Molecular and Functional Characterization of IL-15 in Rainbow Trout *Oncorhynchus mykiss:* A Potent Inducer of IFN-γ Expression in Spleen Leukocytes

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Molecular and Functional Characterization of IL-15 in Rainbow Trout Oncorhynchus mykiss: A Potent Inducer of IFN-γ Expression in Spleen Leukocytes\textsuperscript{1,2}

Tiehui Wang, Jason W. Holland, Allison Carrington, Jun Zou, and Christopher J. Secombes\textsuperscript{3}

IL-15 is a member of the common γ-chain family of cytokines that possess a heterogeneous repertoire of activities on various cells of the immune system. We report here the first functional characterization of a fish IL-15 in rainbow trout. The trout IL-15 gene is 6-kb long and contains six exons and five introns that transcribe into a 1.2-kb mRNA containing seven out-of-frame AUG initiation codons and translate into a 193-aa peptide. Potential sites for transcriptional activators and repressors have been identified in the trout IL-15 gene. Like IL-15 from other species, trout IL-15 is closely linked to an INPP4B gene, but there is also a BCL10 gene located between the IL-15 and INPP4B genes. Three alternative splicing variants of the trout IL-15 gene have also been identified and their expression in vivo was studied. Trout IL-15 expression is present in all the tissues and cell lines studied. Recombinant trout IFN-γ selectively increased IL-15 expression but had little effect on other cytokines such as IL-1β and IL-11. Recombinant trout IL-15 preferentially stimulated splenic leukocytes from healthy fish, where it induced a large increase in IFN-γ expression, with little, if any, effect on IL-1.

**Materials and Methods**

**Cell lines and cell culture**

Four rainbow trout cell lines were used: the mononuclear cell line RTS-11 (37), the gonad cell line RTG-2 (38), the liver cell line RTL (39), and CL-6 (an epithelial liver cell line, a gift from Dr. A. Benmansour, l’Institut National de la Recherche Agronomique, Jouy-en-Josas, France). All trout cells were grown in L-15 medium supplemented with 10% FCS for RTS-11 cells and 10% FCS for all the other cell lines.

**Bacterial challenge, SSH library construction, and analysis**

A virulent strain of *Aeromonas salmonicida* sp. *salmonicida*, MT 423 (30), was used to challenge female rainbow trout of *Oncorhynchus mykiss*, and analysis of a λ genomic clone containing the *IL-15* gene which revealed that *IL-15* is linked closely to a BCL10 gene, which is essential for NF-kB activation by TCRs and BCRs (35), and an INPP4B gene which encodes the inositol polyphosphate 4-phosphatase type II, one of the enzymes involved in phosphatidylinositol signaling pathways (36). Trout IL-15 expression is modulated by recombinant trout IFN-γ (rtIFN-γ) but not rtIL-1β. We next produced and purified bioactive rtIL-15 from *Escherichia coli* and found that rtIL-15 can induce IFN-γ expression in leukocytes from spleen but not from head kidney of normal fish.

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**Table I. Primers used for cloning and expression**

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<th>Name</th>
<th>Gene</th>
<th>Sequence (5' to 3')</th>
<th>Application</th>
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<td>GCTGT ACAAA GCTGA AACAC ACAGG ATG</td>
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<td>IL-15</td>
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<td></td>
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<td>Real-time PCR (with IL-15EF1 or IL-15F)</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>IL-1IR</td>
<td></td>
<td>TGGTG CTCAT CCTGA GGGAG T</td>
<td></td>
</tr>
</tbody>
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β-/γ-chain complex without the α-chain; and 4) IL-15Rα binds, recycles, and presents IL-15 to the opposing cell (18, 19). The interaction of IL-15 with its receptor complex leads to a series of signaling events that include activation of the JAK/STAT pathway, and the PI3K pathway. The β-chain is associated with JAK1, whereas the γ-chain is physically and functionally associated with JAK3, resulting in STAT3 and STAT5 phosphorylation, respectively (20). The PI3K pathway and downstream effectors play a central role in the signaling processes involved in the proliferation and inhibition of apoptosis for every γ-chain-interacting cytokine. IL-15 also has the ability to activate the transcription factors, NF-κB and AP-1 (21).

