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# Identification and Bioactivities of IFN- $\gamma$ in Rainbow Trout *Oncorhynchus mykiss*: The First Th1-Type Cytokine Characterized Functionally in Fish<sup>1</sup>

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IFN- $\gamma$  is one of the key cytokines in defining Th1 immune responses. In this study, an IFN- $\gamma$  homologue has been identified in rainbow trout *Oncorhynchus mykiss*, and its biological activities have been characterized. The trout IFN- $\gamma$  cDNA is 1034 bp in length and translates into a 180-aa protein. The first intron of the trout IFN- $\gamma$  gene contains highly polymorphic GACA mini-satellites and 44-bp DNA repeats, giving rise to at least six alleles. IFN- $\gamma$  is structurally conserved among vertebrates, and a signature motif has been identified. A nuclear localization sequence known to be crucial for IFN- $\gamma$  biological activities is also present in the C-terminal region of the trout IFN- $\gamma$ . The IFN- $\gamma$  expression was induced in head kidney leukocytes by stimulation with PHA or poly(I:C) and in kidney and spleen of fish injected with poly(I:C). rIFN- $\gamma$  produced in *Escherichia coli* significantly stimulated gene expression of IFN- $\gamma$ -inducible protein 10 ( $\gamma$ IP-10), MHC class II  $\beta$ -chain, and STAT1, and enhanced respiratory burst activity in macrophages. Deletion of 29-aa residues from the C terminus containing the nuclear localization sequence motif resulted in loss of activity with respect to induction of  $\gamma$ IP-10 in RTS-11 cells. Moreover, IFN- $\gamma$ -induced  $\gamma$ IP-10 expression was completely abolished by the protein kinase C inhibitor staurosporine, and partially reduced by U0126, a specific inhibitor for ERKs. Taken together, the present study has demonstrated for the first time a functional IFN- $\gamma$  homologue in a fish species, strongly suggesting a conserved Th1 immune response is most likely present in lower vertebrates. *The Journal of Immunology*, 2005, 175: 2484–2494.

Thelper cell 1 (Th1) and Th2 immune responses are elicited by differential synthesis of cytokines in activated CD4<sup>+</sup> cell subsets during pathogen infection. Generally, Th1 cytokines consisting of IL-2, IFN- $\gamma$ , and TNF- $\beta$  orchestrate host defense against intracellular pathogens by activating macrophages, enhancing Ag presentation, and inducing T cell differentiation. In contrast, Th2 cytokines, including IL-4, IL-5, IL-10, and IL-13, mainly coordinate immune responses against extracellular pathogens by enhancing Ab production (1).

Whether lower vertebrates possess Th1 immune responses similar to those in mammals has been a matter of debate for comparative immunologists during the last decade. Evidence gathered in recent years has demonstrated that cell-mediated immune responses are present in lower vertebrates such as fish (2). Many of the key molecules involved in Ag processing and presentation, e.g., MHC class I and II (3, 4), and T cell surface markers such as TCRs (5) and CD8 (6), have also been found in fish. More re-

cently, some of the key cytokines that are known to drive the Th1 response, notably IL-12 and IL-18, have been cloned in fish (7, 8), suggesting CD4 T cells and CD4 subsets may be present in the fish immune system.

IFN- $\gamma$  belongs to the IFN family and is the only type II IFN. It is mainly synthesized and secreted by Th1 cells and NK cells and acts on many cell types, including macrophages, T cells, and NK cells, regulating both innate and cell-mediated immune responses (9). Its active form, a noncovalent homodimer, binds to a receptor complex containing IFN- $\gamma$  receptor R1 and R2, which activates the intracellular JAK/STAT signal pathway to initiate expression of a variety of genes in the nucleus (10). Notably, it drives macrophages to produce toxic agents such as reactive oxygen intermediates and reactive nitrogen intermediates to kill intracellular bacteria, and exerts antiviral activities through inducing the synthesis of a number of host antiviral proteins such as 2',5'-oligoadenylate synthetase, dsRNA-dependent protein kinase PKR, guanylate-binding protein, and adenosine deaminase (10). IFN- $\gamma$  also regulates T cell proliferation and differentiation, mediates leukocyte trafficking, and enhances Ag presentation (10).

IFN- $\gamma$  proteins share low homology among different species, e.g., 50.3% between human and murine and 43.7% between human and chicken (11), accounting for the strict species specificity of biological functions that has been observed. This low homology has contributed to the failure to isolate IFN- $\gamma$  homologues from lower vertebrates during the last decade. By analyzing gene synteny between the *Fugu* and human genome, we have recently discovered a *Fugu* IFN- $\gamma$  homologue, the first IFN- $\gamma$  sequence in a jawed fish (12). Although expected low sequence homology was seen when comparing with other known IFN- $\gamma$  molecules, *Fugu* IFN- $\gamma$  shares some common features such as an identical gene organization, conserved gene synteny in the chromosome, and a

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Table I. *Primer sequences and their use*

Primer Name	Sequence (5'–3')	Use
asF1	AGTTACCGTACACCGATTGAG	IFN- $\gamma$ gene cloning
asR1	TCTGGTTCAGCATCTGGTTG	
F1	CAAGGACCAGCTGTTCAACG	
F2	GACACACCTGCAGAACCTGTG	
R1	CCTGGGCTAAATAGGTCAAG	
R2	CGTGTCTTGGGGAAAACAG	
RF1	GGGATCCGCTCAGTTCACATCAATTAAC	
RR1	CAAGCTTCTACATGATGTGTGATTGAG	
RR3	CAAGCTTATTCTCGTAGATGGTAATGAACTCGG	
M13F	CGTTGTAAAACGACGGCCAG	
M13R	ACACAGGAAACAGCTATGAC	IFN- $\gamma$ polymorphism
F5	GGCTGGATGACTTTAGGATG	
R4	CGTTGAACAGCTGGTCTTG	
Actin-F	ATGGAAGATGAAATCGCC	$\beta$ -actin expression
Actin-R	TGCCAGATCTTCTCCATG	
EF1	GTGAGCAGAGGGTGTGATG	IFN- $\gamma$ expression
ER1	ATGTGTACAATGCAGAGTTG	
IP10-F2	CTGAGCTGTCCATCCAGACTC	$\gamma$ -IP-10 expression
IP10-R1	CTCAATGTGAAGAGGTTTAACACG	
MHCII $\beta$ -F1	AACCGTGGTCAAGAGGTGGTTC	MHCII $\beta$ expression
MHCII $\beta$ -R1	GAGCACCCCAAGAAGACTT	
Mx-F1	ATGCCACCCTACAGGAGATGAT	Mx expression
Mx-R1	CCACAGTGTACATTTAGTTG	
STAT1-F1	GCTCCCGGAATTCAACTATCAACT	STAT1 expression
STAT1-R1	TGCAGGAGGGCTCAGGAAGA	
ELF-F	CCCCCTCAGGACGTTTACAAA	ELF expression by real-time PCR
ELF-R	CACACGGCCACAGGTACA	
ELF probe	6-FAM-ATCGGTGGTA TTGGAAC-MGB	IFN- $\gamma$ expression by real-time PCR
IFN $\gamma$ -F	GCTGTTCAACGAAAACCTGTTT	
IFN $\gamma$ -R	TCACTGTCTCAACGTG	
IFN $\gamma$ probe	6-FAM-GTCCAGAACCACACTCATCCA	
IP10-F	CCAGCCACTCCTGCAGGAACAT	$\gamma$ -IP-10 expression by real-time PCR
IP10-R	ACACTTCTTCCCTTCTCCGTTCTT	
IP10 probe	6-FAM-CAGAGTGACAATGATCTCC	

predicted 6  $\alpha$ -helix structure of the translated protein. Taking advantage of the *Fugu* IFN- $\gamma$  sequence, in this study we describe the identification of the IFN- $\gamma$  gene from another fish species, rainbow trout (*Oncorhynchus mykiss*), whose immune system is relatively well studied, and have characterized the biological activity of the recombinant protein using classical assays in vitro. Involvement of intracellular kinases in IFN- $\gamma$  signal transduction has also been investigated using selective kinase inhibitors.

## Materials and Methods

### Fish

Rainbow trout *O. mykiss* (100–200 g) were purchased from a local Scottish fish farm (Almond Bank) and maintained in 1-m-diameter fiberglass tanks supplied with recirculating freshwater at 9–12°C. Fish were fed twice daily with a commercial pelleted trout diet. Three days before challenge, fish were transferred to a pathogen containment facility. Fish were anesthetized with benzocaine (25  $\mu$ g per liter; BDH) before injection or sacrifice for tissue collection.

### Preparation of primary cultured leukocytes and maintenance of cell lines

The head kidney was collected under sterile conditions from freshly killed rainbow trout and gently pushed through a 100- $\mu$ m nylon mesh (J. Stanier, United Kingdom) with ice-cold Leibovitz medium (L-15; Invitrogen Life Technologies) containing 10 U/ml heparin (Sigma-Aldrich). After washing with L-15 medium, cells were resuspended in L-15 medium containing antibiotics (100  $\mu$ g/ml penicillin and 100 U/ml streptomycin (P/S));<sup>3</sup>

Invitrogen Life Technologies) and cultured at 20°C in the presence or absence of stimulants.

A rainbow trout macrophage cell line RTS-11 was maintained in L-15 medium containing 30% FCS (Labtech International) and antibiotics (P/S) at 20°C (13). Cells were passaged to fresh flasks at 80% confluence and cultured for 2 days before stimulation in the presence of 30% FCS.

