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Salmonids Have an Extraordinary Complex Type I IFN System: Characterization of the IFN Locus in Rainbow Trout *Oncorhynchus mykiss* Reveals Two Novel IFN Subgroups

Jun Zou,* Bartolomeo Gorgoglione,*,†,[1] Nicholas G. H. Taylor,† Thitiya Summathed,* Po-Tsang Lee,* Akshaya Panigrahi,* Carine Genet,‡ Young-Mao Chen,§ Tzong-Yueh Chen,§ Mahmood Ul Hassan,¶ Sharif M. Mughal,‖ Pierre Boudinot,# and Christopher J. Secombes*

Fish type I IFNs are classified into two groups with two (group I) or four (group II) cysteines in the mature peptide and can be further divided into four subgroups, termed IFN-a, -b, -c, and -d. Salmonids possess all four subgroups, whereas other teleost species have one or more but not all groups. In this study, we have discovered two further subgroups (IFN-e and -f) in rainbow trout *Oncorhynchus mykiss* and analyzed the expression of all six subgroups in rainbow trout and brown trout *Salmo trutta*. In rainbow trout RTG-2 and RTS-11 cells, polyinosinic-polycytidylic acid stimulation resulted in early activation of IFN-d, whereas the IFN-e subgroup containing the highest number of members showed weak induction. In contrast with the cell lines, remarkable induction of IFN-a, -b, and -c was detected in primary head kidney leukocytes after polyinosinic-polycytidylic acid treatment, whereas a moderate increase of IFNs was observed after stimulation with resiquimod. Infection of brown trout with hemorrhagic septicemia virus resulted in early induction of IFN-d, -e, and -f and a marked increase of IFN-b and IFN-c expression in kidney and spleen. IFN transcripts were found to be strongly correlated with the viral burden and with marker genes of the IFN antiviral cascade. The results demonstrate that the IFN system of salmonids is far more complex than previously realized, and in-depth research is required to fully understand its regulation and function. *The Journal of Immunology*, 2014, 193: 000–000.

Teleost fish possess multiple type I IFNs to coordinate antiviral defenses (1–3). As in mammals, they are synthesized after viral infections and activate antiviral responses through cell-surface receptors. The IFN genes have been sequenced in many teleost species including Atlantic salmon (4, 5), carp (6), catfish (7), pufferfish (8), goldfish (9), sea bass (10), rainbow trout (11, 12), and zebrafish (12–15). In the last few years, whether fish IFNs are homologs to type I or III IFNs in mammals has been intensively debated, partly due to the fact that fish IFN genes share the same genomic organization with mammalian type III IFN genes, in which four phase 0 introns are present. However, it is now evident that this feature is not only a hallmark for type III IFN genes, and in the amphibian *Xenopus*, both type I and III IFN genes possess this arrangement of introns (16). Most recently, the crystal structures of zebrafish IFNs have been resolved and are shown to exhibit characteristic α helical structures resembling human type I IFNs (17). From an evolutionary perspective, it can be concluded that type I and III IFNs had diverged by the appearance of tetrapods, and the loss of introns in amniote type I IFN genes is likely due to a retrotransposition event (12).

Based on the cysteine patterns in the mature peptide, fish type I IFNs can be divided into two major groups, namely group I and II type I IFNs, with two or four cysteine residues forming one or two pairs of putative intramolecular disulphide bonds, respectively (12). Group I type I IFNs are present in all teleost species studied to date and appear to be fish specific. Group II type I IFNs are limited to salmonid, siluriform and cyprinid species (2, 15). Both groups exert similar antiviral functions but via distinct receptors, contrasting with the findings in mammals in which all type I IFNs share a common receptor complex consisting of IFN-α receptor (IFNAR) 1 and IFNAR2 (15, 18). Studies in zebrafish demonstrate

*Scottish Fish Immunology Research Centre, School of Biological Sciences, University of Aberdeen, Aberdeen AB24 2TZ, United Kingdom; †Centre for Environment, Fisheries and Aquaculture Science, Weymouth Laboratory, Weymouth, Dorset DT48 UB, United Kingdom; ‡Institut National de la Recherche Agronomique, Unité Mixte de Recherche 1313, Unité de Génétique Animale et Biologie Intégrative, Jouy-en-Josas Cedex 78352, France; §Institute of Biotechnology, National Cheng Kung University, Tainan 70101, Taiwan, Republic of China; ¶Zoology Department, Government College University, Lahore 54000, Pakistan; ‖Faculty of Fisheries and Wild Life, University of Veterinary and Animal Sciences, Lahore 54000, Pakistan; and #Institut National de la Recherche Agronomique, Unité de Virologie et Immunologie Moléculaires, Jouy-en-Josas Cedex 78352, France

[1]Current address: Clinical Division of Fish Medicine, University of Veterinary Medicine, Vienna, Austria.

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The sequences presented in this article have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers HF931021, HF931022, HF931030–HF931036, HF931023–HF931038, HF937126, HF937129, HF937130, and HF565489–HF565491.

Address correspondence and reprint requests to Dr. Jun Zou or Dr. Christopher J. Secombes, Scottish Fish Immunology Research Centre, School of Biological Sciences, University of Aberdeen, Aberdeen, AB24 2TZ, U.K. E-mail addresses: j.j.zou@abdn.ac.uk (J.Z.) or c.secombes@abdn.ac.uk (C.J.S.)

Abbreviations used in this article: BAC, bacterial artificial chromosome; CPE, cytopathic effect; CRFB, cytokine receptor family B; EF-1α, elongation factor 1α; EPC, *Epithelioma papillosum* of carp; GH, growth hormone; IFNAR, IFN-α receptor; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; LSD, least significant difference; Mx, Myxovirus resistance; ORF, open reading frame; poly I:C, polyinosinic-polycytidylic acid; PAMP, pathogen-associated molecular pattern; qPCR, quantitative real-time PCR; R848, resiquimod; SAV, salmon α virus; VHSV, viral hemorrhagic septicemia virus; VHSV-G, viral hemorrhagic septicemia virus glycoprotein; VIPERIN, virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible.

that group I (IFN1 and IFN4) and II type I IFNs (IFN2 and IFN3) activate antiviral responses through receptor complexes involving cytokine receptor family B (CRFB) 1/CRFB5 and CRFB2/CRFB5, respectively (15). The genes encoding the type I IFN receptors are shown to be clustered in the same chromosome and are believed to have arisen from a common ancestor by gene/genome duplication events (14).

Phylogenetically, teleost type I IFNs are classified into four subgroups, namely IFN-a, -b, -c, and -d (5, 11), with IFN-a and IFN-d making up the group I type I IFNs and IFN-b and IFN-c the group II IFNs (2, 5, 11). It should be noted that teleost IFN-a and -b are not orthologs of mammalian IFN-α and -β. All four teleost subgroups are present in the Protacanthopterygiian species, such as Atlantic salmon and rainbow trout, which are shown to have the highest number of type I IFN genes. In Atlantic salmon, 11 IFN genes were found in two contigs by screening of a bacterial artificial chromosome (BAC) library. The Ostariophysians, such as zebrafish, carp, and catfish, possess three of the subgroups, IFN-a, -c, and -d, whereas the Acanthopterygiians, such as bass and perch, possess a single subgroup (IFN-d) that can exist as multiple copies (6–10, 13–15).

Expressions of type I IFN subgroups have been relatively well studied, and patterns are diverse among fish species. In general, group I type I IFNs appear to be ubiquitously produced in most cell types and are inducible in response to viral infection and viral RNA analogs. In contrast, group II type I IFNs are constitutively expressed at a very low level, and their induced expression is detectable only in specific leukocyte populations. For example, trout IFN3/IFN-b1, a member of the group II IFNs (IFN-b subgroup), is not expressed in fibroblasts but is induced in head kidney leukocytes after simulation with polyinosinic-polycytidylic acid (poly I:C) (12). Further studies have confirmed that the IFN-bs and -cs are coexpressed by a specific type of leukocyte located in lymphoid tissues (19). Intriguingly, such expression profiles appear to mirror the expression patterns of IFN-α and IFN-β in mammals in which IFN-α is synthesized primarily by leukocytes, whereas IFN-β is made by virtually all cell types. Fish IFNs also respond differentially to TLR ligands. In TO cells, poly I:C, a ligand for fish TLR3, TLR22, RIG-I, and MDA5, significantly upregulates expression of IFN-a but surprisingly not other IFNs (19). In contrast, resiquimod (R848), a TLR7/8 ligand, induces strong expression of IFN-bs. Other ligands for host pattern recognition receptors such as PHA are also potent inducers of fish IFN genes (11). It is known in mammals that IFNs can be regulated through a positive-feedback loop, and such a mechanism is also conserved in fish in which recombinant trout IFN2 (an IFN-a member) strongly induces expression of itself and other IFN genes (11).

Fish group I and II type I IFNs share common antiviral activities. Recombinant proteins of IFN-a, -b, and -c produced in eukaryotic cells or bacteria are potent activators of cellular antiviral genes including Myxovirus resistance (Mx), virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible (VIPERIN), IFN-stimulated gene (ISG) 15, ISG58, protein kinase R, and eukaryotic initiation factor 2α and can protect the host against viral infection (12, 15, 19–22). For example, pretreatment of the salmon TO cells with rIFN-a, -b, or -c led to strong inhibition of the replication of infectious pancreatic necrosis virus and salmon α virus (SAV) and enhanced protection against the viruses (19, 22). Interestingly, IFN-as appear to protect fish at the early stage of SAV infection but are unable to block viral replication once the virus has established infection (22). In contrast, IFN-d has been found to have little or no antiviral activity in both zebrafish and Atlantic salmon (15, 19).

In this study, three BAC clones containing multiple type I IFN genes were obtained in rainbow trout *Oncorhynchus mykiss* and sequenced. This led to identification of 18 full-length genes and 21 partial sequences or pseudogenes and discovery of two novel phylogenetic subgroups (IFN-e and IFN-f) in addition to the four subgroups already known. Expression analysis revealed that the six IFN subgroups, including IFN-e and IFN-f, were differentially modulated in response to stimulation with poly I:C and in vivo during an effective immune response mounted by brown trout (*Salmo trutta*) fighting a viral infection. Our results demonstrate that salmonids have an IFN system that is far more complex than previously realized.