The diverse expression of IL-15, IL-15Rα, and the common β-γ-chains has been suggested to reflect a heterogeneous repertoire of activities mediated by this cytokine on various cells of the immune system. Thus, IL-15 promotes extrathymic development of T and NK cells (22), is a growth, differentiation, activation, and survival factor for NK cells that contributes to increased cytolytic activity of NK cells, stimulation of cytokine (IFN-γ) synthesis, and prolongation of NK cell survival (23, 24). IL-15 is also a potent T cell growth factor that enhances the survival, activation, IFN-γ production, and cytotoxicity of CD8+ T cells and has an important role in regulating CD8+ T cell homeostasis (25). IL-15 regulates both proliferation and Ab production by activated B cells (26), while mast cells proliferate in response to IL-15 and produce functionally active IL-4 (27). Monocytes, macrophages, and dendritic cells are the major source of IL-15 production. IL-15 exerts an autocrine activity on monocytes by inducing IL-8 and MCP-1 production (28) and monocyte differentiation to dendritic cells (29).

The IL-15 gene has been cloned from a number of mammalian species and recently in birds (30). We identified the first fish IL-15 sequence in rainbow trout (Oncorhynchus mykiss), and analysis of a λ genomic clone containing the *IL-15* gene which revealed that *IL-15* is linked closely to a BCL10 gene, which is essential for NF-κB activation by TCRs and BCRs (35), and an INPP4B gene which encodes the inositol polyphosphate 4-phosphatase type II, one of the enzymes involved in phosphatidylinositol signaling pathways (36). Trout IL-15 expression is modulated by recombinant trout IFN-γ (rtIFN-γ) but not rtIL-1β. We next produced and purified bioactive rtIL-15 from *Escherichia coli* and found that rtIL-15 can induce IFN-γ expression in leukocytes from spleen but not from head kidney of normal fish.

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amplification of 5'-end (RLM-RACE) (GeneRacer kit; Invitrogen Life Technologies) as described previously (47). 3'-RACE using forward primers F1 and F2 (Table I, Fig. 1) resulted in a 0.8-kb product that when sequenced contained the C terminus and the 3'-UTR. The resulting 0.9-kb product was cloned and sequenced and found to contain the 5'-UTR and complete coding region.

**Isolation and sequencing of a genomic clone of IL-15**

A rainbow trout genomic library constructed with 

\[ \text{Isolation and sequencing of a genomic clone of IL-15} \]

\[ \text{Expression of IL-15 transcript in vivo} \]

Initial analysis of IL-15 transcript expression by RT-PCR (50) revealed that IL-15 expression is detectable in all the cDNA samples from tissues and cell lines examined. To further quantify the expression of IL-15, six healthy rainbow trout (average, 130 g/fish) were killed and tissues (gills, skin, muscle, liver, spleen, head kidney, intestine, and brain) were removed for RNA preparation using RNA STAT-60 (AMS Biotechnology). The total RNA concentrations were quantified using a Nanodrop Spectrophotometer (NanoDrop Technologies). Thirty micrograms of RNA was reverse transcribed into cDNA using BioScript (Bioline) in 30-μl reactions. The resulting cDNA was diluted in 500 μl of TE buffer (pH 8.0). Four microliters of the resultant cDNA was used for quantitative PCR (Q-PCR) detection of expression of IL-15 (EF1-α, for 4 h with RTS-11 cells) using primers as described in Table I. The Q-PCR was detected with CYBR green (Invitrogen Life Technologies) using DNA Engine Opticon-2 (MJ Research) and quantified by comparing with a serial dilution of standard samples of cloned and linearized plasmid DNA in the same run. The relative expression level in different tissues was obtained by first normalizing the expression level relative to that of EF-1α and then comparing against the average expression level of IL-15 in liver (defined as 1) where the expression level was low but detectable in all fish.