### Cloning

The PCR products were ligated into pGEM T Easy (Promega), and the ligation reaction was transformed into *Escherichia coli* tyrosine-based activation motif competent cells (ActivMotif). Subsequently, plasmid DNA of positive clones was extracted using a Qiagen plasmid miniprep kit (Qiagen) and sequenced by MWG Biotec.

### Sequence analysis

BLAST was used for identification of homologous sequences in the GenBank databases. A multiple alignment was generated using the CLUSTAL W program (version 1.83) (14). Direct comparison of two sequences was performed using the GAP analysis program within the Wisconsin Genetics Computer Group Sequence Analysis Software Package (version 10.0) (15). Prediction of a signal peptide was made using the SignalP program (version 2.0) (16). Modeling of the three-dimensional (3D) structure was performed using the 3D-psm program (17). The hydrophobicity of the human and trout IFN- $\gamma$  was analyzed by the Kyte-Doolittle method (18).

### Extraction of genomic DNA and analysis of polymorphism

Genomic DNA was extracted from tail fin samples using a phenol/chloroform extraction method. Briefly, tail fin was cut into small pieces and incubated in lysis buffer (100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 100  $\mu$ g/ml proteinase K) at 50°C for 3–5 h and extracted twice with an equal volume of phenol/chloroform (24:1, v/v;

<sup>3</sup> Abbreviations used in this paper: P/S, penicillin and streptomycin; 3D, three-dimensional; ELF, elongation factor; EST, expressed sequence tag; ETA, equivalent target amount;  $\gamma$ IP-10, IFN- $\gamma$ -inducible protein 10; MAF, macrophage-activating fac-

tor; NLS, nuclear localization sequence; PKC, protein kinase C; SOD, superoxide dismutase.

Sigma-Aldrich). The aqueous phase was collected, and genomic DNA was precipitated with cold ethanol. The dried DNA pellet was dissolved in TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0).

To study whether the first intron of the IFN- $\gamma$  gene is polymorphic, primers F5/R4 (Table I) located in the predicted exons 1 and 2 were used to amplify the first intron by a hot-start PCR using Biotaq polymerase (Bioline) and PFU polymerase (Promega). The PCR program was as follows: 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 20 s, 62°C for 20 s, and 72°C for 1.5 min, followed by a cycle of 72°C for 5 min. The PCR products were cloned into the pGEM T Easy vector and sequenced.

#### Expression study

For in vitro study of IFN- $\gamma$  expression, head kidney leukocytes were isolated from rainbow trout, as described above, and stimulated for 4 h with 0.1, 1, 10, and 25  $\mu$ g/ml PHA (Sigma-Aldrich) or 0.1, 1, 10, and 100  $\mu$ g/ml poly(I:C) (Sigma-Aldrich). Total RNA was extracted using the RNA-STAT60 reagent (AMS Biotechnology), according to the manufacturer's instructions. The first strand cDNA was synthesized using oligo(dT) primer (Invitrogen Life Technologies) and Bioscriptase (Bioline). The cDNA samples were diluted with TE buffer, and PCR was performed with  $\beta$ -actin primers (Table I) to allow equal amount of templates to be used for detecting IFN- $\gamma$  expression. IFN- $\gamma$  expression was detected by RT-PCR using primers EF1/ER1 (Table I). The PCR program was as follows: 1 cycle of 94°C for 3 min, 25–35 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 20 s, followed by a cycle of 72°C for 5 min.

To verify the results obtained from the semiquantitative RT-PCR analysis, real-time PCR was performed using TaqMan probes designed on a splicing site to amplify exclusively cDNA-encoding rainbow trout elongation factor  $\alpha$ 1 (ELF  $\alpha$ 1, a housekeeping gene) and IFN- $\gamma$ . The probes were labeled with the fluorescent tag 6-FAM (Applied Biosystems). The se-

quence of primers and probes is given in Table I. Calibration was performed on 10-fold serial dilutions of plasmid containing the target sequence. A total of 1  $\mu$ l of first strand cDNA synthesis reaction was added to 10  $\mu$ l of TaqMan 2 $\times$  PCR mix (Applied Biosystems), 900 nM forward and reverse primer, and 250 nM probe in a 20- $\mu$ l reaction. The reaction was transferred into individual wells of a 96-well optical plate (Applied Biosystems), capped, and placed into an ABI-Prism model 7000 sequence detector. PCR conditions were as follows: 1 cycle of 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s, 60°C for 1 min. The fluorescence output for each cycle was measured and recorded upon the completion of the entire run. Cycle threshold values were calculated by the SDS software algorithms (Applied Biosystems). Cycle threshold values from samples were converted into equivalent target amount (ETA) using the statistical standard curve established using the calibrator. The expression levels of IFN- $\gamma$  were expressed relative to that of ELF by calculating the ratio of ETA IFN- $\gamma$  and ETA ELF.

For in vivo study of IFN- $\gamma$  expression, two groups of fish (three fish per group) were injected with PBS (pH 7.0) or poly(I:C) at a dose of 1 mg/200 g fish. Twenty-four hours later, fish were sacrificed and tissues were collected for total RNA extraction using the RNA-STAT60 reagent and cDNA synthesis. Detection of IFN- $\gamma$  expression was performed by RT-PCR, as described previously.

#### Production and purification of rIFN- $\gamma$ proteins

The putative mature peptide of the trout IFN- $\gamma$ , starting from Ala<sup>25</sup>, was predicted by the SignalP program (16) and the multiple alignment generated using the CLUSTAL W program (version 1.83; Ref. 14). The cDNA fragments encoding the putative full-length mature peptide or a mutated mature peptide with deletion of the last 29-aa residues from the C terminus containing the nuclear localization sequence (NLS) were amplified by PCR

**FIGURE 1.** The full cDNA sequence and deduced amino acid sequence of the IFN- $\gamma$  homologue in rainbow trout *O. mykiss*. The motifs (ATTTA) for mRNA instability in the 3' untranslated region are underlined, and the polyadenylation signal (AATAAA) is in bold. In the translated protein sequence, the predicted signal peptide is in italics and the potential glycosylation site is boxed.

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1   ACCGTACACCGATTGAGGACTATTGAGCTTTGAGGGATCTTCAGTGTGACGTTGAGGGCC
61  ATGGATGTGTTATCAAGGGCTGTGATGTGTTTCTGCTTGATGGGCTGGATGACTTTAGGA
1   M D V L S R A V M C F C L M G W M T L G
121 TGGAGTAATGCTGCTCAGTTCACATCAATTAACATGAAGAGAAACATAGACAAACTGAAA
21  W S N A A Q F T S I N M K R N I D K L K
181 GTCCACTATAAGATCTCCAAGGACCAGCTGTTCAACGGAACCCCTGTTTTCCCAAGGAC
41  V H Y K I S K D Q L F N G N P V F P K D
241 ACGTTTGAGGACAGTGAGCAGAGGGTGTGATGAGTGTGGTTCTGGACGTGTACCTGAGT
61  T F E D S E Q R V L M S V V L D V Y L S
301 ATCTTCGGCCAGATGCTGAACCAGACGGGGGACCAGGAAATGATCGAGAGTCTGAAATAT
81  I F G Q M L N Q T G D Q E M I E S L K Y
361 GTCAAGGGGAAAATTCAAGGATCTCCAGAAACACTATTTCTGGGGAGGATACCTGAGCTG
101 V K G K I Q D L Q K H Y F L G R I P E L
421 AGGACACACCTGCAGAACCTGTGGGCCATCGAGACCAGTGACACCACAGTCCAGGGGAAG
121 R T H L Q N L W A I E T S D T T V Q G K
481 GCTCTGTCCGAGTTCATTACCATCTACGAGAAAGCCTCCAACTGGCCCTTAAGTTCCAT
141 A L S E F I T I Y E K A S K L A L K F H
541 CTAAAGAAGGACAACCGCAGGAAGAGACGGCAAGCCCAGAGGCTCAAATCACACATCATG
161 L K K D N R R K R R Q A Q R L K S H I M
601 TAGATGACCAAACGTGTCTACTGTGAAGGCTAGGTGTAGGTGAAGTTACACCGGGAAGT
*
661 TGATCTTGGGTCAGTTTACGATTTACCCCTCTAACGGTGAAGGTTAGGAGGCTGATCCTA
721 GATCTGTACCTAGGGGACCCCCCCCCCGGAGCCACAGTTGAGATACATATTGAAGTAAA
781 AATGTTCTTGATACAAAATACCTCAACTGGATCTATCTATTATATAAATGTATTTATTT
841 AATCTATGAAATCACGCACAATACTCAATGGTATATTATCATTGATAAGAGTATTTATTT
901 TTCTCGAATCAATACATTTATATTTATTTATTAATTATCTTAGAAAATGTTTGTGTTTTTAA
961 TAAATAGTTATTTATTACTAAATCTGTAAATCTACGACTGAATAAACAGACACTGAGGA
021 AAAAAAAAAAAAAA

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Table II. Protein homology between trout IFN- $\gamma$  and other known IFN- $\gamma$  molecules and fish type I IFNs

