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Characterization of the interferon genes in homozygous rainbow trout reveals two novel genes, alternate splicing and differential regulation of duplicated genes

Maureen K. Purcell [a,*], Kerry J. Laing [b], James C. Woodson [a], Gary H. Thorgaard [c], John D. Hansen [a]

[a] U.S. Geological Survey, Western Fisheries Research Center, 6505 NE 65th St., Seattle, WA 98034, USA
[b] Fred Hutchinson Cancer Research Institute, 1100 Fairview Ave N., Seattle, WA 98109, USA
[c] School of Biological Sciences, Washington State University, Pullman, WA 99164, USA

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ABSTRACT

The genes encoding the type I and type II interferons (IFNs) have previously been identified in rainbow trout and their proteins partially characterized. These previous studies reported a single type II IFN (rtIFN-γ) and three rainbow trout type I IFN genes that are classified into either group I (rtIFN1, rtIFN2) or group II (rtIFN3). In this present study, we report the identification of a novel IFN-γ gene (rtIFN-γ2) and a novel type I group II IFN (rtIFN4) in homozygous rainbow trout and predict that additional IFN genes or pseudogenes exist in the rainbow trout genome. Additionally, we provide evidence that short and long forms of rtIFN1 are actively and differentially transcribed in homozygous trout, and likely arose due to alternate splicing of the first exon. Quantitative reverse transcriptase PCR (qRT-PCR) assays were developed to systematically profile all of the rainbow trout IFN transcripts, with high specificity at an individual gene level, in naïve fish and after stimulation with virus or viral-related molecules. Cloned PCR products were used to ensure the specificity of the qRT-PCR assays and as absolute standards to assess transcript abundance of each gene. All IFN genes were modulated in response to *Infectious hematopoietic necrosis virus* (IHNV), a DNA vaccine based on the IHNV glycoprotein, and poly I:C. The most inducible of the type I IFN genes, by all stimuli tested, were rtIFN3 and the short transcript form of rtIFN1. Gene expression of rtIFN-γ1 and rtIFN-γ2 was highly up-regulated by IHNV infection and DNA vaccination but rtIFN-γ2 was induced to a greater magnitude. The specificity of the qRT-PCR assays reported here will be useful for future studies aimed at identifying which cells produce IFNs at early time points after infection.

1. Introduction

The interferons are powerful pleiotropic cytokines that are induced in response to viruses by many different cell types and have diverse roles in regulating the immune system. Mammalian species possess multiple type I IFN genes, which are organized into gene families, such as IFN-α, β, ε, κ, τ and ω [1]. The selective advantage provided by the extensive duplication of type I IFN genes in mammals is not known. Type III IFN is a newly characterized IFN family (IFN-λ) that appears functionally similar to type I IFN and is produced by a variety of cell types [2]. Type I and III IFNs are distinct from type II IFN (IFN-γ), which is produced by a restricted set of immune cells [1]. IFN regulates the transcription of >300 cellular genes, known collectively as the IFN stimulated genes (ISGs) [3,4].

Type I and type II IFN genes have been reported for a range of finfish species, including fugu (*Takifugu rubripes*), common carp (*Cyprinus carpio*), zebrafish (*Danio rerio*), channel catfish (*Ictalurus punctatus*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) [5–13]. It has been hypothesized that the teleost type I IFNs are homologous to mammalian type III IFN [8]; however, since this hypothesis is currently disputed, we will follow Zou et al. [13] in calling this gene family the type I IFNs.

Previous studies have identified three type I IFN genes in rainbow trout (rtIFN1, rtIFN2 and rtIFN3) [13]. The rtIFN1 and rtIFN2 genes are highly similar in sequence, encode a protein with two cysteine residues and are classified as group I type I IFNs [13]. The rtIFN3 gene encodes a protein that possesses 4 cysteine residues and as such, is classified as a type I group II IFN [13]. Recombinant rtIFN1 and rtIFN2 display potent anti-viral activity *in vitro* while recombinant rtIFN3 has minimal anti-viral activity [13]. In contrast to type I IFN, only a single rainbow trout IFN-γ gene has been reported to date [12]. However, other finfish species (zebrafish, channel catfish and common carp) possess at least two IFN-γ genes [6,10,14].