**rIFN-γ and IL-1β stimulation**

The preparations of trout rIFN-γ and rIL-1β were as described by Zou et al. (51) and Hong et al. (52), respectively. RTG-2 and RTS-11 cells were prepared 2 days before stimulation by seeding at 2.5 × 106 cells (RTG-2) or 4 × 106 cells (RTS-11) in 25-cm2 cell culture flasks (Nunc) in 5 ml of complete medium as described above. The cells were stimulated with different amounts of the trout rIFN-γ and rIL-1β, for 4 h with RTS-11 cells and for 5 h with RTG-2 cells, and total RNA was then prepared and cDNA synthesized as described above. The expressions of the IL-15 gene, as well

FIGURE 1. Nucleotide and deduced amino acid sequences of the compiled trout IL-15 cDNA (Accession No. A355868). The upstream ATG codons, the start and stop codons, the two potential N-glycosylation sites (NXX/T), a potential NLS (HRKKH) and the poly(A) signal (AATAAA) are in bold and underlined. The predicted signal peptide is highlighted and in bold italics. The primer binding sites for 3'-RACE (F1 and F2) and 5'-RACE (R1 and R2) are underlined and directions marked by arrows.
FIGURE 2. Multiple alignment of the predicted trout IL-15 translation with known IL-15s based on the gene organization of trout, human, mouse, and rat. Gaps introduced to increase identity are shown by dashes. The exons are numbered according to the trout sequence. The highlighted features include the four conserved cysteine residues forming two potential disulphide bonds, the IL-15R/β3-binding site, the NLS and the 4-aa repeat/insert (LERL/LELR) in the C terminus of trout IL-15.
as the inflammatory cytokine IL-1β (isoform I), and the anti-inflammatory cytokine IL-11 (53), were detected by real-time PCR and normalized to the expression of EF-1. The relative expression of each gene is arbitrary fold change relative to the control.

Northern blot analysis
Total RNA was isolated from the RTS-11 cell line using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. Northern blot analysis was performed as described previously (54). In each experiment, 10–25 μg of total RNA/lane was transferred from a 1.1% formaldehyde-MOPS agarose gel to nylon membranes by capillary action and hybridized overnight at 65°C with a32P-labeled 298-bp cDNA probe purified from a trout IL-15 PCR fragment amplified using primers EF1 and ER1 (Table I). Following stringent washing, membranes were put into an x-ray cassette with intensifying screens and film (Kodak) and exposed for 2 days.

Alternative splicing variants (SVs) of the trout IL-15 gene and their expression
Primers were designed across all the exons of the trout IL-15 gene to amplify PCR products from cDNAs from eight trout tissues (spleen, head kidney, liver, intestine, gills, skin, muscle, and brain) and four trout cell lines (RTG-2, RTS-11, CL-6, and RTL), and the melting curve of the amplified products were examined. The PCR products, amplified using primers IL-15svF and IL-15svR that are located at the first and final exons, respectively, from cell lines CL-6, RTS-11 and liver tissue, have multiple peaks in melting curve analysis. The products were cloned to pGEM-T easy vector (Promega) and four groups of clones with distinct sizes of inserts were sequenced and allowed three alternative SVs to be identified. To study the expression of these SVs, specific primers were synthesized (see Table I) for Q-PCR analysis with the tissue samples described above. IL-15 F+R can only amplify the authentic IL-15 transcript as IL-15F crosses intron II and IL-15R crosses intron IV. IL-15sv1R crossing exons 4 and 2, in combination with IL-15EF1 (located in exon 1), can only amplify transcripts without exon 3 (SV-1). IL-15sv2F spanning exon 2 and exon 3, and IL-15sv3F spanning exons 1 and 3, when used in combination with IL-15R, can only amplify SV-2 and SV-3, respectively. A common reference was constructed by mixing equal amounts of plasmids of each variant and was used for Q-PCR quantification.