Species	Identity	Similarity
IFN- $\gamma$ molecules		
Human	16.4	37.3
Mouse	15.9	34.3
Pig	23.2	39.9
Rabbit	20.5	40.5
Sheep	23.7	44.7
Chicken	21.3	44.1
Duck	23.4	43.8
<i>Fugu</i>	31.0	48.5
Zebrafish	35.4	56.9
Fish type I IFNs		
Trout IFN1	12.9	26.3
Trout IFN2	13.8	27.1
Salmon IFN1	13.3	26.2
Salmon IFN2	12.9	25.3
<i>Fugu</i>	14.1	27.8
Tetraodon	18.7	35.4
Zebrafish	12.1	24.2
Catfish	16.1	31.8

using primer pairs RF1/RR1 and RF1/RR3, respectively. The fragments were separated on a 1.5% agarose gel, purified using a Qiagen gel extraction kit (Qiagen), and digested with restriction enzymes *Bam*HI and *Hin*dIII. The digested fragments were inserted into the pQE30 expression vector (Qiagen) at the restriction enzyme sites *Bam*HI and *Hin*dIII, and the resultant plasmids (pQE30-IFN- $\gamma$  for the full-length mature peptide and pQE30-IFN- $\gamma$ 1 for the truncated mature peptide) were sequenced for confirmation of the IFN- $\gamma$  gene sequence. The resultant recombinant proteins were named rIFN- $\gamma$  and rIFN- $\gamma$ 1, respectively. To allow expression of soluble proteins, the plasmids were transformed into *E. coli* M15 cells (Qiagen). Induction and purification of recombinant protein were performed, as described previously (19). To eliminate the potential contamination of bacterial endotoxins such as LPS during protein preparation, the purified recombinant protein was loaded onto a polymyxin B column (Sigma-Aldrich) and the collected samples were stored at  $-80^{\circ}\text{C}$  before use. The LPS concentration in the purified proteins was measured using a Pyrogen-5000 kit (Cambrex), with LPS standards being replaced by a series of dilutions of known LPS concentration. Purity of the rIFN- $\gamma$  was checked on a 4–12% precast SDS-PAGE gel (Invitrogen Life Technologies) stained with Brilliant Blue G (Sigma-Aldrich), and concentration was measured by comparing the protein band density with a standard protein (trypsin inhibitor; Sigma-Aldrich) in the same SDS-PAGE gel using an Ultraviolet Products gel imaging system and Ultraviolet Products gelworks ID advanced software. Western blot analysis was performed to confirm the identity of the rIFN- $\gamma$  using a chromogenic Western blot immunodetection kit (Invitrogen Life Technologies), according to the manufacturer's instructions. Penta-His Ab (Qiagen) was used as the primary Ab for Western blot detection.

#### Induction of gene expression in RTS-11 cells stimulated by rIFN- $\gamma$ proteins

The trout RTS-11 cells were incubated for 6 h with PBS or elution buffer as controls; different stimulants at optimal doses including 10  $\mu\text{g}/\text{ml}$  LPS (*E. coli* 0127:B8; Sigma-Aldrich), 10  $\mu\text{g}/\text{ml}$  PHA, 50  $\mu\text{g}/\text{ml}$  poly(I:C), 50 ng/ml rIL-1 $\beta$  (19), and 50 ng/ml rTNF2 (20); and a range of concentrations (0.1, 1, 10, 100 ng/ml) of rIFN- $\gamma$  or rIFN- $\gamma$ 1 proteins. Cells were harvested and RNA was extracted for expression analysis of IFN- $\gamma$ -inducible protein 10 ( $\gamma$ IP-10) (21), Mx protein (22), and STAT1 (GenBank accession no. U60331). The primers or probes used for gene expression are given in Table I. To establish the time course for rIFN- $\gamma$  induction, the RTS-11 cells were stimulated with 10 ng/ml rIFN- $\gamma$  and harvested at 0.5, 1, 2, 4, 8, 24, 48, and 72 h poststimulation. Detection of expression of  $\gamma$ IP-10, Mx protein, STAT1, and MHC class II  $\beta$ -chain was analyzed by RT-PCR with the same conditions used as in the IFN- $\gamma$  expression study, except for the cycling numbers or real-time PCR, as described previously.

#### Respiratory burst assay

Because previous studies have demonstrated that the trout macrophage cell line RTS-11 is unable to elicit a respiratory burst (13), freshly isolated

kidney cells were used in the present study to determine whether rIFN- $\gamma$  can enhance respiratory burst activity. Fish were anesthetized with benzocaine (25  $\mu\text{g}$  per liter; BDH) and killed. The head kidney was removed aseptically and passed through a 100- $\mu\text{m}$  nylon mesh using L-15 medium (Invitrogen Life Technologies) supplemented with 0.1% FCS (Labtech International) and P/S. The resulting cell suspensions were diluted in L-15, layered onto a 51/34% discontinuous Percoll gradient, and centrifuged at  $400 \times g$  for 30 min at  $4^{\circ}\text{C}$ . Head kidney leukocytes at the 51/34% Percoll interface (enriched for macrophages) were collected and washed in L-15 containing P/S, as above, by centrifugation at  $400 \times g$  for 20 min at  $4^{\circ}\text{C}$ . The resulting cell pellets were resuspended in 2 ml of L-15, as above. A viable cell count was determined by trypan blue (Sigma-Aldrich) exclusion, and the suspension was adjusted to  $2 \times 10^7$  cells/ml. One hundred microliters were seeded into each well of a 96-well culture plate, and 4 h later attached macrophages were incubated with 100  $\mu\text{l}$  of rIFN- $\gamma$  in L-15, as above, at a final concentration of 0.1, 1, 10, or 100 ng/ml at  $20^{\circ}\text{C}$  for 4 h. Each condition was prepared in triplicate.

Cytochrome *c* (Sigma-Aldrich) was dissolved at 2 mg/ml in phenol red-free HBSS. To an appropriate amount of this solution, PMA (Sigma-Aldrich) was added to make a final concentration of 1  $\mu\text{g}/\text{ml}$ . To half of this solution, superoxide dismutase (SOD; Sigma-Aldrich) was added to make a final concentration of 300 U/ml. After the appropriate incubation time, the macrophage monolayers were washed twice with phenol red-free HBSS and 100  $\mu\text{l}$  of cytochrome *c* containing either PMA or PMA and SOD was added to the appropriate wells. Readings were taken immediately on a multiscan spectrophotometer at 550 nm every 30 s for 30 min, with the PMA plus SOD wells being used as blanks. Results were tested for normality and thereafter analyzed using one-way ANOVA and Tukey's pairwise comparison test.

#### Inhibition of recombinant IFN- $\gamma$ -induced $\gamma$ IP-10 gene expression by kinase inhibitors

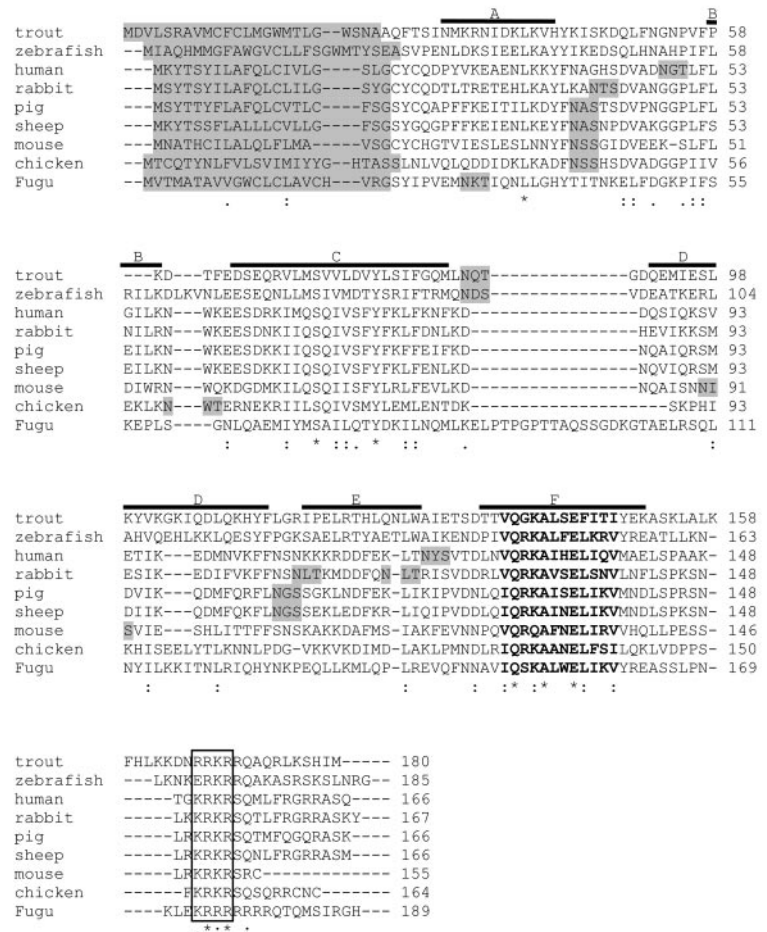
Staurosporine, an inhibitor for protein kinase C (PKC), was purchased from Sigma-Aldrich. Selective inhibitor for MAPKs, U0126, was purchased from Promega. RTS-11 cells were passaged to fresh flasks at 80% confluence and cultured for 2 days. To study IFN- $\gamma$ -induced signaling in RTS-11 cells, the cells were incubated with different doses of U0126 (Promega) or staurosporine (Sigma-Aldrich) for 0.5 h and then stimulated with 10 ng/ml rIFN- $\gamma$  for 2 h. Cells were then harvested for extraction of total RNA, and semiquantitative PCR was performed to detect  $\gamma$ IP-10 gene expression using primers IP-10-F2/IP-10-R1.