Materials and Methods

Sequence analysis

Three clones were obtained by screening of a rainbow trout BAC library using IFN gene probes (23). Nucleotide sequences of the BAC clones were analyzed for putative transcripts using the GenScan program (http://genes/mit.edu/GENSCAN.html). The identities of the predicted transcripts were confirmed by Blastp search using default parameters on the National Center for Biotechnology Information Web site (http://blast.ncbi.nlm.nih.gov). In the case in which partial IFN sequences were found, manual editing was performed by comparing the predicted amino acid sequences with known rainbow trout and Atlantic salmon (*S. salar*) IFN sequences (http://ca.expasy.org/tools). The presence of signal peptides was predicted by the SignalP program (version 3.0, http:www.cbs.dtu.dk/services/SignalP). A multiple alignment was generated using the CLUSTAL W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) program and subsequently applied to construct phylogenetic trees by the neighbor-joining method using Molecular Evolutionary Genetics Analysis software version 5.05 (http://www.megasoftware.net/). Homology between sequences was calculated using the Matrix Global Alignment Tool (Matgat, version 2.0) (24).

The fish IFN sequences retrieved from the databases for analysis included: *Carassius auratus* (caau): AAR20886; *Ctenopharyngodon idella* (ctid): DQ357216; *Cyprinus carpio* (cyca): BAG68521; *Danio rerio* (dare): NP_997523 (IFN1), NP_001104552 (IFN2), NP_001104553 (IFN3), NP_001155212 (IFN4); *Dicentrarchus labrax* (dila): CAQ17043; *Epinephelus coioides* (epco): KC495072; *Epinephelus septemfasciatus* (epse): BAJ79339; *Gasterosteus aculeatus* (gaac): CAM31706 (IFN1); *Hyporthodus septemfasciatus* (hyse): BAJ79339; *Ictalurus punctatus* (icpu): NP_001187180 (IFN), NP_001187228 (IFN2); *Paralichthys olivaceus* (paol): AET71736; *Oncorhynchus mykiss* (onmy): CAM28541 (IFN-a1/IFN1), NP_001153977 (IFN-a2/IFN2), NP_001153974 (IFN-b1/IFN3), NP_001158515 (IFN-b2/IFN4), NP_001152811 (IFN-d1/IFN5); *Oryzias latipes* (orla): CAM32419; *Oreochromis niloticus* (orni): XP_003448784 (IFN1), XP_003453450 (IFN2); *S. salar* (sasa): NP_001117182 (IFN-a1), NP_001117042 (IFN-a2), ACE75687 (IFN-a3), ACE75691 (IFN-b1), ACE75693 (IFN-b2), ACE75689 (IFN-b3), ACE75692 (IFN-c1), ACE75693 (IFN-c2), ACE75688 (IFN-c3); EG884136 (IFN-d1); *Sparus aurata* (spau): CAP72359; *Takifugu rubripes* (taru): CAM82751; and *Tetraodon nigroviridis* (teni): CAD67762.

For prediction of the possible binding sites of the immune-related transcription factors in the putative IFN promoters, a region of 2 kb preceding the translation initiation codon (ATG) was analyzed by the MatInspector program (http://www.genomatix.de/). Only the binding sites on the forward strand with a cutoff of >0.800 were recorded.

To confirm the presence of IFN-e and IFN-f in other salmonid species, an analysis of a member of the Salmo genus was performed, namely the brown trout (*S. trutta*). Based on the trout sequences, IFN primers (Table I) were synthesized to amplify IFN-e, IFN-f, and other IFN genes in *S. trutta*. The PCR products were sequenced, and their phylogenetic relationship with Atlantic salmon and trout molecules was determined (data not shown).

Analysis of IFN gene expression in rainbow trout

Primers were designed to amplify the total transcript level of each subgroup and tested for quantitative real-time PCR (qPCR) analysis based on the genomic sequences from the BAC clones (Table I). The PCR products amplified were confirmed by sequencing. To determine if the new IFN genes discovered were inducible following stimulation with viral pathogen-associated molecular patterns (PAMPs), a trout fibroblast cell line (RTG-2) and macrophage cell line (RTS-11) were incubated with the synthetic dsRNA poly I:C (Sigma-Aldrich). RTG-2 cells were maintained in L-15 medium supplemented with 10% FBS (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin at 20°C. Two days before stimulation, cells were plated into fresh flasks at a density of 5×10^6 cells/25-cm^2 flask. The cells were then stimulated with poly I:C dissolved in PBS for 0.5, 2, 6, 24, and 72 h at a dose of 100 μg/ml, known to induce

IFN expression in previous studies (12). Control groups were treated with an equal volume of PBS buffer. For IFN-a2/IFN2 stimulation of RTS-11 cells, the cells with incubated with 20 ng/ml recombinant trout IFN-a2/IFN2 or protein elution buffer (control) for 6 h (12). After stimulation, media was removed from the flasks, and 1 ml TRIzol reagent (Invitrogen) was added to lyse the cells by pipetting up and down the cell lysate several times. The cell lysates were transferred to 1.5-ml microcentrifuge tubes for RNA extraction according to the manufacturer's instructions. Three replicate flasks of cells were used for each treatment. For analysis of gene expression in RTS-11 cells, the cells were maintained in L-15 medium supplemented with 30% FBS and penicillin/streptomycin and stimulated with poly I:C as for RTG-2 cells. For preparation of primary head kidney cells, the head kidney tissue was collected from three freshly killed rainbow trout under sterile conditions and gently pushed through a 100-μm nylon mesh with ice-cold L-15 medium containing 0.1% heparin (12). The cells were washed twice with L-15 medium and cultured in L-15 medium supplemented with 10% FBS and penicillin/streptomycin at 20°C in the presence or absence of poly I:C. For R848 treatment, R848 (Sigma-Aldrich) was dissolved in DMSO and added to the culture medium at a final concentration of 10 μg/ml. The control was treated with an equal volume of DMSO.

The extracted total RNA from each sample was treated with RNase-free DNase I (Fermentas) and reverse transcribed into cDNA using a first-strand cDNA synthesis kit (Fermentas) according to the manufacturer's instructions. The synthesized cDNA was diluted with TE buffer (10 mM Tris and 1 mM EDTA [pH 8]) and kept at −20°C for qPCR analysis.

The primers used for qPCR analysis are listed in Table I. The primers for detecting expression of the IFN-a subgroup cannot discriminate the alternatively spliced transcript variants, but amplify the overall transcript level of IFN-a members. qPCR was performed on a LightCycler 480 II (Roche) using the following program: 1 cycle of 95°C for 10 min; 45 cycles of 95°C for 5 s, 63°C for 15 s, 72°C for 30 s, and 80°C for 5 s for fluorescent detection. A standard curve was established using a series of 10-fold dilutions of the purified PCR products of the corresponding amplicons. The expression data of individual genes was normalized to that of the reference gene elongation factor 1α (EF-1α). Fold change of gene expression level was then obtained by comparing the normalized gene expression level of treated groups with the appropriate untreated groups (defined as 1). The data were statistically analyzed using the one-way analysis of covariance method and a post hoc least significant difference (LSD) test, as described previously (25).

Analysis of gene expression in brown trout during viral hemorrhagic septicemia infection

To gain an insight into the relative expression of the six IFN subgroups during viral infection, fish exposed to viral hemorrhagic septicemia virus (VHSV) were analyzed. Due to the availability of pathogen-free brown trout, ideal for pathogen-exposure trials, the work in this study was performed with this species. This necessitated the development of primers for subgroups a–d in this species, as done for IFN-e and IFN-f above. The brown trout were hatched and reared under pathogen-free conditions, certified by introducing disinfected eggs to a recirculating system sterilized by biofilters (Centre for Environment, Fisheries and Aquaculture Science, Weymouth, U.K.). Fish were kept on a 12-h day light/dark photoperiod with 30-min dusk and dawn and fed with a standard trout diet at 1% bodyweight/d. The persistence of the pathogen-free condition was assessed by standard bacteriological, virological, parasitological, and routine histological screening techniques carried out on randomly sampled fish prior to the prechallenge acclimation.

One hundred fifty-five brown trout (50–100 g) were randomly transferred to six 30L tanks, with three replicate tanks allocated for each treatment group. Five days prior to challenge, the temperature was adjusted to 12°C to increase fish susceptibility to the virus. Fish were bath challenged for 4 h at 5.56×10^5 $TCID_{50}$/ml using a suspension of VHSV-Ia, isolate J167 (26). Viral titration was performed as described previously on monolayers of *Epithelioma papillosum* of carp (EPC) cells by recording the appearance of cytopathic effects (CPE) (27). Control fish were mock challenged with the sterile medium used to suspend the virus.

Five fish from each tank (three control and three challenged tanks) were killed at days 1, 3, 7, and 14 post–viral/sham exposures. Kidney and spleen tissues were dissected and incubated in RNAlater (Ambion) for 24 h at 4°C before storing at −80°C. The VHSV infection on sampled fish was confirmed at the individual level, and the viral burden assessed by recording of the CPE for EPC cells using pools of tissues known to be the main target organs for VHSV, including brain, heart, kidney, spleen (data not shown), and viral genes.

Kidney and spleen tissues (~100 mg) were used for total RNA extraction as described above, without DNase treatment. Five micrograms total RNA was reverse transcribed using Bioscript reverse transcriptase (Bioline), and the resulting cDNA volume adjusted with TE buffer (10 mM Tris and 1 mM EDTA [pH 8]) and stored at −20°C prior to qPCR analysis. Individual cDNA samples were screened to confirm VHSV presence using primers targeting the VHSV transmembrane glycoprotein (G) gene (F/R-1028) as described previously (28). Only VHSV-positive samples were selected for gene expression analysis; VHSV was not detectable in any of the unexposed fish. qPCR was performed on a LightCycler480 (Roche) using the primers listed in Table I. Transcript concentrations were calculated using a standard curve created specifically for each gene, normalized to brown trout EF-1α (HF563594), and the fold change for the gene expression data calculated and analyzed as described above. To study the involvement of the IFN pathway, the expression of IFN regulatory factor (IRF) 3 and IRF7 were studied, as key genes involved in the IFN cascade. To study the production of key effectors of the antiviral response we also studied the transcription of Mx and VIPERIN proteins as key ISGs. Parametric correlation analysis between individual viral burden, assessed by detecting the VHSV glycoprotein (VHSV-G) expression (by RT-qPCR) in kidney and spleen cDNA to the expression of each type I IFN gene during the course of VHSV infection, was also carried out. In addition, general linear model analysis (analysis of covariance) was used to assess the significance of the factorial interaction between days post–VHSV infection (time) and the individual viral burden.