rtIL-15 production and purification
The cDNA sequence encoding the mature trout IL-15 was amplified from a cDNA clone using Pfu DNA polymerase (Promega) with primers IL15RF: (g ctg tac aaa GCT GAA ACA CAC GGG ATG) and IL15RR (cg tgt aca TTA ACT GAC AGT TTG CCC TAT TC, the BsrGI site was underlined). The resulting amplification was digested with BsrGI and ligated to pET160/DEST (Invitrogen Life Technologies) that was also digested with BsrGI and dephosphorylated. Plasmid DNA was prepared and sequenced to confirm that the construct produced a 162-aa fusion recombinant protein of 18.7 kDa with a N-terminal 6xHis tag for purification and a Lumio tag for specific detection of recombinant protein using Lumio Green Detection kit (Invitrogen Life Technologies).

A plasmid pET160/IL15 was subsequently transformed into BL21 star (DE3; Invitrogen Life Technologies) and the expression of rtIL-15 was induced by 1 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h at 37°C with vigorous shaking. The rtIL-15 was purified under denaturing conditions using a His-Selected Nickel Affinity Gel (Sigma-Aldrich), refolded on the column (55), and eluted with PBS containing 200 mM imidazol. The buffer of the eluted rtIL-15 was changed to PBS containing 40% glycerol using an iCON concentrator (9 kDa cut-off; Pierce), sterilized by 0.22-μm filtration and stored at −20°C ready for use. The induction of expression and purification of the rtIL-15 was analyzed using a 4–12% SDS-PAGE gel and detected first with the Lumio reagent (Invitrogen Life Technologies) and subsequently by staining with Brilliant Blue R (Sigma-Aldrich).
Preparation of spleen and head kidney leukocytes and stimulation with rtIL-15

Spleen and head kidney were aseptically removed from freshly killed trout (300–500 g) and placed in universal tubes containing incomplete medium (L-15 plus 100 U/ml penicillin plus 100 μg/ml streptomycin plus 0.5% FBS). The tissues were gently pushed through sterile 100-μm mesh screens and the screens were rinsed with incomplete medium. The resulting cell suspension was layered on 51% Percoll (Pharmacia) and centrifuged at 400 × g for 20 min. Cells at the medium/51% Percoll interface were collected, washed twice in incomplete medium by centrifugation at 200 g for 5 min, and the leukocytes were then resuspended in complete medium (the same as incomplete medium except with 10% FCS) at 0.5–1 × 10^6 cells/ml. Six milliliters of cells were seeded into 25-cm² cell culture flasks, and incubated at 20°C. For rtIL-15 stimulation, rtIL-15 was added to freshly prepared or in vitro (overnight) cultured leukocytes and incubated at 20°C for different times before total RNA preparation and cDNA synthesis as described above. The expression of IFN-γ, IL-1β, and IL-11 were detected by real-time PCR as described above.

Statistical analysis

Real-time quantitative PCR measurements were analyzed using the non-parametric Mann-Whitney tests within the SPSS package 15.0, with p < 0.05 between treatment groups and control groups considered significant.

Results

Cloning and sequence analysis of trout IL-15

A SSH clone from a gill SSH library, prepared from bacterial challenged rainbow trout, was identified with homology to mammalian IL-15. Based on this clone, primers were designed (Table I) and two overlapping PCR products were obtained using 3’- and 5’-RACE, that contained the full-length trout IL-15 cDNA (Fig. 1). The transcript contains 1,207 bp with an open reading frame of 193 aa. The 202-bp 5’-UTR contains seven out-of-frame AUG initiation codons while the 423-bp 3’-UTR contains a polyadenylation signal 16 bp upstream from the poly(A) tail. The translated molecule is 22,715 Da with a theoretical isoelectric point of 6.19, and contains two potential N-linked glycosylation sites (Fig. 1). An unusually long LSP of 65 aa was predicted (56). Thus, the predicted mature trout IL-15 generated following cleavage of the signal peptide is 128 aa with a calculated molecular mass of 15,050 Da and a theoretical isoelectric point of 5.16.