## Results

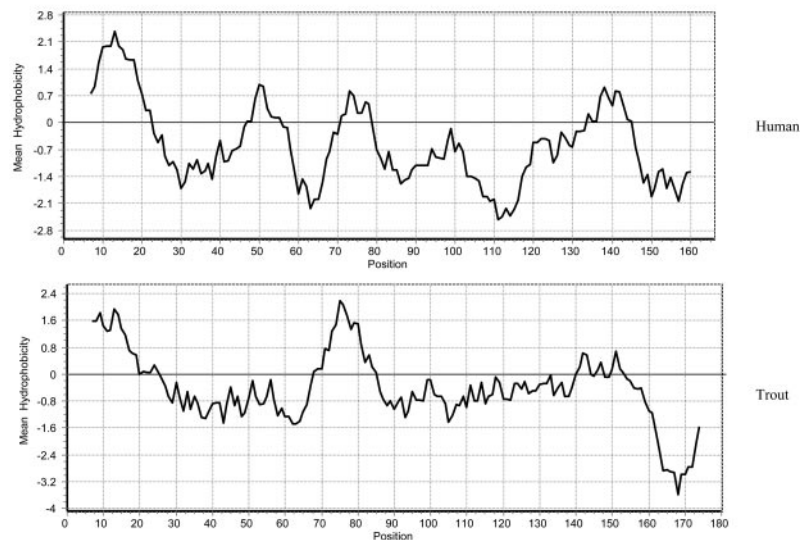
#### Cloning and sequencing of trout IFN- $\gamma$

We recently identified an IFN- $\gamma$  homologue in the Japanese puffer fish *Fugu rubripes* (12). Using the *Fugu* IFN- $\gamma$  protein sequence to search the salmonid expressed sequence tag (EST) database by BLAST ((http://snoopy.ceb.uvic.ca)), an Atlantic salmon EST (ssal.hmfif-002.028-1) sequence with 40.7% predicted amino acid similarity to the *Fugu* molecule was obtained. Because rainbow trout and Atlantic salmon are salmonids whose genomes share significant homology, primers (asF1 and asR1) (Table I) designed from the salmon EST sequence were synthesized to clone the trout IFN- $\gamma$  cDNA sequence by PCR, using a cDNA library derived from head kidney cells stimulated with PHA (23). A partial sequence of the trout IFN- $\gamma$  cDNA (329 bp) was obtained by a seminested PCR approach using primer pairs asF1/M13F and asF1/asR1 for the first and second round PCR, respectively (Table I). Subsequently, the 5' cDNA sequence was generated by seminested PCR using primers R1/M13R and R2/M13R (Table I). Similarly, the 3' end region was cloned using primers F1/M13F and F2/M13F. The compiled full-length cDNA sequence of the trout IFN- $\gamma$  is 1034 bp, and translates into a 180-aa precursor molecule with a 24-aa signal peptide predicted by the SignalP program (Fig. 1). As in mammalian and avian IFN- $\gamma$  genes, the 3' untranslated region of the trout IFN- $\gamma$  gene contains multiple mRNA instability motifs (ATTTA) and is AT rich (66%), particularly in the last 200-bp region, where AT accounts for 89%.

A

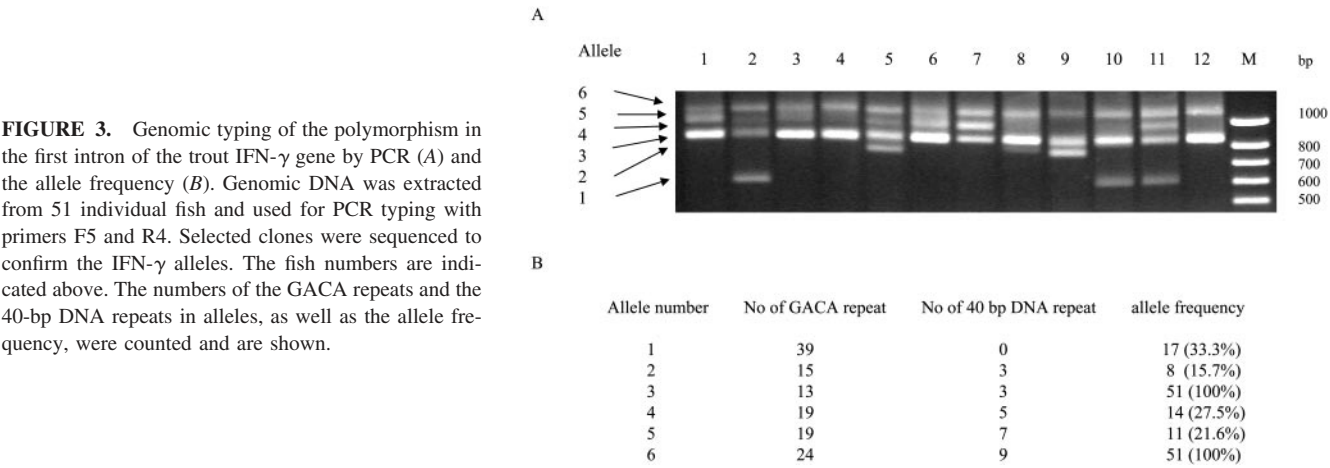


B



Type I IFN has recently been sequenced in several fish species (24–27). Not surprisingly, the trout IFN- $\gamma$  has very low amino acid similarity (26.3–27.1%) with the two trout type I IFNs (GenBank accession nos. AJ580911 and AJ582754) known to date (Table II). Higher similarity is seen to IFN- $\gamma$  in birds (43.8–44.1%) and mammals (34.3–44.7%). In addition to the *Fugu* IFN- $\gamma$  sequence mentioned above, recently, an IFN- $\gamma$  molecule in zebrafish *Danio rerio* has also been deposited in GenBank (GenBank accession no.

AB126869). It is apparent that trout IFN- $\gamma$  has the highest homology with the two fish IFN- $\gamma$  molecules, 56.9% with zebrafish and 48.5% with *Fugu*. As shown in the multiple alignment, the IFN- $\gamma$  proteins are significantly divergent, and only 8 aa were absolutely conserved (Fig. 2A). Highest conservation lies in the regions involved in formation of the secondary structure, containing helix C and helix F. In helix F, an apparent signature motif ([IV]-Q-X-[KQ]-A-X<sub>2</sub>-E-[LF]-X<sub>2</sub>-[IV]) is conserved among known IFN- $\gamma$



molecules (Fig. 2A). Despite the limited sequence homology among known IFN- $\gamma$  molecules, 3D modeling of the structure revealed six putative  $\alpha$ -helices, similar to that of the characterized human IFN- $\gamma$  (data not shown) (28). One noticeable difference was that, in general, fish IFN- $\gamma$  has a longer loop between helices C and D than other known IFN- $\gamma$ . In mammals, IFN- $\gamma$  is a glycosylated protein. The predicted mature peptide of trout IFN- $\gamma$  contains a potential glycosylation site (NQT) located within the loop between helices C and D in the multiple alignment, which is also seen in the zebrafish molecule (Fig. 2A). A single potential glycosylation site was also noted in the *Fugu* sequence, although further upstream in comparison with trout and zebrafish. In contrast, avian and mammalian IFN- $\gamma$  molecules contain at least two glycosylation sites, one of which is conserved in the loop between helices A and B (Fig. 2A). The putative mature peptide of the trout IFN- $\gamma$  contains 9 arginines and 18 lysines, making it hydrophilic and cationic as with other IFN- $\gamma$  proteins (Fig. 2B). Interestingly, all IFN- $\gamma$  proteins have a highly polycationic C-terminal tail containing multiple basic amino acids (arginine and lysine), which has been demonstrated to be critical for the biological activity in mammals. In particular, a motif RRKR similar to the NLS of the human and mouse molecule (KRKR) is conserved (Fig. 2A). The highly hydrophilic C-terminal tail was also confirmed by hydrophobicity plot analysis (Fig. 2B).

*Intron 1 comprises DNA repeats and is polymorphic*

In mammals, the first intron of the IFN- $\gamma$  gene contains polymorphic microsatellite repeats that are closely associated with disease susceptibility (29, 30). To examine whether this is also true in rainbow trout, the first intron of the IFN- $\gamma$  gene was amplified by PCR with primers F5 and R4 using genomic DNA extracted from different individual fish and sequenced. Figure 3 shows that at least six alleles were present, containing multiple copies of minisatellites (GACA), followed by insertion of a DNA repeat of 40 bp (GACAGAACAGGGAGATCTCCTTCCTAATGGGAGGCCAA CAGTAGGACA) between the GACA minisatellites. Figure 3B summarizes the frequency of the alleles obtained from 51 fish and the numbers of the GACA minisatellites and the 40-bp DNA repeats for each allele.

*Modulation of IFN- $\gamma$  expression by cytokines and other stimulants*

IFN- $\gamma$  is a Th1 cytokine that is mainly produced by T and NK cells upon stimulation by mitogens such as PHA or viral infection (31). Modulation of the trout IFN- $\gamma$  expression was studied in primary head kidney cells isolated from healthy fish. As shown in Fig. 4,