Results

Gene identification and subgroup assignment

Three BAC clones of ~116, 142, and 149 kb were obtained from library screening and analyzed for the presence of IFN genes using bioinformatics tools. This led to the identification of 39 partial or complete copies of IFN genes (Fig. 1). Among them, 18 copies encoded complete open reading frames (ORFs) of IFN genes, whereas the rest were partial or pseudogenes. The complete IFN ORFs are deposited under accession numbers HF931021–38 (Table II). In addition to the IFN genes, the three BAC clones contained numerous transposon elements separating the IFN genes but interestingly no other functional genes were found. Clone RT292E06 contained 17 IFN genes, including 9 full-length IFN ORFs belonging to the IFN-a, -b, -c, and -f subgroups (see below for assignment to subgroups), respectively. Clone RT282J16 had 10 IFN genes with 4 full-length members of the IFN-e subgroup (IFN-e2, -e3, -e4, and -e5) and 1 full-length member of the IFN-a subgroup (IFN-a3). Clone RT303F02 had eight partial/pseudo-IFN genes, three full-length IFN-e subgroup members, and one IFN-f. All of the full-length IFN ORFs were predicted to be interrupted by four phase 0 introns, the same genomic organization shared by all known teleost type I IFN genes. The ORFs of some members of the IFN-e and IFN-f subgroups, including IFN-e1, IFN-e2, IFN-e5–e7, and IFN-f1 (deposited under GenBank accession numbers: HF931030, HF931032, and HF932034–HF931036) were sequenced by cDNA cloning, confirming the intron/exon organization of these genes predicted by the GenScan program.

To analyze the evolutionary relationship of the newly identified rainbow trout IFNs with other known fish molecules, a phylogenetic tree was constructed with the full-length ORFs using the neighbor-joining method (Fig. 2). To our surprise, the tree clearly displayed two novel phylogenetic IFN subgroups, which we term IFN-e and -f. The IFN-e subgroup consists of seven full-length ORFs, four of which are located in clone RT282J16 and three in clone RT303F02. This subgroup belongs to the teleost group I type I IFN subfamily with two conserved cysteine residues seen in the predicted mature peptide. Moreover, within the IFN-e subgroup, two further separate clades are apparent, containing IFN-e1–e4 and IFN-e5–e7, respectively. Regarding the IFN-f subgroup, two members were found in two different BAC clones (RT292E06 and RT303F02). The two IFN-f proteins contain four cysteine residues in the mature peptide and are thus members of the teleost group II type I IFN subfamily. It is likely

Table I. Primers used for qPCR analysis

Gene	Primer Name	Forward Primer (5′–3′)	Primer Name	Reverse Primer (5′–3′)
Rainbow trout				
EF-1α	EF1a-F	CAAGGATATCCGTGGCA	EF1a-R	ACAGCGAAACGACCAAGAG
IFN-a	IFNa-F	GATGCTGAGTTTGAGGACAAAGTC	IFNa-R	GTTTCATGGCAGGTGATACACAGGA
IFN-b	IFNb-F	TTTACAAGAACACAGAGGGTGAGGA	IFNb-R	ACACCGCCCACAACACATAAC
IFN-c	IFNc-F	GGCGAATCACAGTTGAGCAGCA	IFNc-R	TCCACACTGCCCATCATACATTTGC
IFN-d	IFNd-F	TGATGGCCTTCAGTCATGTGTTAG	IFNd-R	TGCATGAGCACTGTATGCCATTCT
IFN-e	IFNe-F	GTCGATCATTTCAAGGATGACGA	IFNe-R	AGCTCTTATAGCCTTTACACATTGG
IFN-f	IFNf-F	CAACGCTTTCAAAAGAGGAGGTGGA	IFNf-R	GCAGACGCATGTCTCTGACACATTC
Brown trout				
EF-1α	EF1α-F	CAAGGATATCCGTCGTGGCA	EF1α-R	ACAGCGAAACGACCAAGAGG
VHSV-Gp	VHSV G F-1028	CTCATTTCTCCTCTCAAAGTTTCG	VHSV G R-1028	CCGTCTGTGTTGTTGTCTACC
IFN-a	IFNa-054F	CTGTTTGATGGGAATATGAAATCTGC	IFNa-054R	CCTGTGCACTGTAGTTCATTTTTCTCAG
IFN-b	IFNb-056F	CTGCTCTCAGATATGGGTGGAATCT	IFNb-056R	CACCGCCTACGACGCATAACTC
IFN-c	IFNc-1F	GGCAGCAGTGGTGCTAAGGCTAT	IFNc-R	ATCCACACTGCCCATCATACA
IFN-d	IFNd-F1	GGACCTGTACAAGCAAGCTGAAT	IFNd-R3	GCACTGTATGCCATTCTTTTAAGG
IFN-e	IFNe-F1	GAGCTGGACCAATGCGTAAAGG	IFNe-R1	ATGTGTTTCAGCACCACGTTC
IFN-f	IFNf-F1	GACCTATTCGGAATGTGTGAGA	IFNf-R1	TGATGCTCCCCATTTCAGCT
IRF3	IRF3-145F	ACTGGTCATGGTCGAGGTGGT	IRF3-145R	CACAAGTCCATCATCTCCTGCAG
IRF7	IRF7-F1	CTGCCTGCCGCCCACTCATCT	IRF7-R1	TTGGGGCATCTTCCTGGGTTCG
Mx	MX-1F1	CCTCCTGAAATCAGCGAAGAC	MX-1R1	GAGTCTGAAGCATCTCCCTCTG
VIPERIN	Vig1-F	AGAACTCAACCCTGTACGCTGGA	Vig1-R	GGCAATCCAGGAAACGCATATATTC

that the IFN-f subgroup possesses more members because several incomplete IFN-f ORFs were found in the BAC clones (data not shown). To verify that the IFN-e and IFN-f genes were present in other salmonid species, primers based on the rainbow trout genes were used to amplify the corresponding genes from brown trout, a species within the Salmo genus. The sequences confirmed that these subgroups were present in this species and likely present in all salmonids (data not shown). The sequences are deposited in GenBank with accession numbers: IFN-e1, HF937128; IFN-e5, HF937129; and IFN-f1, HF937130 (http://www.ncbi.nlm.nih.gov/genbank).

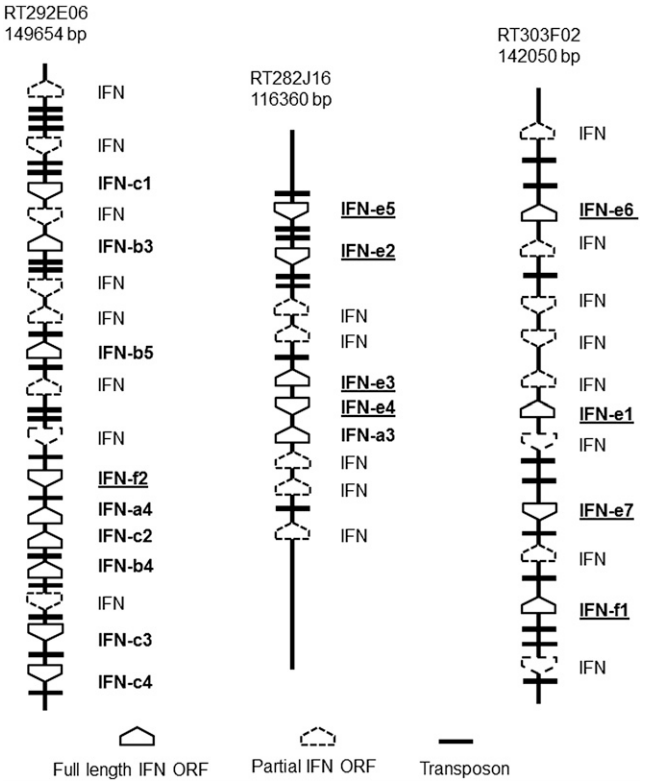

FIGURE 1. Diagram showing the location of IFN ORFs and partial/pseudo-ORFs in three rainbow trout BAC clones. The IFN-e and IFN-f are underlined, and names of the genes represented by full-length ORFs are in boldface.

The present study shows that the trout group I type I IFNs consist of three subgroups: IFN-a, -d, and -e. The trout IFN-a subgroup has at least four members, including two reported previously (referred to as IFN-a1 and -a2) and two in BAC clones RT292E06 and RT282J16 (referred to as IFN-a3 and -a4). It should be noted that IFN-a3 and trout IFN-a1/IFN1 have a single amino acid difference, but their intron sequences are more divergent. Both molecules branch with the group containing salmon IFN-a1 and -a2. Trout IFN-a4 forms a clade with salmon IFN-a3 with a bootstrap value of 76%, indicating they are evolutionarily related. The only member of the IFN-d subgroup (IFN5/IFN-d1) (11, 19) is not present in the BAC clones. The IFN-e subgroup is the largest subgroup with at least seven members, and surprisingly, all of these IFN molecules have been missed in previous studies.

Similarly, the trout group II type I IFNs also comprise three subgroups: IFN-b, -c, and -f. The IFN-b subgroup has five members, including the previously reported trout IFN3 (IFN-b1) and IFN4 (IFN-b2) and three additional molecules found in the current study (IFN-b3, -b4, and -b5). The IFN3 (IFN-b1) and -4(IFN-b2) genes were not found in the BAC clones, whereas the other three genes are located in BAC clone RT292E06, with -b3 separated from -b5 by two partial IFN ORFs. The IFN-c subgroup recently reported in Atlantic salmon (5) is also present in rainbow trout, with four copies found in BAC clone RT292E06. The IFN-f subgroup consists of two members, located in BAC clones RT292E06 and RT303F02. Like the IFN-b subgroup that is found only in salmonids, IFN-f has not been reported in any nonsalmonid fish to date.

Sequence analysis

The amino acid homology of the full-length rainbow trout proteins was analyzed using the Matgat program (Table III). Within each subgroup, the sequence identity between members was high, ranging from 65.2–97.8% aa identity. In contrast, the intergroup sequence identity is rather low, ranging from 18.5–39.7%. The highest identity was seen between subgroups -a and -d, which are both group I IFNs. Subgroup -e molecules also had relatively high identity to subgroup -d. Within the group II subgroups, -b and -c had relatively high identity, but subgroup -f members were highly divergent from all the subgroups.

Table II. Rainbow trout and brown trout type I IFNs, accession numbers, BAC location, and size

Group	GenBank Accession No.	BAC Location	Precursor (aa)	Mature Peptide (aa)
Rainbow trout				
Group I type I IFN				
IFN-a1 (IFN1)	CAM28541	NA	175	152
IFN-a2 (IFN2)	NP_001153977	NA	175	154
IFN-a3	HF931021	RT282J16	175	152
IFN-a4	HF931022	RT292E06	175	152
IFN-d1 (IFN5)	NP_001152811	NA	178	159
IFN-e1	HF931030	RT303F02	186	164
IFN-e2	HF931031	RT282J16	186	164
IFN-e3	HF931032	RT282J16	186	164
IFN-e4	HF931033	RT282J16	181	159
IFN-e5	HF931034	RT282J16	186	164
IFN-e6	HF931035	RT303F02	186	164
IFN-e7	HF931036	RT303F02	186	164
Group II type I IFN				
IFN-b1 (IFN3)	NP_001153974	NA	184	164
IFN-b2 (IFN4)	NP_001158515	NA	183	163
IFN-b3	HF931023	RT292E06	180	160
IFN-b4	HF931024	RT292E06	183	163
IFN-b5	HF931025	RT292E06	187	167
IFN-c1	HF931026	RT292E06	187	167
IFN-c2	HF931027	RT292E06	186	166
IFN-c3	HF931028	RT292E06	187	167
IFN-c4	HF931029	RT292E06	196	176
IFN-f1	HF931037	RT303F02	177	153
IFN-f2	HF931038	RT292E06	184	160
Brown trout (partial sequences)				
IFN-a	HF565489			
IFN-b	HF565491			
IFN-c	HF937129			
IFN-d	HF565490			
IFN-e	HF937126			
IFN-f	HF937130			

NA, not available.