In view of the gene organization of trout IL-15 (described later), a multiple amino acid alignment with other known IL-15 molecules, including mammalian, bird, and other recently identified fish IL-15, was constructed according to the encoding exons (Fig. 2). It showed that good conservation was seen in the mature peptide, including the four cysteine residues forming the two potential disulphide bonds and one of the two IL-15Rα binding sites identified in the human IL-15 molecule (57) which were conserved in all the IL-15 molecules. Although the mammalian IL-15 molecules are highly homologous, there are apparently several unique features in the other vertebrate IL-15 molecules. These include the even longer signal peptide in fish and birds compared with the unusually long 48-aa mammalian signal peptide, as a result of the longer coding region in the first coding exon. There is also a 4-aa repeat/insert (e.g., LERL/LELI in the trout sequence) in the C terminus of the fish IL-15s (Fig. 2).

Although there is no nuclear localization signal (NLS) found in other IL-15 molecules, a “pat4” type NLS (58) (HRKK or RKKK, Figs. 1 and 2) was identified in the trout IL-15 molecule by PSORT (59). A Hopp-Woods hydrophilicity analysis (60) predicted that the pat4 NLS in the fish IL-15 sequence is in the hydrophilic region and should be accessible (61). The trout IL-15 sequence shows low homology to other IL-15 molecules with an overall amino acid sequence identity/similarity of 24%/44% (Fig. 3). It also shows a similar homology to the avian IL-2s and some of the mammalian IL-2s. To define the relationship of trout IL-15 to other IL-15/IL-2 molecules, a phylogenetic tree was constructed by the neighbor-joining method and bootstrapped 1000 times. This tree (Fig. 3) clearly grouped the trout and chicken IL-15s with the mammalian IL-15s and separated from all of the IL-2 molecules.
Gene organization of the trout IL-15 and gene synteny analysis

A 10,042-bp sequence containing the complete gene/genomic sequence that is identical with the trout IL-15 cDNA sequence and its 5'- and 3'-flanking region was fully sequenced and deposited with the EMBL, GenBank, and DDBJ nucleotide sequence databases under Accession No. AJ628345. The trout IL-15 gene is 6,073-bp long and contains six exons and five introns (Fig. 4). All the intron/exon boundaries in the trout sequence conformed strictly to the known GT/AG donor/acceptor site rule and all the introns are phase 0 except intron II that is phase I. The trout IL-15 sequence was compared with the four known IL-15 genes from human (Ensembl Gene ID: ENSG00000164136), mouse (Ensembl Gene ID: ENSMUSG00000031712), rat (Ensembl Gene ID: ENSRNOG00000033439), and chicken (Ensembl Gene ID: ENSGALG0000009870) (Table II and Fig. 4). The IL-15 genes from other species are longer, with >32 kb compared with only 6 kb in trout, and have one to three more exons and large introns in the 5'-UTR. Nevertheless, all the IL-15 genes transcribed to mRNAs with comparable size, although different SVs do exist. The IL-15 proteins are encoded by the final six exons in all the species. The trout IL-15 gene is located on chromosome 1 and was originally found to be involved in the t(1;14)(p22;q32) MALT lymphoma translocation (62). Human BCL10 has a four exon/three intron structure (Ensembl Gene ID ENSG00000142867). There is no report that BCL10 is linked to the IL-15 gene in other species.

The relationship between the IL-15 gene and INPP4B gene is shown schematically in Fig. 5. Thus, the trout IL-15 gene is closely linked to the INPP4B gene compared its mammalian counterparts. In addition, the trout IL-15 gene is also linked with a BCL10 gene that is not linked to mammalian IL-15. These linkages between IL-15 and INPP4B genes, and IL-15 and BCL10 genes have also been confirmed by PCR using primers located in the coding regions of these genes, and genomic DNA from 15 fish (data not shown).