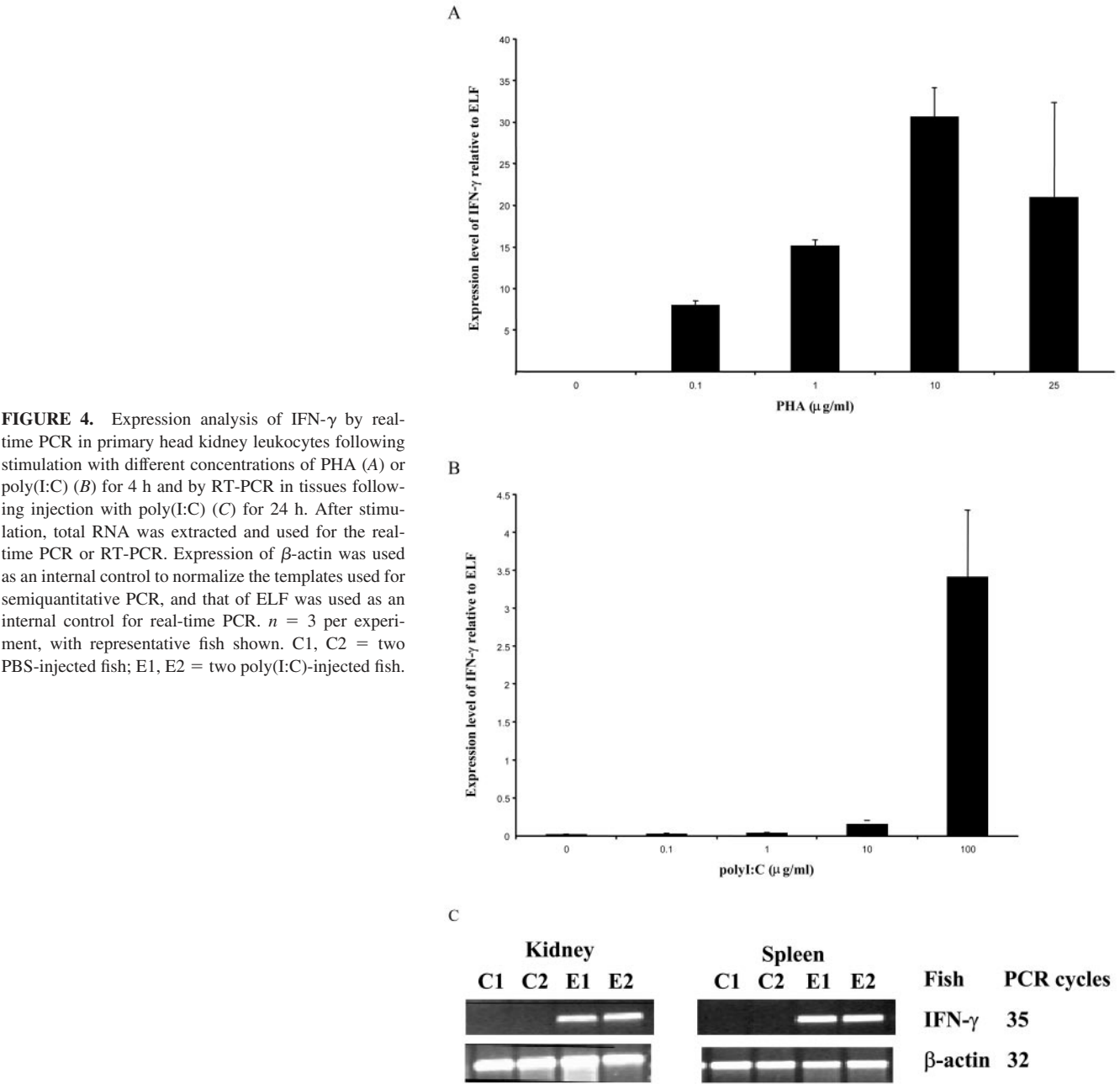
IFN- $\gamma$  was not expressed in control cells, but was induced by PHA at doses of 0.1, 1, 10, and 25  $\mu$ g/ml (Fig. 4A) or by poly(I:C) at doses of 10 and 100  $\mu$ g/ml (Fig. 4B) 4 h after stimulation. Apparently, PHA was more potent than poly(I:C) in inducing IFN- $\gamma$  expression in primary head kidney cells. A further experiment was conducted to determine whether administration of poly(I:C) could up-regulate IFN- $\gamma$  expression in vivo. Twenty-four hours after i.p. injection of poly(I:C) (1 mg of poly(I:C) per 200 g of fish), IFN- $\gamma$  mRNA was detected in kidney and spleen with no IFN- $\gamma$  transcripts observed in fish injected with PBS (Fig. 4C).

*rIFN- $\gamma$  activates gene expression and respiratory burst activity in macrophages*

To determine the biological activities of trout IFN- $\gamma$ , the putative mature peptide was expressed as an N-terminal 6-histidine-tagged fusion protein in *E. coli* M15 cells and purified under native conditions by affinity chromatography (Fig. 5). The rIFN- $\gamma$  protein was confirmed by Western blot analysis using a mAb against the histidine tag in which a single band was detected (Fig. 5). The purified recombinant proteins contained  $\sim$ 33 ng/ml LPS according to the endotoxin measurement. To investigate the importance of the C-terminal region on IFN- $\gamma$  bioactivities, a truncated IFN- $\gamma$  mutant was generated by deletion of the last 29-aa residues of the C terminus and recombinant protein produced (data not shown).

It is known in mammals that IFN- $\gamma$  is a potent activator of macrophages and induces expression of many immune genes, including cytokines such as  $\gamma$ IP-10 (a CXC chemokine initially identified as an IFN- $\gamma$ -induced protein), and signal transduction molecules such as STAT1 (9, 10). A  $\gamma$ IP-10 homologue was recently cloned in rainbow trout by our group (21), and hence considered in this study a key marker gene to determine the biological activities of trout rIFN- $\gamma$ . As shown in Fig. 6A, 6 h of stimulation with 0.1 ng/ml rIFN- $\gamma$  resulted in weak induction of  $\gamma$ IP-10 expression, while incubation with  $\geq$ 1 ng/ml rIFN- $\gamma$  significantly induced  $\gamma$ IP-10 expression. Deletion of 29-aa residues from the C terminus containing the NLS motif abolished the biological activities of rIFN- $\gamma$  (Fig. 6B). The effect of other stimulants on  $\gamma$ IP-10 expression was also evaluated (Fig. 6A) with no stimulating effect observed for 10  $\mu$ g/ml LPS, 50  $\mu$ g/ml poly(I:C), 50 ng/ml rIL-1 $\beta$ , and 50 ng/ml rTNF2. A significant increase of  $\gamma$ IP-10 transcripts was only seen in cells stimulated by 10  $\mu$ g/ml PHA. Transcription of one of the key intracellular signaling molecules for rIFN- $\gamma$ , STAT1, was also up-regulated by rIFN- $\gamma$  in a dose-dependent manner. However, unlike  $\gamma$ IP-10, STAT1 was also up-regulated by stimulation with LPS and poly(I:C), and to a lesser extent rIL-1 $\beta$ . Not surprisingly, Mx protein, an antiviral protein mainly induced





**FIGURE 4.** Expression analysis of IFN- $\gamma$  by real-time PCR in primary head kidney leukocytes following stimulation with different concentrations of PHA (A) or poly(I:C) (B) for 4 h and by RT-PCR in tissues following injection with poly(I:C) (C) for 24 h. After stimulation, total RNA was extracted and used for the real-time PCR or RT-PCR. Expression of  $\beta$ -actin was used as an internal control to normalize the templates used for semiquantitative PCR, and that of ELF was used as an internal control for real-time PCR.  $n = 3$  per experiment, with representative fish shown. C1, C2 = two PBS-injected fish; E1, E2 = two poly(I:C)-injected fish.

by type I IFNs, was weakly induced by rIFN- $\gamma$  at doses of 10 and 100 ng/ml, but was markedly induced by poly(I:C). Study of the time course of  $\gamma$ IP-10 induction by rIFN- $\gamma$  showed that  $\gamma$ IP-10 expression was transiently increased by rIFN- $\gamma$ , at a dose of 10 ng/ml, with expression first detectable at 0.5 h and peak expression seen between 2 and 24 h after stimulation (Fig. 7). In contrast, analysis of induced MHC expression showed that a significant increase of MHC class II  $\beta$ -chain transcripts was not seen before 24 h poststimulation, much later than that for  $\gamma$ IP-10.

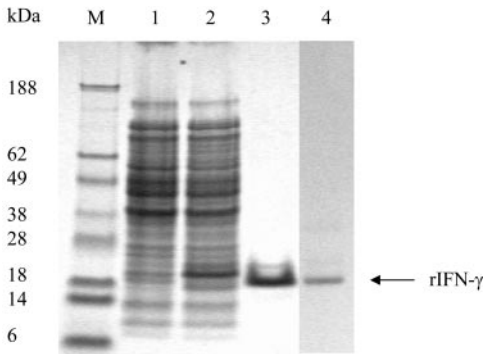
A further experiment was conducted to investigate whether rIFN- $\gamma$  enhances production of reactive oxygen species in macrophages, using the respiratory burst assay. Macrophages isolated from the head kidney were stimulated with different doses of rIFN- $\gamma$  before measuring the respiratory burst levels. Four hours preincubation of head kidney macrophages with 100 ng/ml rIFN- $\gamma$  significantly ( $p < 0.05$ , ANOVA and Tukey's pairwise comparison) increased respiratory burst activity over that seen in cells

preincubated in medium alone (Fig. 8). This enhancing effect was not due to the contamination of LPS, because a control experiment confirmed the LPS content (33 ng/ml) in the recombinant protein had no impact on respiratory burst level (data not shown).

*Inhibition of recombinant IFN- $\gamma$ -induced  $\gamma$ IP-10 gene expression by kinase inhibitors*

It has been demonstrated in mammals that IFN- $\gamma$  exhibits biological functions via a JAK/STAT signaling pathway (10). Several protein kinases have been implicated in phosphorylation of intracellular signaling proteins such as JAKs and STATs. Among these, MAPKs are the most studied candidate kinases. To date, three distinct MAPK signal transduction pathways have been characterized and involve ERKs, JNK/stress-activated protein kinase, or P38 protein kinase (10). In mammals, specific inhibitors have been developed to study the signaling pathways by selectively blocking





**FIGURE 5.** Production and purification of trout rIFN- $\gamma$  in *E. coli*. The plasmid pQE30-IFN- $\gamma$  expressing the putative mature IFN- $\gamma$  was transformed into *E. coli* M15 cells, and production of rIFN- $\gamma$  was induced by isopropyl  $\beta$ -D-thiogalactoside. Bacterial lysate or purified rIFN- $\gamma$  was separated in a 4–12% SDS-PAGE gel under denaturing conditions and confirmed by Western blot analysis. Lanes 1–3, Lysate from uninduced cells, lysate from isopropyl  $\beta$ -D-thiogalactoside-induced cells, and purified rIFN- $\gamma$ . Lane 4, 100 ng of purified rIFN- $\gamma$  detected by Western blot analysis using a mAb against the histidine tag.

