtttggc~~aatcatg~~ -220  
 ctattgtg~~caagagaggggggagggcctcc~~agaaat~~caaac~~at~~acacatt~~tactggaaa -160  
 GC Box  
 cagtgt~~aatgacgccatcattcacc~~acccactcctctt~~ataaaa~~cctccacctcacctc -100  
 TATA  
~~acaaa~~g~~aaacgtctctttgggtct~~aa~~atttctcgtttg~~cactagaagctttgattat -40  
 HEAT SHOCK IRF-1/2 ISRE  
 ttttaacacgagggt~~aaat~~aagtagctat~~attcgcttgcc~~ATG +1

**B**

ctatgttgcaacgttattat~~aaaacgattttggagaatgatgatccg~~caacagcgcattt -700  
 TCF II  
 cagtat~~cagaagt~~~~aaaa~~gaaat~~acagccaaactcacctg~~acactgccttttttagacctta -640  
 IRF-1/2  
 taaactgtcgagt~~acc~~~~aaaaa~~act~~aat~~~~caaaa~~t~~ggaatgaa~~acat~~aaaat~~~~aaagat~~atata -580  
 NF- $\kappa$ B  
~~aaaactactcattgcatgg~~tgggtgtt~~gtaggctagtttaggtaggaccatgttatcg~~tcc -520  
 CHOP GATA-1  
 ctagacaagttcaat~~aggggagcttattgagctagatgtctcatgtcttg~~cgc~~aatgacg~~ -460  
 TCF II  
 tacgtggcttctccgctgcct~~accagatatgc~~atctgggtgttgcggattttat~~ttat~~aaaa -400  
 ttgggtgcagctttgaattatt~~at~~ttttttttttgtgggtatgattgctagttagcctattgca -340  
 CCAAT  
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 GATA-1  
 atatacagtttaactggtttacactccc~~aaaagctaaaac~~~~aaaa~~agttagaca~~accctc~~ -220  
 IRF-1/2 ISRE Sp1  
 ccctattgagcaagagagtctct~~aggggaatcaacatttaca~~atttactgg~~aaaccgtg~~ -160  
 cttatgcctgcaaccttcgcccgcgcctgct~~at~~at~~aggtacgttttgccttcac~~ctttcc -100  
 TATA IRF-1/2 ICSEB  
 tcacaaaattaccattttcttcggctcgct~~cttttttcgtttgcccgagaagctttgtgtt~~ -40  
 NF-AT HEAT SHOCK  
 ttttaacactaagct~~aaat~~aagaatctgtattcgcttgccATG +1

**A.****B.**