Analysis of trout IL-15 promoter

A sequence fragment containing 750-bp 5'-flanking region, the first exon and part of the first intron was analyzed for transcription

<table>
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<th>Mouse</th>
<th>Chicken</th>
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<td>2</td>
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<td>5</td>
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<td>1,253</td>
<td>815</td>
<td>1,186</td>
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</table>

Table II. Number of base pairs in the exons and introns of the IL-15 genes in trout, human, mouse, rat, and chicken

Numbers refer to the noncoding region of the exons. All numbers without superscripts in the exon size panel refer to the sizes of the coding regions. Numbers in bold and underlined indicate the second coding exon.

* The phase I intron in the coding region. Note that all the introns in the coding region are phase 0 except the second intron within the coding region of the gene that is phase I.

* Numbers refer to the 5'-flanking region of the exons. All numbers without superscripts in the exon size panel refer to the sizes of the coding regions. Numbers in bold and underlined indicate the second coding exon.
factor binding sites using a SIGNAL SCAN program. The important transcription factor binding sites identified include 3 AP-1 and 1 AP-3 sites, 1 α-IFN.2 site (63), 13 IFN-γ-responsive element (γ-IRE) sites (64), 10 GM-CSF sites (repeated sequence CATT(A/T) required for GM-CSF promoter activity) (65), 2 GCF (66), glucocorticoid receptor (GR), NF-IL-6 and NF-κB binding sites (67), 1 c-Myc and 1 Myb (68) binding site (Fig. 6A). Like mammalian IL-15 genes (69, 70), the trout IL-15 gene also has no conventional TATA box upstream of the transcription start site.

Comparative promoter analysis showed that most of these potential transcription factor binding sites found in the trout IL-15 gene are also present in other species (Fig. 6B). Multiple potential binding sites for γ-IRE, SP1, and NF-IL-6 were present in the promoters of all three species analyzed. There were two potential NF-κB binding sites present in the 5’-flanking region of the human IL-15 gene, but only one in trout and none in zebrafish using the first 1000 bp (including the first exon) of trout, zebrafish (BX957281), and human (NT_016354.18) are shown.

FIGURE 6. Analysis of the promoters of IL-15 genes. A. Sequence analysis of the promoter of the trout IL-15 gene; 750 bp of the 5’-flanking region, the first exon (shaded), and part of the first intron of the trout IL-15 gene are shown. The transcription factor binding sites α-IFN.2 (AARK GA), AP-1 (TGANTMA), AP-3 (TGTGWW WW), c-Myb (CMGTTR), c-Myc (TCTCT TA), γ-IRE (CWKKANNY), GCF (SGGSS SC), GM-CSF (CATTW), GR (TGTCCT), NF-IL-16 (TKNNNGAAK), NF-κB (GGGR NTYYCY) are highlighted and underlined except the GM-CSF site within the γ-IRE site that is also italicized. Arrow indicates the translation start. B. Comparative analysis of potential transcription factor binding sites among IL-15 genes from trout, humans, and zebrafish. The potential binding sites for NF-κB (⊗), γ-IRE (■), SP1 (△), and NF-IL-6 (□) in the first 1000 bp of sequence (including the first exon) of trout, zebrafish (BX957281), and human (NT_016354.18) are shown.

FIGURE 7. In vivo expression of the IL-15 transcript. The relative expression level of IL-15 in different tissues was normalized first with the expression level of EF-1α, and then compared with the average expression level in liver where the expression level was low and defined as 1. The results represent the mean ± SEM of six fish.
Further analysis revealed a single potential NF-κB binding site 3.5 kb upstream of the first exon of the zebrafish IL-15 gene (data not shown). Multiple sites for AP-1, GR, and GCF are also present in the human and zebrafish promoters.