The predicted mature peptide sequences of trout type I IFNs were aligned with that of zebrafish IFNs for which the crystal structure has been determined (Fig. 3) (17). The six α-helices are arranged in an antiparallel bundle manner to form the core structure in human IFN-α2, and these regions are well conserved in fish IFNs (17, 29, 30), although the primary sequences of type I IFNs share limited homology among the subgroups (data not shown). Among the six helices, helix F is the most conserved and is one of the two key regions shown to interact with the receptor chain IFNAR2 in humans (31). The two cysteine residues (C1 and C3) forming a putative intramolecular disulphide bond in both group I and II IFNs in fish reside at the N-terminal region preceding helix A and the C terminus of helix D, respectively. The other two cysteine residues (C2 and C4) that form another intramolecular disulphide bond in group II IFNs are located within the AB loop (C2) and in the region adjacent to the N terminus of helix F, respectively (Fig. 3). Subgroup -e molecules have a 5-aa insertion (RK, T, L, P, and R/P/K) upstream of helix A relative to the IFN-a and IFN-d proteins, and the DE loop is longer than in the other two subgroups. In the group II molecules, it is apparent that different subgroups have a variable number of amino acid residues upstream of C1, with one in IFN-f, three in IFN-b, and four in IFN-c. In addition, there is a 4-aa deletion in the IFN-c molecules just downstream of C1 and a ≥4-aa insertion between helices B and C. Relative to the other group II molecules, the IFN-f sequences have two small deletions at the N terminus, the second in the AB loop, and curiously, the alanine in the CAWE motif in helix F is replaced by a serine. The amino acid residues involved in interaction with the receptors have been extensively analyzed in human IFN-α2 where helices A and E (equivalent to helix F in zebrafish IFNs), and the AB loop, are known to bind to the high-affinity binding receptor IFNAR2, whereas helices B and C form the core structure that engages the low-affinity receptor IFNAR1 (32). Interestingly, the corresponding regions of IFN subgroups show little sequence conservation (data not shown).

Analysis of the regulatory elements in the IFN promoter

Analysis of the 5′ flanking regions was undertaken to see if presence of the transcription factors in the IFN promoters gives any clues to the regulation of gene expression. In our previous studies, the IFN-d gene promoter was previously shown to contain multiple binding sites for several transcription factors such as IRFs, STATs, and NF-κB (11). The regions analyzed with the MatInspector program included 2 kb of the 5′ flanking sequence of the translation initiation codon (ATG) from the IFN genes available in the BAC clones. The promoter regions of IFN-e5, -e6, and -e7 are highly homologous with identity of >95.5%, and hence, only the IFN-e5 promoter was selected for analysis. IFN-a3 was not analyzed because it had only 1073 bp available in the 5′ flanking region. Fig. 4 shows the patterns of the transcription factors are remarkably different among individual genes. The canonical TATA box required for initiation of transcription of most genes in vertebrates is present in the majority of the IFN genes except for the IFN-c (1–3) genes. IFN synthesis is known to be tightly regulated via the signaling pathways involving the IRFs and NF-κB after sensing of viral PAMPs, and the JAK/STAT pathway controls the cellular actions triggered by IFNs. The IRF binding sites can be found in all of the genes excluding IFN-c2, whereas the NF-κB binding sites are seen in some of the IFN genes including IFN-a4, -b4, -c4, -c5, -e1, -e2, -e3, -e4, and -e5.

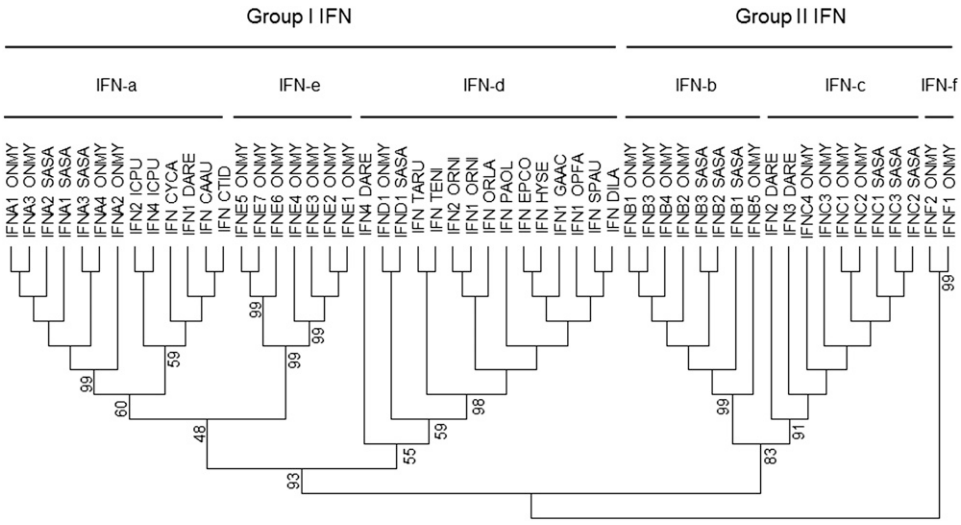

FIGURE 2. Phylogenetic tree analysis of fish type I IFN family members. Full-length IFN protein sequences were used for construction of the tree by the neighbor-joining method within the Mega4 program using the following parameters: pairwise deletion for gaps and missing data, Poisson correction for amino acid substitutions, and uniform rates among sites. The tree was bootstrapped 10,000 times, and the bootstrap values of the major branches are shown as percentages. CAAU, *Carassius auratu*; CTID, Ctenopharyngodon idella; CYCA, *Cyprinus carpio*; DARE, *Danio rerio*; DILA, *Dicentrarchus labrax*; EPCO, *Epinephelus coioides*; GAAC, *Gasterosteus aculeatus*; HYSE, *Hyporthodus septemfasciatus*; ICPU, Ictalurus punctatus; ONMY, *Oncorhynchus mykiss*; OPFA, *Oplegnathus fasciatus*; ORLA, *Oryzias latipes*; ORNI, *Oreochromis niloticus*; PAOL, *Paralichthys olivaceus*; SASA, *Salmo salar*; SPAU, *Sparus aurata*; TARU, *Takifugu rubripes*; and TENI, *Tetraodon nigroviridis*.

Interestingly, multiple STAT binding sites are predicted mainly in group II type I IFN gene promoters. Furthermore, myeloid and lymphoid lineage-related transcription factors including members of the GFI, GATA, PAX, and NFAT family could also be identified in the IFN gene promoters. For example, the IFN-f2 promoter contains two binding sites for GFI1 and PAX5, which are expressed in myeloid cells and early B cells, respectively.

Expression kinetics of IFN subgroups in RTG-2, RTS-11, and head kidney cells

To test the primers for detection of gene expression within each subgroup (Table I), multiple sequence alignment was performed to search for a region with few mismatches among subgroup members. In contrast, one of the two primers was selected to span two exons to ensure that contamination of genomic DNA in RNA preparation can be excluded from PCR amplification. To verify whether the primers were able to amplify multiple members within each subgroup, sequencing of the PCR products derived from HK cells stimulated with poly I:C for 6 h revealed that the transcripts of multiple IFN members were amplified from the same pair of primers and that the primers were subgroup specific as expected (Table IV).

It has been shown that the previously known IFN subgroups (IFN-a, -b, -c, and -d) are induced after poly I:C stimulation or viral infection in salmonids (5, 11, 12, 19, 22). In the current study, expression of all six IFN subgroups was examined initially in two trout cell lines, RTG-2 and RTS-11, and primary head kidney leukocytes in response to stimulation with poly I:C, R848, or IFN-a2. In unstimulated cells, group I IFNs, especially IFN-a and -d, were constitutively expressed, whereas transcripts of group II IFNs were hardly detectable (Fig. 5A), in line with previous studies that group II IFNs are produced by limited leukocyte populations (12, 19). Poly I:C stimulation resulted in a marked induction of group I IFN transcripts in both RTG-2 (Fig. 5B) and RTS-11 (Fig. 5C) cells after 2 h stimulation, with a 5–15-fold increase seen for IFN-a and -d, and had no effect on IFN-b and -c expression. However, unlike the other two members of group II IFN subgroup (IFN-b and -c), IFN-f was upregulated by poly I:C in RTG-2 and RTS-11cells. It is worth noting that IFN-d is the earliest inducible IFN gene in response to poly I:C, with increased transcripts detected at 0.5 h after stimulation. In contrast to the gene expression changes in RTG-2 and RTS-11 cells, head kidney leukocytes were more responsive to poly I:C treatment, with all six subgroups upregulated 2 h after stimulation (Fig. 5D). However, IFN-a, -b, and -c exhibited by far the greatest induction, with >100-fold increase of expression. In contrast, stimulation of head kidney leukocytes with R848, an agonist of TLR7/8, resulted in much weaker induction of all of the IFN subgroups with no change of expression for IFN-e (Fig. 5E). It is interesting that in RTS-11 cells, the IFN-e and IFN-f genes did not respond to stimulation with IFN-a2/IFN2 which was shown to induce IFN-a and IFN-d expression in RTG-2 cells (11).

Table III. Amino acid identity (%) among rainbow trout IFN subgroups

	IFN-a	IFN-b	IFN-c	IFN-d	IFN-e	IFN-f
IFN-a	**75.7–97.1**	28.4–31.9	25.4–29.2	36.5–39.7	26.4–30.2	21.8–25.5
IFN-b		**95.1–97.8**	30.6–35.7	31.4–32.6	19.8–23	24.7–27.7
IFN-c			**84.0–94.1**	27.4–28.7	19.8–23.8	20.6–23.7
IFN-d				**100**	31.4–33.2	21.9–22.8
IFN-e					**65.2–97.8**	18.5–20.2
IFN-f						**75**

Identity among members of each subgroup is shown in bold.