Infectious hematopoietic necrosis virus (IHNV) is a negative-sense single stranded RNA virus within the genus *Novirhabdovirus* [15].

* Corresponding author. Tel.: +1 206 526 6282x252; fax: +1 206 526 6654.
E-mail address: mpurcell@usgs.gov (M.K. Purcell).

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We have previously shown in trout that IHNV infection or vaccination with a DNA vaccine encoding the IHNV glycoprotein rapidly induces the type I and II IFN pathways [16,17]. However, in these previous studies the rtIFN1 gene (alias IFN-α1) was the only type I IFN investigated. The goal of the present study was to develop highly specific quantitative reverse transcriptase PCR (qRT-PCR) assays for all known rainbow trout IFN genes and systematically profile these genes following vaccination, IHNV infection and poly I:C stimulation. During the course of this study, we found evidence of additional transcript forms. To eliminate possible allelic variants, we amplified and sequenced the IFN genes in homozygous rainbow trout. Here, we confirm the identification of two new rainbow trout IFN genes (a second type I group II IFN (rtIFN4) and a second IFN-γ gene (rtIFN-γ2)), and report the development and validation of specific qRT-PCR assays for rainbow trout IFN gene families. Transcriptional profiles of these genes following viral infection or stimulation with viral-related molecules are presented.

2. Materials and methods

2.1. Homozygous rainbow trout

Specific pathogen free homozygous rainbow trout from the Hot Creek and OSU strains were obtained from Washington State University trout hatchery. The trout lines were created by androgenesis as previously described [18]. The trout were transferred to Western Fisheries Research Center, reared in sand-filtered, UV-treated freshwater at 15 °C and fed a commercial semi-moist pellet diet (Bioproducts). Three unhandled homozygous trout were euthanized with buffered tricaine methanesulfonate (MS222; Argent Chemical Laboratories) and anterior kidney, heart, posterior kidney, spleen, gill, skin, anterior intestine, posterior intestine, liver, muscle, ovary and brain were removed, sampled into RNAlater (Qiagen Inc.) and stored at −80 °C until extraction.

2.2. DNA vaccination and infection of homozygous trout

The construction and efficacy of the pIHNw-G DNA vaccine have been previously reported [19–22]. Hot Creek trout, each weighing approximately 83 g, were injected with the IHNV DNA vaccine (pIHNw-G) vaccine plasmid or controls as previously described [17]. Briefly, trout were intra-muscularly (I.M.) injected with 50 μl volumes of phosphate buffered saline (PBS), 10 μg of pcDNA3.1 vector DNA diluted in PBS or 10 μg of pIHNw-G vaccine in PBS. Each fish was sampled at 7 days post-vaccination (p.v.). Tissues from the I.M. site and anterior kidney were snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

IHNV infection of homozygous trout, weighing approximately 300 g, has been previously described [16]. Briefly, 1×10^6 plaque forming units (pfu) of IHNV (WRAC strain; ATCC VR-1392) in 100 μl PBS was injected intraperitoneally (I.P.); control fish received only the PBS. Five fish were sacrificed from each group at days 3, 7 and 28 post-injection and the anterior kidney was sampled, frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.3. Poly I:C stimulation of anterior kidney leukocytes in vitro from non-homozygous fish

Rainbow trout were obtained from Clear Spring Foods Inc. (Buhl, ID) and held at a constant temperature of 15 °C until use, as described above. Poly I:C stimulation of anterior kidney leukocyte obtained from non-homozygous trout has been previously described [23]. Briefly, anterior kidney tissue from five individual animals was aseptically removed and leukocytes were enriched by centrifugation in a discontinuous (34%/51%) Percoll gradient (Sigma–Aldrich) [24]. The leukocytes were adjusted to 2×10^7 cells/mL and 100 μL of cells were plated into a 96 well tissue-culture plate and 5 μg/mL of poly I:C (Sigma–Aldrich) were added to the appropriate wells and incubated for 6, 12 or 24 h; a 50 μg/mL dose was incubated for 24 h only.