**Trout IL-15 is broadly expressed in tissues and cell lines**

RT-PCR was initially used to examine the expression of the trout IL-15 gene in tissues and cell lines and revealed that IL-15 expression was detectable in all the tissues and cell lines examined. The expression of IL-15 in vivo was further quantified by real-time PCR using tissue samples from six individual fish. The expression of the housekeeping gene EF-1α among all the tissues was almost constant in the same tissue among individuals but its expression was lower in muscle and brain as indicated by a higher crossing point (ct) (of 13–14) relative to the other tissues (count of ~11). The expression of IL-15 was detectable by Q-PCR in all the tissues examined. IL-15 was most highly expressed in spleen, with considerable levels in gills, head kidney, and intestine (Fig. 7). Because the expression of IL-15 was normalized to the expression of EF-1α and EF-1α expression in muscle and brain was low, this explains why the relative expression of IL-15 in muscle and brain appears high.

**Trout IL-15 is up-regulated by rtIFN-γ but not by IL-1β**

Multiple γ-IRE and NF-κB sites present in the promoter region of the trout IL-15 gene imply that trout IL-15 could be modulated by IFN-γ and IL-1β, as IL-1β can activate NF-κB in trout cells (47).
To test this hypothesis, rIFN-γ and IL-1β (isorm 1) were added to RTS-11 cells for 4 h and to RTG-2 cells for 5 h and the expression of IL-15, as well as IL-1β and IL-11, were detected by real-time PCR. rIFN-γ increased the expression of IL-15 × 5- to 6-fold at all the doses (1–100 ng/ml) tested in both cell lines (Fig. 8, A and C). No difference in the expression of IL-1β was detectable in either cell line stimulated with IFN-γ (Fig. 8, A and C); however, IFN-γ decreased the expression of IL-11 in the monocyte/macrophage RTS-11 cells (Fig. 8C). rIL-1β only had a limited effect on the expression of IL-15, with a 2-fold increase of IL-15 expression seen in RTG-2 cells, and <2-fold increase in RTS-11 cells at all the tested concentrations (Fig. 8, B and D). The effectiveness of IL-1β stimulation was confirmed by the up-regulation of the expression of IL-1β itself and IL-11 seen in both cell lines (Fig. 8, B and D). Thus, IFN-γ selectively up-regulates the expression of IL-15 but IL-1β only has a limited role on IL-15 expression in RTG-2 and RTS-11 cells. Isolated head kidney leukocytes showed the same pattern of IFN-γ and IL-1β action on the expression of the IL-15 transcript (data not shown).

Alternative SVs of the trout IL-15 gene and their expression

Although there is always a major band amplified by PCR from the cDNAs from tissues and cell lines examined, melting curve analysis revealed that additional peaks were amplified from some cDNAs, e.g., cDNAs from CL-6, RTS-11, and liver. Sequence analysis of the PCR products using primer pair IL-15svR and R revealed three alternative SV existed for the trout IL-15 gene (Fig. 9A). IL-15 SV-1 has the third exon spliced out and thus the transcript is 118 bp shorter. IL-15 SV-2 has an additional exon located in the second intron (E2'). This exon has 94% identity to the cloned genomic sequence reported in this article and also present in other unrelated fish genes with similar identities (e.g., Ay78595, Salmo salar zonadhesin-like gene). This sequence may represent an old reposition. The IL-15SV-2 cDNA sequence only shares 97% identity to the IL-15 cDNA when the additional E2‘ is removed. Thus, the transcript of IL-15SV-2 may be from a pseudogene of IL-15. IL-15 SV-3 has the second exon spliced out but the fifth intron retained. All the alternative splicings result in a premature stop of translation of the putative IL-15 protein.