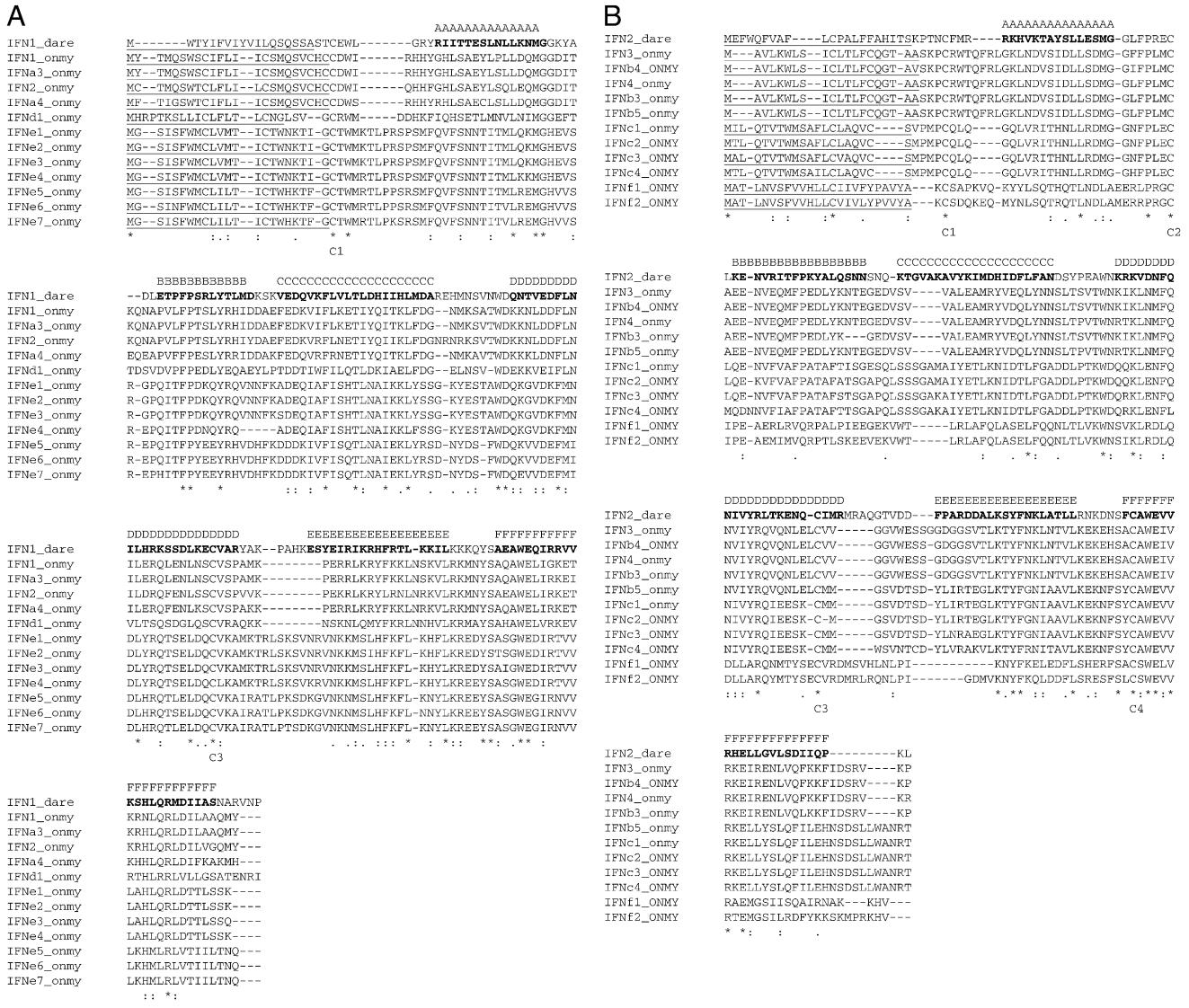

FIGURE 3. Multiple alignment of rainbow trout group I (**A**) and II (**B**) IFNs. The signal peptide (underlined) sequences of trout IFNs were predicted using the SignalP program. Identical amino acids among all sequences are indicated by asterisks, whereas those with high or low similarity are indicated by a colon (:) and period (.), respectively. The α helices (a–f) of zebrafish IFNs are indicated in boldface. Conserved cysteine residues are marked and numbered.

Expression of IFNs in brown trout during VHSV infection

To directly study the impact of viral infection on IFN subgroup expression, a viral challenge of brown trout was undertaken, due to the availability of pathogen-free stock for this experiment. Initially, primers to the IFN subgroups a–d were developed to allow analysis of all six subgroups. The amplicons obtained by PCR were sequenced, deposited in GenBank (with accession numbers: IFN-a, HF565489; IFN-b, HF565490; IFN-c, HF937126; and IFN-d, HF565491), and their relationships with rainbow trout and Atlantic salmon molecules verified by phylogenetic analysis as for the brown trout IFN-e and IFN-f genes above (data not shown). In healthy tissues, relatively high levels of constitutive expression of group I type I IFNs (IFN-a, -d, and -e) were detected when compared with that of group II IFNs (IFN-b, -c, and -f). The IFN-a subgroup showed the highest constitutive expression among all of the subgroups in all tissues screened (Fig. 6). VHSV infection was quantified in sampled brown trout tissues by viral titration on the EPC cell line, with CPE apparent from 3 d postinfection. qPCR detection of the VHSV-G protein was apparent earlier, by 1 d postinfection, but peaked at day 3 in both kidney and spleen (Fig. 7A, 7B). VHS resulted in differential expression kinetics of type I IFN subgroups in brown trout kidney and spleen (Fig. 8). Among the group I IFNs, IFN-d and -e exhibited similar patterns with a moderate fold change and significant induction only detectable during the early stages of the infection (days 1 and 3) and mostly in the kidney (Fig. 8D, 8E). In contrast, IFN-a exhibited a different pattern; it was activated from day 3 and the induction was sustained for up to day 14 (the last time point analyzed), although its expression level had drastically decreased at this time (Fig. 8A). Among the group II IFNs, a remarkable increase of IFN-b and IFN-c transcripts (IFN-c peaked at 1934-fold in spleen and 1623-fold in kidney) was detected in both kidney and spleen at days 3 and 7, with an expression pattern comparable to IFN-a (Fig. 8B, 8C). A unique induction profile was seen for IFN-f transcripts, which, specifically in the kidney, increased early (day 1) and continued to increase up to day 7 (Fig. 8F). Finally, genes known to regulate expression of the type I IFN genes, such as IRF3 and IRF7, and the ISGs Mx and VIPERIN were found to be strongly induced from days 3 until 14 in both kidney and spleen (Fig. 9).

Correlation analysis confirmed, although limited to transcriptomic data, a strong relationship between the expression of both

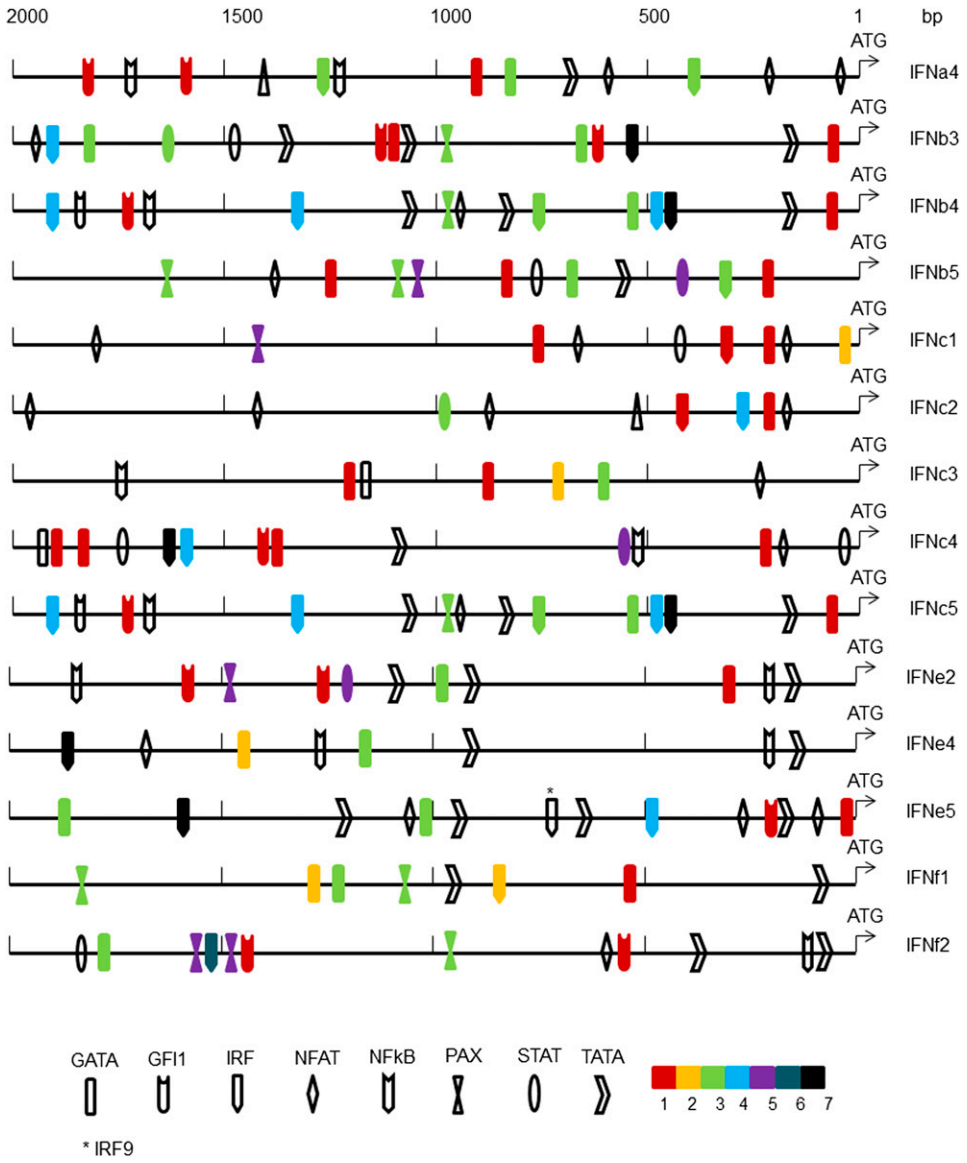

FIGURE 4. Analysis of the binding sites of immune-related transcription factors in the 2-kb sequences of the 5′ flanking region preceding the translation initiation start (ATG) of rainbow trout were analyzed by the MatInspector program (http://www.genomatix.de/), and the predicted motifs for immune-related transcription factors in the forward strand with scores with >0.80 are shown. The color indicates the isoform present.

type I IFN groups and viral burden at an individual level. IFN-a, -b, and -c were all significantly correlated with VHSV burden in both tissues studied, with IFN-c being the most strongly correlated (r = 0.907 and 0.916 in kidney and spleen, respectively). However, IFN-d, -e, and -f showed significant correlations only in the kidney, in agreement with the fold change analysis. To study the clinical progression of the disease, time postinfection was used as a measure. Several IFN gene transcripts were positively modulated during the progression of the infection, but only IFN-a, -c, and -d showed a time-dependent modulation in both kidney and spleen (Table V). Interestingly, IFN-b, which showed a large upregulation from day 3 (Fig. 8B) and strong correlation with viral burden in both tissues, was not significantly influenced by disease progression (i.e., was modulated in a time-independent manner) (Table V). Correlation analysis also showed some strong relationships between the expression of the IFNs and genes involved in the antiviral IFN pathway. IRF3 and IRF7 were strongly correlated with the expression of both type I IFN groups but only in kidney for IFN-d, -e, and -f. ISGs showed the same pattern of strong correlations with the expression of brown trout IFNs, with VIPERIN having the strongest correlation with IFN-c (r = 0.928 and 0.902 in kidney and spleen, respectively). Once again, a significant correlation of IRFs and ISGs with expression of IFN-d, -e, and -f was significant only in kidney tissue.