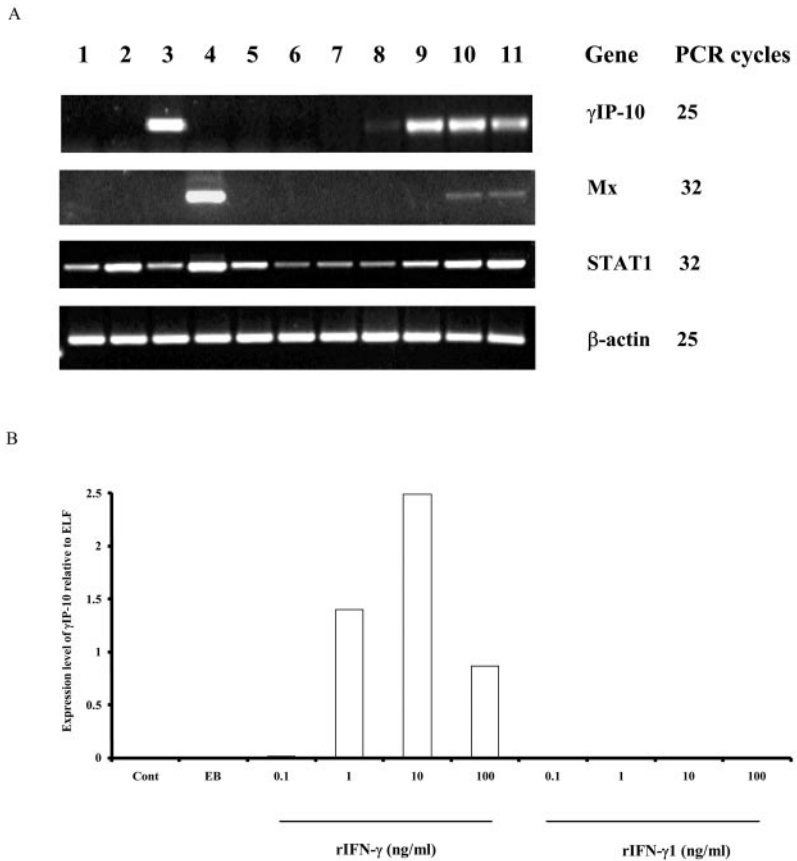
the activation of the kinases; for example, U0126 specifically inhibits signaling events elicited by ERKs. To investigate the involvement of ERKs in IFN- $\gamma$  signaling, U0126 was added to the culture medium of macrophages 0.5 h before stimulation with 10 ng/ml rIFN- $\gamma$ . Two hours after IFN- $\gamma$  stimulation, cells were harvested and analyzed for detection of  $\gamma$ IP-10 expression by RT-PCR. Figure 9A shows that IFN- $\gamma$ -induced  $\gamma$ IP-10 expression in RTS-11 cells was partially inhibited by incubation with 10  $\mu$ M U0126, with lower doses being ineffective.

Recently, it has been suggested that PKC is also involved in activation of STAT1, although the exact mechanism is still unclear. To examine the roles of PKC in IFN- $\gamma$ -induced gene expression, a nonselective inhibitor of PKC, staurosporine, was used to block rIFN- $\gamma$  activities in macrophages. RTS-11 cells were treated for 0.5 h with different doses of staurosporine and subsequently stimulated with 10 ng/ml rIFN- $\gamma$  for 2 h. Treatment of the cells with 0.5  $\mu$ M staurosporine dramatically decreased  $\gamma$ IP-10 expression, with both 1 and 5  $\mu$ M completely blocking IFN- $\gamma$ -induced  $\gamma$ IP-10 expression (Fig. 9B), demonstrating that PKC is essential for the activation of the  $\gamma$ IP-10 gene by IFN- $\gamma$  in trout macrophages.

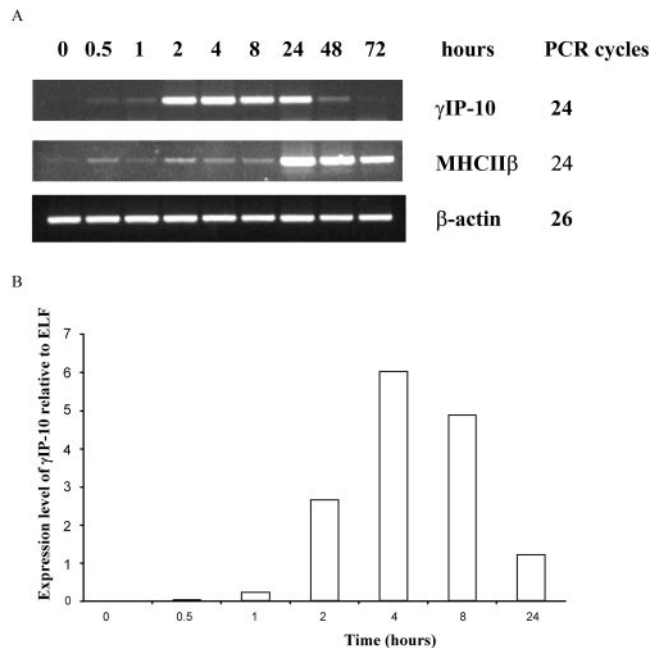
**Discussion**

IL-2 and IFN- $\gamma$ , mainly produced by CD4<sup>+</sup> cells, are regarded as the hallmarks of the Th1 immune response in birds and mammals. Whether lower vertebrates such as fish have developed the equivalent of a Th1 immune system has been questionable until recently, when an IFN- $\gamma$  gene was identified by synteny in the puffer fish, *F. rubripes*. In this study, an IFN- $\gamma$  homologue has been identified from rainbow trout *O. mykiss*, and for the first time in fish some of its biological activities have been characterized. This study demonstrates that trout IFN- $\gamma$  is functionally similar to its mammalian counterpart, and that at least some of the intracellular signaling pathways are conserved.

Trout IFN- $\gamma$  shares modest sequence similarity with the zebrafish (56.9%) and *Fugu* (48.5%) molecules and lower similarity with avian (43.8–44.1%) and mammalian (34.3–44.7%) IFN- $\gamma$  proteins. Only 6 identical aa residues were found to be absolutely conserved in the multiple alignment (Fig. 2A). Significant sequence divergence between species may explain the fact that IFN- $\gamma$  functions in a highly species-specific manner. Despite the



**FIGURE 6.** Dose response of rIFN- $\gamma$  proteins in inducing gene expression in RTS-11 cells. RTS-11 cells were stimulated for 6 h with different stimulants, and RNA was extracted for analyzing gene expression by RT-PCR (A) or real-time PCR (B). A, Lanes 1–11, PBS, 10  $\mu$ g/ml LPS, 10  $\mu$ g/ml PHA, 50  $\mu$ g/ml poly(I:C), 50 ng/ml rIL-1 $\beta$ , 50 ng/ml rTNF2, elution buffer, and 0.1, 1, 10, 100 ng/ml rIFN- $\gamma$ .



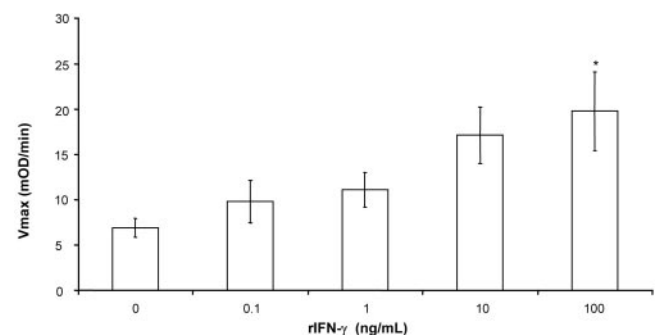
**FIGURE 7.** Time course of rIFN- $\gamma$  in inducing gene expression in RTS-11 cells. The RTS-11 cells were stimulated with 10 ng/ml rIFN- $\gamma$  for 0.5, 1, 2, 4, 8, 24, 48, and 72 h, and harvested for RT-PCR analysis of gene expression of  $\gamma$ IP-10 and the MHC class II  $\beta$ -chain (A). The expression level of  $\gamma$ IP-10 was subsequently confirmed by real-time PCR (B).

low similarity of the IFN- $\gamma$  molecules, the overall tertiary structure of six  $\alpha$ -helices seems to be conserved from fish to mammals. Although no signature motif sequence exists in the PROSITE database, a conserved motif containing the consensus pattern [IV]-Q-X-[KQ]-A-X<sub>2</sub>-E-[LF]-X<sub>2</sub>-[IV] can be seen in helix F among all known IFN- $\gamma$  molecules in the multiple alignment. All IFN- $\gamma$  proteins are also highly hydrophilic and cationic (charge ranges from +3 to +13), with the C-terminal region containing multiple arginine and lysine residues (the key basic amino acids that contribute to the cationic charge). Indeed, in the human molecule, the C-terminal cationic motif KRKR has been shown to act as an NLS and is essential for IFN- $\gamma$  function. Deletion of 29-aa residues from the C terminus of trout IFN- $\gamma$  abolished the ability of rIFN- $\gamma$  in inducing  $\gamma$ IP-10 expression, in agreement with other studies in mammals demonstrating deletion or modification of the NLS motif results in significant loss of the biological activities of the recombinant protein (32) and blockage of the nuclear translocation of STAT1 $\alpha$  induced by exogenous IFN- $\gamma$  (33). A peptide of the IFN- $\gamma$  C-terminal region containing the NLS has agonist activity and can induce MHC class II expression and an antiviral state when internalized in human or murine macrophages (34). A similar motif is also present in fish IFN- $\gamma$ , as shown in the multiple alignment (Fig. 2A), and strongly suggests a NLS motif is required for IFN- $\gamma$  function in lower vertebrates.