2.4. RNA extraction, cDNA synthesis and sequencing

RNA was extracted using the RNeasy Mini-Kit with in-column DNAse I treatment following manufacturer's instructions (Qiagen Inc.). RNA was used to synthesize cDNA as previously described [25] using 1 μg RNA to initiate cDNA synthesis. Primers designed against previously characterized rainbow trout IFN sequences [12] (Table 1) were used to amplify the genes or gene products from Hot Creek homozygous trout RNA. All conventional PCRs were performed using the following parameters: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min for all primers except those for amplifying IFN-γ intron 1 [13], which used an annealing temperature of 63 °C. Amplified products were subjected to electrophoresis on a 1% agarose gel (1.5% agarose gel for intron primers) and PCR products were cloned using the Topo TA cloning kit (Invitrogen). Clones were screened by PCR with the M13 forward and reverse primers and recombinant clones were sequenced as previously described [25]. Sequences were aligned visually with Sequencher V.4.5 (Gene Codes Inc.). Multiple alignments of cDNA and amino acid sequences

Table 1
Sequencing primer sets, real-time PCR primer and probe sequences and GenBank accession numbers.

Transcript	Name	Sequence(5′-3′)	Accession
Sequencing primers			
IFN1-Long	911-F28	gaagactacggaacaacatttcg	AJ580911
	911-R1132	tgtacaaaatacgttttattcaca	
IFN1-Short	890-F1	gaaactcatctggataactaacagcga	AY788890
	890-R737	acatgtacaaaagggaaatacgaaata	
IFN1-Promoter	SasaIFNA1/A2 F2	accaaggcctgtatttattaagcat	DQ354152
	OmyIFN1 R119	gcttaagtgaccgaagtggtgtt	FJ477854
IFN2	754-F1	tggaaaagctaaaagcaaaataaac	AJ582754
	754-R805	gcacaatacatttttattcacaatttc	
IFN3	738-F1	agctgttccattcaagttctt	AM235738
	738-R805	gttgtacgtatttttttatttaatt	
IFN-γ	IFN-γ F7	caccgattgaggactattgag	AJ616215
	IFN-γ R640	ctacacctagccttcacagtaga	
IFN-γ Intron 1	IFN-γ F5	ggctggatgactttaggatg	AM489415
	IFN-γ R4	cgttgaacagctggtccttg	
qRT-PCR Assays			
IFN1-Long	IFN1L 138F	cacgcgaagttattagcagttgaa	FJ184370
	IFN1L 253R	aaattatagttgaaccacaatgaaatattattc	
	IFN1L 164T	6′FAM-caaagctcgcgaatagcctattc tcgc-TAMRA	
IFN1-Short	IFN1S 24F	gcgaaacaaactgctatttacaatgtata	FJ184371
	IFN1S 119R	tcacagcaatgacacacgctc	
	IFN1S 58T	6′FAM-cagagctggagttgtatttttctta ttatttgcagtatgc-TAMRA	
IFN1-Total	IFNIT 131R	ttcttgaagtaccgtttcagtctcctct	FJ184370
	IFN1T 28F	ctggacgatttcctcaacattctagaa	FJ184371
	IFN1T 69T	6′FAM-ccttaattcctgtgtatcacctgccat gaaacc-TAMRA	
IFN2	IFN2 5F	aaagctaaaagcaaaataaacagctctt	FJ184369
	IFN2 107R	cagcaatgacacacactctgca	
	IFN2 47T	6′FAM-tgcagagttggacgtgtctttttcttat tctttg-TAMRA	
IFN3/4[a]	IFN3 270F	tggaggctatgcgatatgtgg	FJ184372
	IFN3 370R	acgatatatgacgttttggaacatgtt	
	IFN3 317T-MGB	MGB-6′FAM-tctgtcacgtggaacaa-NFQ	
IFN-γ1	IFN-γ1 299F	tcggccagatgctgaacc	FJ184374
	IFN-γ1 402R	cctccccaggaaatagtgtttc	
	IFN-γ1 333T-MGB	MGB-6′FAM-aatgattgagagtct gaaata-NFQ	
IFN-γ2	IFN-γ2 207F	gttgatgagtgtggttctggacg	FJ184375
	IFN-γ2 329R	ttctgggtctcctgaaccttcc	
	IFN-γ2 279T	6′FAM-agtgagggagaggctggaccag gtcaag-TAMRA	

[a] This assay was designed against IFN3 but does not discriminate between IFN3 and IFN4 (FJ184373).