Table IV. Sequence confirmation of IFNs amplified by qPCR in trout HK cells

	1	2	3	4	5	6	7
IFN-a	4	3	7	ND			
IFN-b	10 (1 and 4)	3 (2 and 5)	0	10 (1 and 4)	3 (2 and 5)		
IFN-c	13	2 (2 and 3)	2 (2 and 3)	ND			
IFN-e	ND	ND	ND	ND	6 (5 and 6)	6 (5 and 6)	8
IFN-f	ND	13					

Numbers in parentheses indicate the IFN genes are identical in the region amplified by qPCR.
ND, not detected.

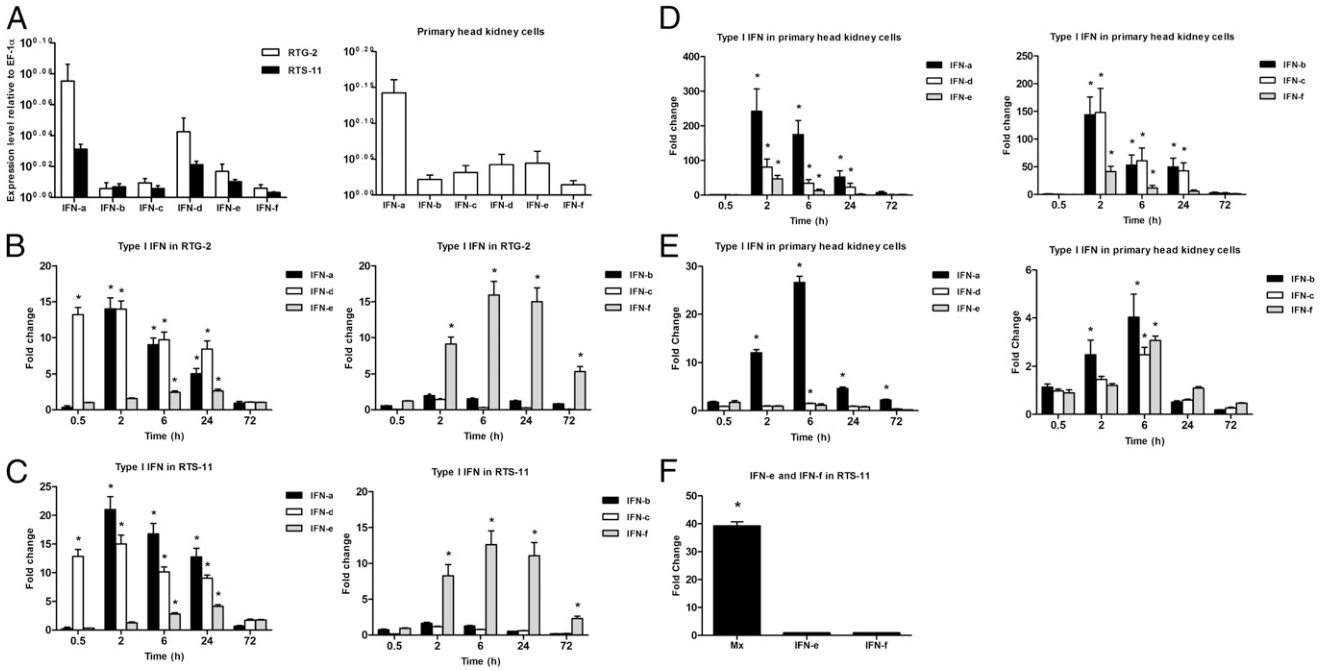

FIGURE 5. Expression of trout IFN subgroups in RTG-2 cells, RTS-11 cells, and primary head kidney leukocytes. (**A**) Constitutive expression in control cells. The RTG-2 cells (**B**), RTS-11 cells (**C**), and primary head kidney leukocytes (**D**) were stimulated with 100 μg/ml poly I:C and used for qPCR analysis. In addition, primary head kidney leukocytes (**E**) were treated with 10 μg/ml R848 and the RTS-11 cells (**F**) with trout IFN-a2/IFN2 for 6 h. Gene expression levels of the IFN genes were normalized to that of EF-1α and the normalized expression levels compared between the treated groups and the appropriate control group (which was set to 1) to obtain the relative fold changes. The data are presented as group means ± SEM of three independent flasks of cells or cells from three fish. $*p < 0.05$.

Discussion

The teleost fish type I IFN family is classified into two groups based on the cysteine patterns in the mature peptide, with group I and II containing either two or four cysteines, respectively (12). Recent studies have shown that they can be further divided into four phylogenetic subgroups, termed IFN-a, -b, -c, and -d (5, 11). In the current study, two additional novel IFN subgroups (termed IFN-e and IFN-f) were identified in rainbow trout by screening of a BAC library. The IFN-e subgroup consists of seven members and belongs to the group I IFNs, whereas two IFN-fs were identified and are group II IFNs. The six subgroups of IFN genes are regulated differently, as seen in the differential expression in cultured cells following stimulation and in lymphoid tissues during viral infection. Analysis of the IFN gene promoters revealed marked differences in the regulatory elements for immune-related transcription factors, giving a clue to the different signaling pathways used to induce these genes. Overall, our data demonstrate that the salmonid IFN system is more complex than previously realized.

The present study demonstrates that salmonids have three subgroups of group I IFNs (IFN-a, -d, and -e) and three subgroups of group II IFNs (IFN-b, -c, and -f). It is believed that type I IFNs evolved from an ancestral molecule containing four cysteines and appeared during evolution of the Gnathostomes (jawed vertebrates) and RAG-mediated adaptive immunity (8, 12, 33). Although it is unclear if the complexity of the salmonid IFN system is unique in the teleosts, it is apparent that some of these subgroups were present in early teleosts and may have been lost in certain teleost lineages during evolution. For example, the Ostariophysians (e.g., zebrafish, carp, and catfish) have three subgroups (IFN-a, -c, and -d) (12, 14, 15). In stark contrast, the Acanthopterygians possess a single subgroup (IFN-d) (10, 34). Because Ostariophysians are quite basal teleosts (35), it suggests that at least subgroups -a and -c have been lost in the Acanthopterygians. Also, our identification of the IFN-e and IFN-f subgroups has allowed a reanalysis of the IFN genes present in other teleost groups. In the Atlantic cod (*Gadus morhua*), a Paracanthopterygian species, we found three IFN genes in the genome (accession numbers CAEA01552993, CAEA01421839, and CAEA01011836), two of which appear to be members of the IFN-f subgroup and one a member of the IFN-a subgroup (J. Zou, unpublished observations). Hence, IFN-f may have been lost from some teleost groups as well.

The IFN-e molecules are closely related to the IFN-a molecules, suggesting that they may have diverged from the -a subgroup much later in evolution, perhaps in the salmonid lineage. Similarly, with regard to the group II IFNs, a common ancestral gene may have given rise to the IFN-f subgroup, and the progenitor of IFN-bs and IFN-cs, which segregated later, again potentially in salmonids. This notion is supported by the fact that IFN-bs and IFN-cs are clustered in the salmon and trout genome (5) and reside in the same locus with the growth hormone (GH) gene 1. Similar gene synteny with the GH1 gene is also seen in zebrafish and Chinook salmon and appears to be conserved more generally in teleosts, cartilaginous fish, and coelacanth (15, 36). Salmonids are thought to have undergone an extra whole-genome duplication event compared with other teleost species and often have multiple paralogous genes. For example, an additional GH paralogue (GH2) exists and is located in a different chromosomal locus that exhibits little conservation of the functional genes with the GH1 locus and, to date, no clear linkage to IFN genes (36). One of the notable characteristic features of the fish IFN/GH1 locus is that it is transposon rich, which may contribute to the instability of the region and could be the driving force for frequent insertions of duplicated IFN genes, expanding subgroup number, or giving rise to new subgroups locally. However, whether the newly discovered IFN genes in BAC clones RT282J16 and RT303F02 are located in

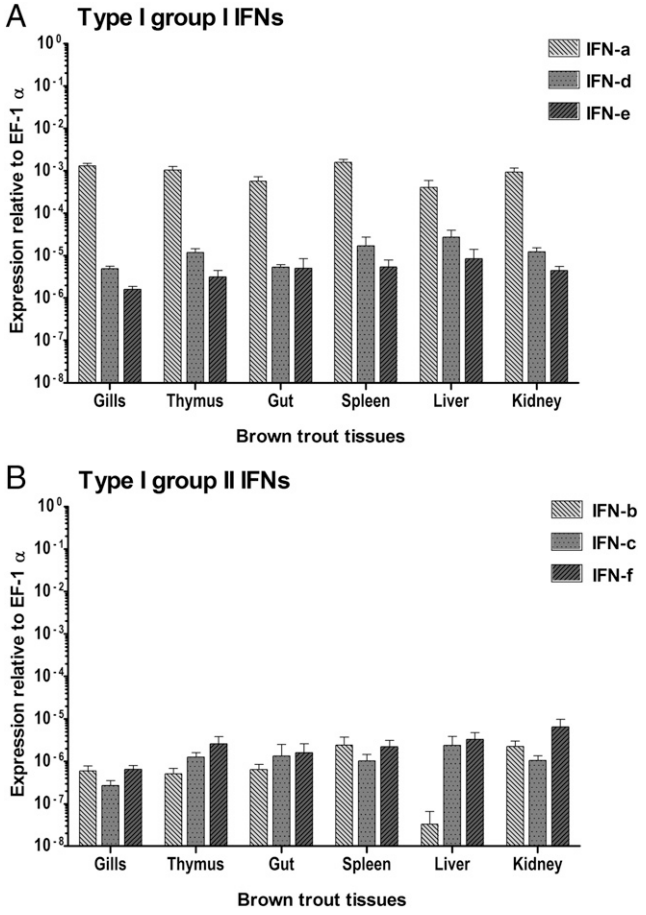

FIGURE 6. Constitutive expression profiles of type I IFN genes in brown trout detected by qPCR. (**A**) Type I group I IFNs: IFN-a, IFN-d, and IFN-e. (**B**) Type I group II IFNs: IFN-b, IFN-c, and IFN-f. Tissues including gills, thymus, gut, spleen, liver, and kidney were sampled from six healthy fish. Transcript concentrations were calculated using a standard curve specifically obtained for each primer pair. Data are normalized to the expression of a reference gene EF-1α and presented as group means ± SEM.

a region associated with the GH1 or GH2 gene or an unrelated locus remains to be determined. It must be noted that zebrafish IFNφ4/IFNd1 is on chromosome 12 and is not linked to other IFN genes or to the GH gene (15).