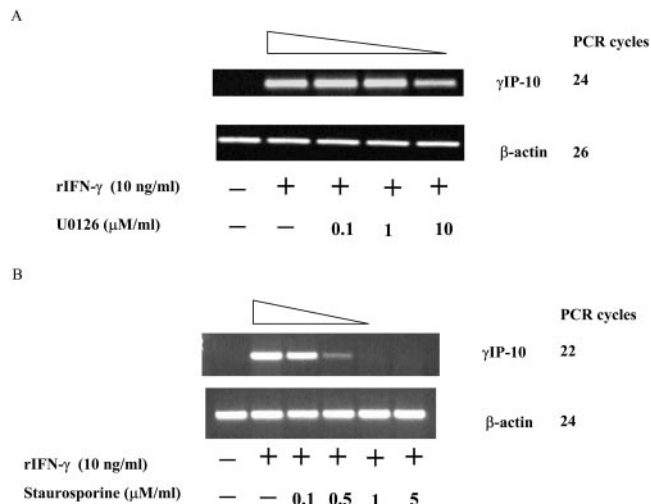
Dinucleotide microsatellites (CA) have been described in the first intron of the human IFN- $\gamma$  gene, leading to the identification of five alleles with 11–15 CA repeats (35). Among the alleles, 1 with 12 CA repeats is implicated in disease susceptibility to allograft fibrosis (35, 36). In Soay sheep, a microsatellite polymorphism in the IFN- $\gamma$  gene has been shown to be associated with resistance to gastrointestinal nematodes in a naturally parasitized population (30). However, not all animals possess a polymorphic region in the first intron of IFN- $\gamma$  gene, as seen in chicken and *Fugu* (12, 37). To examine whether the first intron of the trout IFN- $\gamma$  gene contained polymorphic microsatellite repeats, 51 fish

were typed by PCR. Six major PCR products were seen, and sequencing of the PCR products revealed that at least six alleles were present and contained various numbers of a tetramicrosatellite repeat (GACA). Strikingly, following the consecutive minisatellite repeats, a 40-bp DNA sequence was inserted and repeated several times. Whether this highly polymorphic intron correlates to IFN- $\gamma$  production and disease susceptibility or resistance in this species will be of interest to investigate further, to establish its potential as a gene marker to select fish stocks. Studies in birds and mammals have shown that, unlike type I IFN genes, which have multiple copies clustered in the chromosome, IFN- $\gamma$  exists as a single copy gene (10). In *Fugu* whose entire genome has recently been sequenced, a single IFN- $\gamma$  homologue was identified (12). Synteny analysis of the surrounding genes reveals strong conservation between *Fugu* and humans. However, in trout it is quite possible that two IFN- $\gamma$  genes are present, due to chromosomal duplication, an ancient event in salmonids, although only a single IFN- $\gamma$  sequence was described in the present study. With respect to this, of the six trout IFN- $\gamma$  alleles identified in this study, two were seen in all individuals and some fish possessed three or four alleles, suggesting these six alleles may correlate to two possible IFN- $\gamma$  genes.

IFN- $\gamma$  orchestrates a variety of immune responses by initiating intracellular gene expression. It is well established that IFN- $\gamma$  is a potent activator for macrophages, and hence the reason it was initially termed as a macrophage-activating factor (MAF). MAF activity has been well known in fish for many years (38), and partially purified MAF was known to have antiviral activity and to be acid sensitive, suggesting it could be a type II IFN (39). As expected, like IFN- $\gamma$  in other species, trout IFN- $\gamma$  significantly increased respiratory burst activity in rainbow trout macrophages, suggesting its involvement as a MAF to increase macrophage microbicidal activity in fish. In addition to its role in activation of macrophages, IFN- $\gamma$  enhances Ag presentation and attracts leukocytes to the infected tissue sites mainly through induction of gene expression of a variety of immune factors, including MHC class II and chemokines such as  $\gamma$ IP-10, known to be a T cell attractant (10). Trout IFN- $\gamma$  may also play an important role in mediating leukocyte trafficking, as the recombinant protein was shown to significantly induce  $\gamma$ IP-10 expression in macrophages. The fact that bacterially produced rIFN- $\gamma$  was active in terms of its ability to induce gene expression and enhance respiratory burst activity in macrophages demonstrates that glycosylation may not be essential for IFN- $\gamma$  biological activity, although a potential glycosylation site was present in the mature peptide region, in agreement with studies in other species. For example, in the human molecule, it has been demonstrated that glycosylation had no impact on the



**FIGURE 8.** Respiratory burst activity of trout head kidney macrophages after stimulation with various concentrations of rIFN- $\gamma$  for 4 h. Data are means  $\pm$  SE of triplicate wells. \*,  $p < 0.05$  relative to macrophages incubated with no added rIFN- $\gamma$  (0).



**FIGURE 9.** Suppression of IFN- $\gamma$ -induced  $\gamma$ IP-10 expression in RTS-11 cells by a selective ERK inhibitor U0126 (A) and a PKC inhibitor staurosporine (B). The RTS-11 cells were incubated with various doses of U0126 or staurosporine for 0.5 h, and then stimulated with 10 ng/ml rIFN- $\gamma$  for 2 h. RNA was extracted and used for RT-PCR expression analysis of  $\gamma$ IP-10.

biological activities of IFN- $\gamma$ , but might affect the protein  $t_{1/2}$  (40, 41).

The intracellular events involved in IFN- $\gamma$  signaling are beginning to be unveiled in mammals (9, 10). It is known that activation of STAT1 by protein kinases is required for regulation of IFN- $\gamma$ -responsive genes, through translocation of a phosphorylated STAT1 homodimer and subsequent binding to the IFN- $\gamma$  activation site or IFN-stimulated response elements in the gene promoters (10). ERKs are important signaling molecules in the MAPK pathway and have recently been implicated in phosphorylation of STAT1 in IFN- $\gamma$ -activated gene expression in murine macrophages (42). In the present study, a specific inhibitor for ERKs (U0126) was used to study involvement of ERKs in IFN- $\gamma$  signaling. In trout macrophages, IFN- $\gamma$ -induced  $\gamma$ IP-10 expression was inhibited by the ERK-specific inhibitor U0126, supporting the notion that ERKs can be activated by IFN- $\gamma$ . However, IFN- $\gamma$ -induced  $\gamma$ IP-10 expression is not solely dependent on activation of ERKs, as it was only partially inhibited by U0126, suggesting involvement of the ERK-independent signaling pathways.

PKC, a family of serine/threonine-directed protein kinases that consists of at least 12 isoforms, plays an important role in regulating cellular proliferation, transformation, and differentiation, and studies in mammals have demonstrated PKC is involved in mediating IFN- $\gamma$  signal transduction (43). Treatment of cultured human keratinocytes with two specific inhibitors of PKC, H-7 and calphostin C, suppresses IFN- $\gamma$ -induced  $\gamma$ IP-10 expression (44). In human monocytes, IFN- $\gamma$  activates calcium-independent PKC, and recent studies have shown IFN- $\gamma$  induced nuclear translocation and activation of two PKC isoforms, PKC  $\alpha$  and  $\beta$ 1 (45). Furthermore, selective inhibition of PKC $\delta$ , an isoform of the PKC proteins, abolished phosphorylation of STAT1 specifically on Ser<sup>727</sup>, which is an essential event for IFN- $\gamma$  signaling to induce gene expression by activation of IFN- $\gamma$  activation site elements in the promoters of IFN- $\gamma$ -inducible genes (10). In the present study, it was indirectly established that PKC is activated by IFN- $\gamma$  in macrophages using the nonselective PKC inhibitor staurosporine. Thus, IFN- $\gamma$ -induced  $\gamma$ IP-10 expression in trout macrophages is dependent on PKC activation, as treatment of the macrophages with 1  $\mu$ M staurosporine ablated the stimulating effects of rIFN- $\gamma$ .

Such data also suggest that fish may possess PKC homologues equivalent to those in mammals, although no fish PKC homologue has yet been characterized.

In conclusion, functional studies in trout have established that the gene first discovered in *Fugu* by synteny analysis is indeed equivalent to IFN- $\gamma$ . Clearly, this molecule has evolved early and raises the possibility of Th1 responses being present in lower vertebrates. This finding will have a major impact on the ability to measure specific cell-mediated immune responses at this level of phylogeny.

## Disclosures

The authors have no financial conflict of interest.