One interesting question to be answered is why salmonids have retained so many type I IFNs in their immune system, somewhat similar to the situation in mammals, as seen on chromosome 9 in humans where >20 type I IFN genes/ pseudogenes are present. Although it is not clear whether all IFN molecules found in the genome are functional, evidence gathered to date suggests all subgroups are expressed and some are induced in cells/tissues in response to viral infection or immune stimulation, indicating they are involved in activating host antiviral immunity (2, 3, 19, 22). Nevertheless, many IFN pseudogenes also exist, as evidenced by the finding that incomplete IFN sequences are found in the trout BAC clones in the current study. This is typical of the birth-and-death model of gene family evolution in which duplication events combined with pseudogenization-deletion of individual members contributes to the rapid diversification of gene families (37). It would be of interest to find out whether the complexity of the salmonid IFN system is linked in any way to their complicated life cycle. Some salmonid species live in freshwater and seawater environments at different life stages, where they may be exposed to different pathogens. Furthermore, adaptation to a freshwater or seawater (e.g., smoltification) environment causes considerable stress and physiological changes, which could have a profound impact on the immune system. Several studies have already shown that the salmon immune system is compromised during smoltification, and fish are particularly vulnerable to infections as early as postsmolts (38, 39).

The sequence homology between the trout IFN subgroups is relatively low, ranging from 18.5–39.7% aa identity. This is perhaps not surprising because low homology exists between IFN-α and β in humans. However, despite divergent primary sequences, the IFN proteins appear to be structurally conserved in vertebrates, as shown in a recent study using the x-ray diffraction approach in which both zebrafish group I and II IFNs were found to have a similar α-helical topology to human type I IFNs. Human IFN-α (containing four cysteine residues) and -β (containing two cysteine residues) share a common receptor, and both activate antiviral gene expression via the JAK/STAT pathway (40). This feature contrasts with the finding in zebrafish that the two cysteine-containing group I and four cysteine-containing group II IFNs use distinct receptors to trigger cellular responses (15). One of the IFN receptors (IFNAR) in zebrafish, termed CRFB5/IFNAR1, is shared by both group I and II IFNs, whereas CRFB1/IFNAR2-1 and

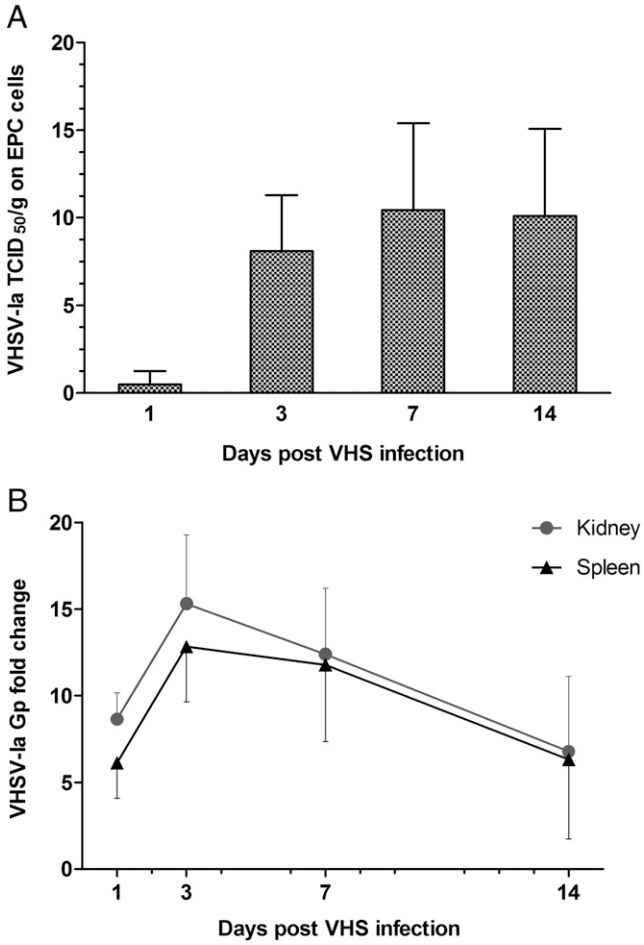

FIGURE 7. Viral detection of VHSV in experimentally infected brown trout (*S. trutta*). Fish were challenged with VHSV (isolate J167) at 5.56×10^5 $TCID_{50}$/ml. (**A**) Viral titration of CPE on EPC cells using pooled organs (brain, heart, kidney, and spleen). (**B**) qPCR detection of VHSV-Ia transmembrane glycoprotein (primers based on JN180851) from kidney and spleen. Data are presented as group means + SEM (A) or ± SEM (B). The VHSV expression was calculated by normalizing the results to the expression of the brown trout reference gene EF-1α.

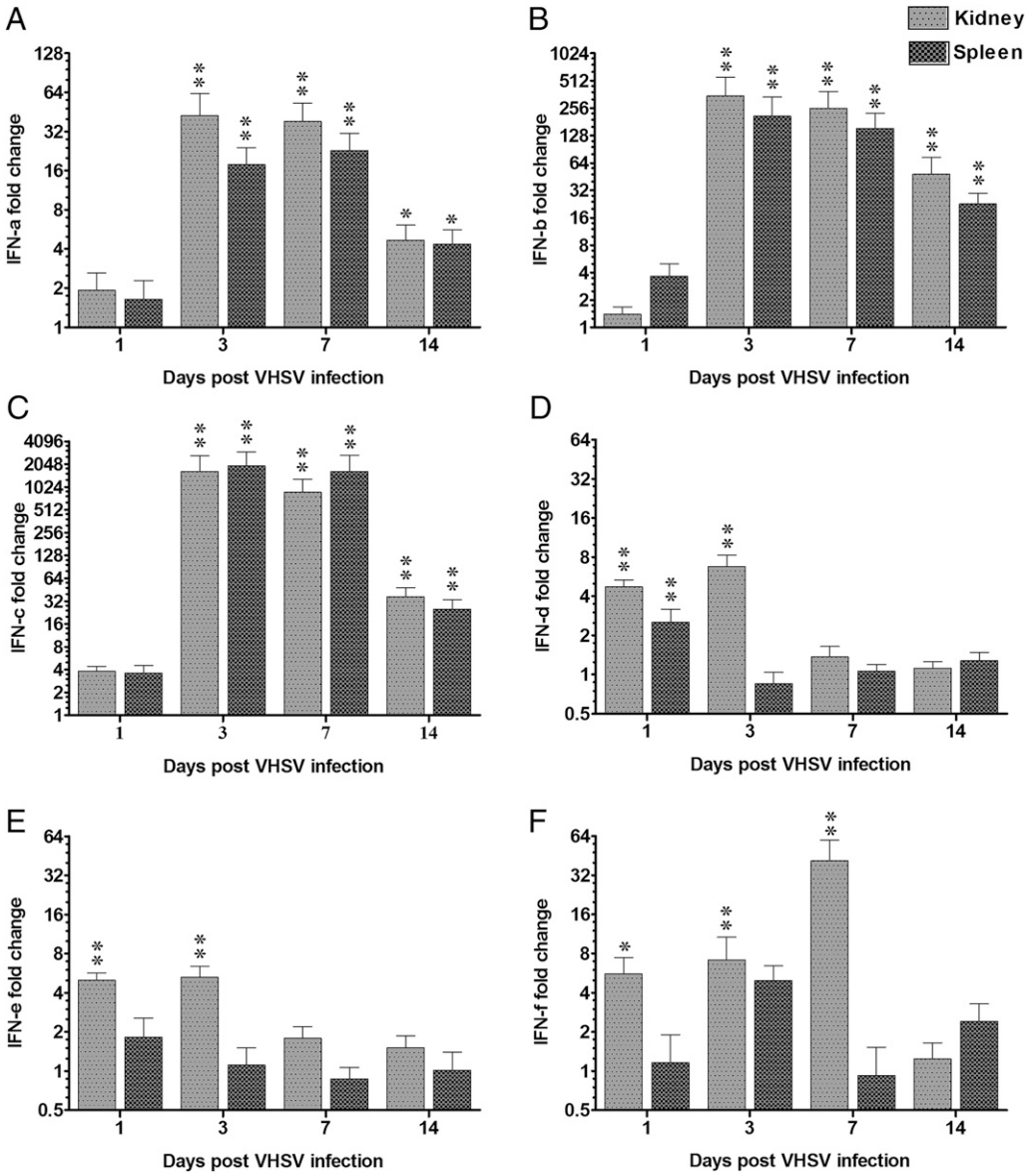

FIGURE 8. Expression kinetics profiles of type I IFNs in kidney and spleen of brown trout following bath challenge with VHSV. (**A**) IFN-a. (**B**) IFN-b. (**C**) IFN-c. (**D**) IFN-d. (**E**) IFN-e. (**F**) IFN-f. qPCR-detected transcript levels were normalized to the expression of a reference gene, EF-1α, and presented as group means + SEM. The p value of an LSD post hoc test after a one way-ANOVA between the VHSV-infected group and the corresponding control (not shown in the graph) is shown above the bars as $*p < 0.05$, $**p < 0.01$.

CRFB2/IFNAR2-2 show ligand specificity, interacting with group I and II IFNs, respectively. Intriguingly, in some fish lineages (e.g., tetraodon and fugu) in which only the group I IFN subgroup (IFN-d) is present, three receptors homologous to zebrafish CRFB1/IFNAR2-1 and CRFB2/IFNAR2-2 exist (8, 14, 15). One possible explanation for this is that fish IFNs can bind to different receptor complexes, perhaps with different affinity. The receptors of type I IFNs have yet to be fully characterized in salmonids (41). The diversity of the IFNs now known to exist in salmonids will undoubtedly add to the complexity in elucidating the interaction of IFN ligands with their cognate receptors and the elicited cellular responses in this group of fish.

The expression patterns of type I IFNs in fish are beginning to emerge. Group I IFNs appear to be constitutively expressed in most cell types and in fish tissues, as shown in the current study in which the expression levels of the three group I (IFN-a, -d, and -e) subgroups are much higher than group II IFNs in both kidney and spleen in brown trout (Fig. 6). This suggests that group I IFNs (at least IFN-a) play an important role in maintaining a constant antiviral state of host cells ready to defend against viral infection and may also act as the first wave of IFNs to amplify the IFN response. Previous work demonstrates that group I IFNs are upregulated in response to viral infection or stimulation with viral PAMPs, mimicking the expression pattern of human IFN-β, which is ubiquitously expressed, although they are not authentic orthologs of human IFN-β (5, 11, 12, 19, 42). The IFN-d expression and function remain controversial (10, 11, 19, 34). Our previous studies have shown that IFN-d is inducible in trout RTG-2 cells by a variety of viral PAMPs and rIFNs and in salmon TO cells following infection with SAV (11). However, a recent study found that IFN-d was not upregulated in fish tissues and in TO cells following stimulation with viral PAMPs such as poly I:C (a known ligand of TLR3, TLR22, RIG-I, and MDA5 in fish) and R848 (19), and no antiviral activity was detected for the rIFN-d. In Acanthopterygians such as rock bream *Oplegnathus fasciatus* and sea bass *Dicentrarchus labrax*, which possess only the IFN-d

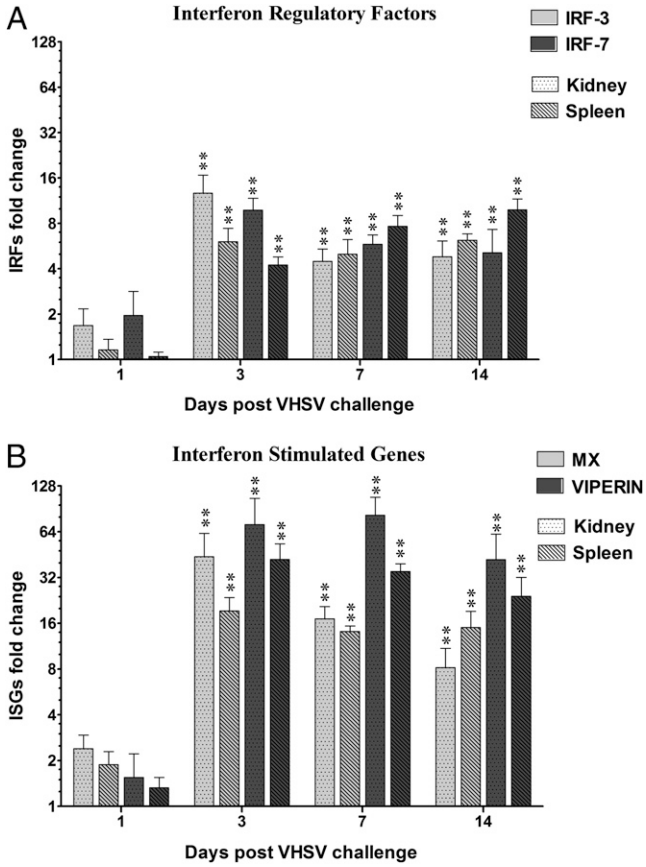

FIGURE 9. Expression kinetics of the immune genes involved in type I IFN response in brown trout kidney and spleen following bath challenge with VHSV. (**A**) IRF3 and IRF7. (**B**) ISGs: Mx and VIPERIN. Fish were challenged with VHSV (isolate J167) at 5.56×10^5 $TCID_{50}$/ml. qPCR-detected transcript levels were normalized to the expression of a reference gene, EF-1α, and presented as group means ± SEM. The p value of an LSD post hoc test after a one-way ANOVA between the VHSV-infected group and the corresponding control (not shown in the graph) is shown above the bars as $^{**}p < 0.01$.

subgroup, a typical IFN response to bacterial and viral PAMPs is seen, and the recombinant rock bream IFN-d protein was able to trigger antiviral gene expression (10, 34). Our results that IFN-d expression increased several fold in brown trout kidney and spleen at day 1 after VSHV infection (Fig. 8) are in agreement with the above observations. However, it must be noted that regulation of type I IFN expression is complex, the cell types and treatments differ in the reported studies, and hence, further investigations are required to draw firm conclusions (10, 11, 19, 20, 34).

With regard to the group II IFNs, some genes, if not all, are shown to be produced in limited cell (leukocyte) types (19). Thus, trout IFN-b1/IFN3 transcripts are not detectable in RTG-2 cells or RTS-11 cells after stimulation with poly I:C but significant induction is seen in head kidney leukocytes (12). Transcripts of salmon IFN-b increased markedly in primary head kidney leukocytes after stimulation with poly I:C but curiously not in head kidney–derived TO cells treated in this way (5, 43). Differential expression of the newly discovered trout IFN-es and IFN-fs was also found in the current study, in which the IFN-f expression increased 10–15-fold in both cell lines after stimulation with poly I:C (Fig. 5) (44–47). This contrasts with much weaker induction of IFN-es after treatment with either poly I:C or R848. In VHSV-challenged brown trout, the magnitude of IFN transcript increase was markedly

Table V. Correlation analysis between type I IFN gene expression during VHSV infection in brown trout (*S. trutta*) and the expression of IRFs (IRF3 and IRF7) and ISGs (Mx and VIPERIN)

Correlations	Analysis		Gene Versus VHSV Burden		Time versus Burden		IRF3		IRF7		Mx		VIPERIN	
			Kidney	Spleen	Kidney	Spleen	Kidney	Spleen	Kidney	Spleen	Kidney	Spleen	Kidney	Spleen
Type I, group I (two cysteines)	IFN-a	Pearson correlation	0.873**	0.896**	0.007	0.035	0.828**	0.723**	0.722**	0.641**	0.832**	0.812**	0.894**	0.858**
		Significance (two -tailed)	0.000	0.000			0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	IFN-d	Pearson correlation	0.585**	0.080	0.001	0.025	0.527**	0.191	0.420**	0.114	0.354*	0.057	0.408**	0.043
		Significance (two -tailed)	0.000	0.598			0.000	0.203	0.004	0.449	0.016	0.707	0.005	0.778
	IFN-e	Pearson correlation	0.612**	−0.039	0.027	0.287	0.513**	0.025	0.456**	-0.038	0.399**	−0.077	0.474**	−0.050
		Significance (two-tailed)	0.000	0.796			0.000	0.867	0.001	0.800	0.006	0.613	0.001	0.739
Type I, group II (four cysteines)	IFN-b	Pearson correlation	0.816**	0.841**	0.229	0.610	0.729**	0.706**	0.684**	0.729**	0.799**	0.857**	0.813**	0.854**
		Significance (two-tailed)	0.000	0.000			0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	IFN-c	Pearson correlation	0.907**	0.916**	0.001	0.001	0.785**	0.687**	0.724**	0.689**	0.886**	0.874**	0.928**	0.902**
		Significance (two-tailed)	0.000	0.000			0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	IFN-f	Pearson correlation	0.740**	0.140	0.032	0.512	0.547**	−0.068	0.458**	−0.126	0.569**	0.093	0.676**	0.097
		Significance (two-tailed)	0.000	0.355			0.000	0.652	0.001	0.403	0.000	0.539	0.000	0.520

Time versus burden is p value based on Fisher F-distributions using general linear model analysis (analysis of covariance) on the gene expression to assess the significance of the interaction between days postinfection with the effect of individual pathogen burden as covariate.
$^{*}p < 0.05$, $^{**}p < 0.01$.

different between the subgroups, with an extraordinary elevation of IFN-b and -c expression in both kidney and spleen (Fig. 8B, 8C), in agreement with the observation that high IFN-b/IFN-c–producing cells are present in fish lymphoid tissues (19). However, it was IFN-c that had the strongest correlation with viral load and ISG expression. Interestingly, the correlation analysis also confirmed that IFN-d, -e, and -f only had a significant correlation with viral load in the kidney, confirming their restricted site of expression and probable cell source.

Distinct expression kinetics of the IFN subgroups in lymphoid organs after viral infection was also apparent in the current study. In general, IFN-d, -e, and -f were early responders to VHSV infection, whereas the most responsive genes (IFN-a, -b, and -c) were activated later (Fig. 8A–C). It is probable that the early induction of IFN-d, -e, and -f (at day 1) is facilitated by the activation of the constitutively expressed IRF proteins or by an IRF3/IRF7-independent pathway because induced expression of IRF3 and IRF7 occurred later (at day 3) (Fig. 8). In humans, IFN-α4 and IFN-β have been described as the early responsive IFN genes following activation of TLR3 by viral PAMPs (48, 49). Interestingly, constitutive expression of IRF3 but not IRF7 has been reported in fish cultured cells and tissues, and the signaling pathway regulating IFN production by IRFs seems to be evolutionary conserved in vertebrates (50–52). It has been suggested that fish IRF3 and IRF7 may play distinct roles in mediating expression of the IFN subgroups. For example, salmon IRF3 has been confirmed as one of the main regulators of IFN-a1 production, whereas IRF7 plays a lesser role (53).

The differential responsiveness of trout IFN genes to viral PAMPs and infection may be explained by the distinct regulatory elements predicted in the gene promoters. The binding sites for immune-related transcription factors (such as NF-κB, IRF3, and IRF7), known to be instrumental for activation of IFN genes (51, 53), differ considerably among the individual genes (Fig. 4). The gene promoters containing IRF3, IRF7, or NF-κB binding sites are likely to respond to signaling pathways that activate these molecules. Thus, the IFN-a4, -b4, -b5, -c5, -e1, and e3 genes may be regulated preferentially by IRF3, IFN-b3, -b4, -c4, -c5, -e1, -e3, -e4, and -e5 genes by IRF7 and IFN-a4, -b4, -c3, -c4, -c5, -e1, -e2, -e3, -e4, and -f2 genes by NF-κB, respectively. It has been well established that type I IFNs induce expression of hundreds of ISGs, including the IFN genes themselves, through a STAT1/2-dependent pathway in both mammals and fish (9, 18, 54). Thus, the genes containing STAT1 and STAT2 binding sites such as IFN-b3, -b5, -c1, and -c4 could be the potential targets for the early responsive IFNs. Remarkably, this coincides with the late and high response of IFN-b and IFN-c in lymphoid organs of fish infected with virus (Fig. 7). It is also noteworthy that the binding sites for IRF3, IRF7, and NF-κB are also predicted in the IFN-d gene promoter (11, 33). Furthermore, the IFN-b and IFN-c genes that are upregulated specifically in lymphocytes and lymphoid organs contain binding sites for leukocyte lineage transcription factors such as GATA3 for T cells, PAX5 for B cells, and GFI1 for myeloid/dendritic cells. Intriguingly, a PAX5 binding site is present only in the 5′ flanking region of group II IFN genes. Some fish IFN-a genes use alternative transcription start sites. This process producing different protein isoforms with or without a leader peptide was reported for rainbow trout IFN-a1/IFN1 (41, 42) and was regulated during responses to viruses. This mechanism brings an additional level of complexity to the control of fish type I IFN genes. Further experimental characterization of the role of these mechanisms will help clarify the control of IFN production in fish and characterize their immune functions in antiviral defense.

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Disclosures

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