## References

- Romagnani, S. 1999. Th1/Th2 cells. *Inflamm. Bowel Dis.* 5: 285–284.
- Nakanishi, T., U. Fischer, J. M. Dijkstra, S. Hasegawa, T. Somamoto, N. Okamoto, and M. Otatake. 2002. Cytotoxic T cell function in fish. *Dev. Comp. Immunol.* 26: 131–139.
- Hansen, J. D., P. Strassburger, and P. L. Du. 1996. Conservation of an  $\alpha$ 2 domain within the teleostean world, MHC class I from the rainbow trout *Oncorhynchus mykiss*. *Dev. Comp. Immunol.* 20: 417–425.
- Rodrigues, P. N., T. T. Hermens, J. H. Rombout, E. Egberts, and R. J. Stet. 1995. Detection of MHC class II transcripts in lymphoid tissues of the common carp (*Cyprinus carpio* L.). *Dev. Comp. Immunol.* 19: 483–496.
- Partula, S., A. De Guerra, J. S. Fellah, and J. Charlemagne. 1996. Structure and diversity of the TCR  $\alpha$ -chain in a teleost fish. *J. Immunol.* 157: 207–212.
- Hansen, J. D., and P. Strassburger. 2000. Description of an ectothermic TCR coreceptor, CD8 $\alpha$ , in rainbow trout. *J. Immunol.* 164: 3132–3139.
- Yoshiura, Y., I. Kiryu, A. Fujiwara, H. Suetake, Y. Suzuki, T. Nakanishi, and M. Otatake. 2003. Identification and characterization of *Fugu* orthologues of mammalian interleukin-12 subunits. *Immunogenetics* 55: 296–306.
- Zou, J., S. Bird, J. Truckle, N. Bols, M. Horne, and C. Secombes. 2004. Identification and expression analysis of an IL-18 homologue and its alternatively spliced form in rainbow trout (*Oncorhynchus mykiss*). *Eur. J. Biochem.* 271: 1–11.
- Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to interferon- $\gamma$ . *Annu. Rev. Immunol.* 15: 749–795.
- Schroder, K., P. J. Hertzog, T. Ravasi, and D. A. Hume. 2004. Interferon- $\gamma$ : an overview of signals, mechanisms and functions. *J. Leukocyte Biol.* 75: 163–189.
- Digby, M. R., and J. W. Lowenthal. 1995. Cloning and expression of the chicken interferon- $\gamma$  gene. *J. Interferon Cytokine Res.* 15: 939–945.
- Zou, J., Y. Yoshiura, J. Dijkstra, M. Sakai, M. Otatake, and C. Secombes. 2004. Identification of an interferon  $\gamma$  homologue in *Fugu*, *Takifugu rubripes*. *Fish Shellfish Immunol.* 17: 403–409.
- Ganassin, R. C., and N. Bols. 1998. Development of a monocyte/macrophage-like cell line, RTS11, from rainbow trout spleen. *Fish Shellfish Immunol.* 8: 457–476.
- Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.
- Needleman, S. B., and C. D. Wunsch. 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. *J. Mol. Biol.* 48: 443–453.
- Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 10: 1–6.
- Kelley, L. A., R. M. MacCallum, and M. J. Sternberg. 2000. Enhanced genome annotation using structural profiles in the program 3D-PSSM. *J. Mol. Biol.* 299: 499–520.
- Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157: 105–132.
- Hong, S., J. Zou, M. Crampe, S. Peddie, G. Scapigliati, N. Bols, C. Cunningham, and C. J. Secombes. 2001. The production and bioactivity of rainbow trout (*Oncorhynchus mykiss*) recombinant IL-1 $\beta$ . *Vet. Immunol. Immunopathol.* 81: 1–14.
- Zou, J., S. Peddie, G. Scapigliati, Y. Zhang, N. C. Bols, A. E. Ellis, and C. J. Secombes. 2003. Functional characterization of the recombinant tumor necrosis factors in rainbow trout, *Oncorhynchus mykiss*. *Dev. Comp. Immunol.* 27: 813–822.
- Laing, K. J., N. Bols, and C. J. Secombes. 2002. A CXC chemokine sequence isolated from the rainbow trout *Oncorhynchus mykiss* resembles the closely related interferon- $\gamma$ -inducible chemokines CXCL9, CXCL10 and CXCL11. *Eur. Cytokine Network* 13: 462–473.
- Collet, B., and C. J. Secombes. 2001. The rainbow trout (*Oncorhynchus mykiss*) Mx1 promoter: structural and functional characterization. *Eur. J. Biochem.* 268: 1577–1584.
- Zou, J., P. S. Grabowski, C. Cunningham, and C. J. Secombes. 1999. Molecular cloning of interleukin 1 $\beta$  from rainbow trout *Oncorhynchus mykiss* reveals no evidence of an ice cut site. *Cytokine* 11: 552–560.

24. Altmann, S. M., M. T. Mellon, D. L. Distel, and C. H. Kim. 2003. Molecular and functional analysis of an interferon gene from the zebrafish, *Danio rerio*. *J. Virol.* 77: 1992–2002.
25. Long, S., M. Wilson, E. Bengten, L. Bryan, L. W. Clem, N. W. Miller, and V. G. Chinchar. 2003. Identification of a cDNA encoding channel catfish interferon. *Dev. Comp. Immunol.* 28: 97–111.
26. Robertsen, B., V. Bergan, T. Rokenes, R. Larsen, and A. Albuquerque. 2003. Atlantic salmon interferon genes: cloning, sequence analysis, expression, and biological activity. *J. Interferon Cytokine Res.* 23: 601–612.
27. Zou, J., S. Bird, and C. Secombes. 2004. Fish cytokine gene discovery and linkage using genomic approaches. *Mar. Biotechnol.* 6: S533–S539.
28. Ealick, S. E., W. J. Cook, S. Vijay-Kumar, M. Carson, T. L. Nagabhushan, P. P. Trotta, and C. E. Bugg. 1991. Three-dimensional structure of recombinant human interferon- $\gamma$ . *Science* 252: 698–702.
29. Awad, M., V. Pravica, C. Perrey, A. El Gamel, N. Yonan, P. J. Sinnott, and I. V. Hutchinson. 1999. CA repeat allele polymorphism in the first intron of the human interferon- $\gamma$  gene is associated with lung allograft fibrosis. *Hum. Immunol.* 60: 343–346.
30. Coltman, D. W., K. Wilson, J. G. Pilkington, M. J. Stear, and J. M. Pemberton. 2001. A microsatellite polymorphism in the  $\gamma$  interferon gene is associated with resistance to gastrointestinal nematodes in a naturally-parasitized population of Soay sheep. *Parasitology* 122: 571–582.
31. De Ley, M., J. van Damme, H. Claeys, H. Weening, J. W. Heine, A. Billiau, C. Vermylen, and P. de Somer. 1980. Interferon induced in human leukocytes by mitogens: production, partial purification and characterization. *Eur. J. Immunol.* 10: 877–883.
32. Subramaniam, P. S., M. G. Mujtaba, M. R. Paddy, and H. M. Johnson. 1999. The carboxyl terminus of interferon- $\gamma$  contains a functional polybasic nuclear localization sequence. *J. Biol. Chem.* 274: 403–407.
33. Subramaniam, P. S., J. Larkin III, M. G. Mujtaba, M. R. Walter, and H. M. Johnson. 2000. The COOH-terminal nuclear localization sequence of interferon  $\gamma$  regulates STAT1 $\alpha$  nuclear translocation at an intracellular site. *J. Cell Sci.* 113: 2771–2781.
34. Szente, B. E., J. M. Soos, and H. W. Johnson. 1994. The C-terminus of IFN $\gamma$  is sufficient for intracellular function. *Biochem. Biophys. Res. Commun.* 203: 1645–1654.
35. Pravica, V., A. Asderakis, C. Perrey, A. Hajeer, P. J. Sinnott, and I. V. Hutchinson. 1999. In vitro production of IFN- $\gamma$  correlates with CA repeat polymorphism in the human IFN- $\gamma$  gene. *Eur. J. Immunogenet.* 26: 1–3.
36. Pravica, V., I. J. Brogan, and I. V. Hutchinson. 2000. Rare polymorphisms in the promoter regions of the human interleukin-12 p35 and interleukin-12 p40 subunit genes. *Eur. J. Immunogenet.* 27: 35–36.
37. Kaiser, P., H. M. Wain, and L. Rothwell. 1998. Structure of the chicken interferon- $\gamma$  gene, and comparison to mammalian homologues. *Gene* 207: 25–32.
38. Graham, S., and C. J. Secombes. 1988. The production of a macrophage-activating factor from rainbow trout *Salmo gairdneri* leukocytes. *Immunology* 65: 293–297.
39. Graham, S., and C. J. Secombes. 1990. Cellular requirements for lymphokine secretion by rainbow trout *Salmo gairdneri* leukocytes. *Dev. Comp. Immunol.* 14: 59–68.
40. Bocci, V., A. Pacini, G. P. Pessina, L. Paulesu, M. Muscettola, and G. Lunghetti. 1985. Catabolic sites of human interferon- $\gamma$ . *J. Gen. Virol.* 66: 887–891.
41. Gray, P. W., D. W. Leung, D. Pennica, E. Yelverton, R. Najarian, C. C. Simonsen, R. Derynck, P. J. Sherwood, D. M. Wallace, S. L. Berger, et al. 1982. Expression of human immune interferon cDNA in *E. coli* and monkey cells. *Nature* 295: 503–508.
42. Blanchette, J., M. Jaramillo, and M. Olivier. 2003. Signalling events involved in interferon- $\gamma$ -inducible macrophage nitric oxide generation. *Immunology* 108: 513–522.
43. Deb, D. K., A. Sassano, F. Lekmine, B. Majchrzak, A. Verma, S. Kambhampati, S. Uddin, A. Rahman, E. N. Fish, and L. C. Platanias. 2003. Activation of protein kinase C $\delta$  by IFN- $\gamma$ . *J. Immunol.* 171: 267–273.
44. Boorsma, D. M., J. Flier, E. N. van den Brink, S. Sampat, H. L. Walg, R. Willemze, C. P. Tensen, and T. J. Stoof. 1999. IP-10 mRNA expression in cultured keratinocytes is suppressed by inhibition of protein kinase-C and tyrosine kinase and elevation of cAMP. *Cytokine* 11: 469–475.
45. Mazzi, P., M. Donini, D. Margotto, F. Wientjes, and S. Dusi. 2004. IFN- $\gamma$  induces gp91<sup>phox</sup> expression in human monocytes via protein kinase C-dependent phosphorylation of PU.1. *J. Immunol.* 172: 4941–4947.