The authentic IL-15 mRNA is expressed at a higher level relative to the alternative SVs in all eight tissues examined, although the three alternative SVs are all detectable in the tissue samples (Fig. 9B). SV-1 and SV-2 are expressed at 1.8 ± 0.3, and 4.8 ± 1.6(mean ± SD)% of the level of the authentic IL-15 mRNA (Fig. 9C). SV-3 is expressed at a relatively higher level and closer to the authentic IL-15 mRNA expression level. Northern blot analysis revealed that only one major band was detectable using RNA prepared from RTS-11 cells (Fig. 9D).

**Production and purification of rIL-15**

To study the function of trout IL-15, a fusion recombinant protein, containing a N-terminal 6 his-tag for purification, a Lumio tag for detection, and the predicted mature trout IL-15 in the C-terminal was produced in *E. coli* as an inclusion body. The rIL-15 was highly induced by IPTG induction, purified under denaturing conditions and then renatured on the column (Fig. 10).

**rIL-15 selectively up-regulates the expression of IFN-γ in leukocytes from spleen but not head kidney**

Mammalian IL-15 stimulates macrophages and NK cells to produce IFN-γ (10). rIL-15 (200 ng/ml) was added to the macrophage cell line RTS-11, and head kidney leukocytes enriched for macrophages, but no effect on the IFN-γ transcript was detectable (data not shown). However, addition of rIL-15 to leukocytes prepared from spleen consistently showed an increase of IFN-γ expression (Fig. 11). In contrast, rIL-15 had no
significant effect on the expression of IL-1β in spleen or head kidney leukocytes.

The up-regulation of IFN-γ by rtIL-15 was studied in detail in splenic leukocytes. Overnight cultures of splenic leukocytes were stimulated with 200 ng/ml rtIL-15 and the stimulation was terminated at 1, 3, 6, 9, 12, and 24 h after addition of rtIL-15. The up-regulation of IFN-γ expression by rtIL-15 was seen after 1 h stimulation with rtIL-15 and reached a plateau at 3 h that lasted for at least a day after stimulation (Fig. 12). The modulation of IFN-γ expression varied among individuals, as shown in a dose response study where cells from different fish were stimulated individually with rtIL-15 for 6 h (Fig. 13). Nevertheless, splenic leukocytes from all the fish (17 fish) tested showed an increase of IFN-γ expression after rtIL-15 stimulation. The increase of IFN-γ expression was also confirmed at the protein level by Western blotting using a mAb to rtIFN-γ.

FIGURE 13. Dose-dependent up-regulation of IFN-γ expression by rtIL-15. Splenic leukocytes were incubated with different amounts of rtIL-15 for 6 h and total RNA was prepared for real-time RT-PCR analysis as described in Fig. 11. The relative expression of three individual fish is shown.

Discussion

In this study, we have first characterized the IL-15 gene in rainbow trout (O. mykiss). We then investigated the expression and modulation of IL-15 transcript expression in different tissues and cell lines. We have also identified three alternative SVs of the trout IL-15 gene in rainbow trout. Overnight cultures of splenic leukocytes were stimulated with 200 ng/ml rtIL-15 and the stimulation was terminated at 1, 3, 6, 9, 12, and 24 h after addition of rtIL-15. The up-regulation of IFN-γ expression by rtIL-15 was seen after 1 h stimulation with rtIL-15 and reached a plateau at 3 h that lasted for at least a day after stimulation (Fig. 12). The modulation of IFN-γ expression varied among individuals, as shown in a dose response study where cells from different fish were stimulated individually with rtIL-15 for 6 h (Fig. 13). Nevertheless, splenic leukocytes from all the fish (17 fish) tested showed an increase of IFN-γ expression after rtIL-15 stimulation. The increase of IFN-γ expression was also confirmed at the protein level by Western blotting using a mAb to rtIFN-γ, where IFN-γ was only detected in rtIL-15-stimulated splenic leukocytes (our unpublished data). rtIL-15 was also found to up-regulate the expression of the IL-12β chain in splenic leukocytes (our unpublished data).

In this study, we have first characterized the IL-15 gene in rainbow trout (O. mykiss). We then investigated the expression and modulation of IL-15 transcript expression in different tissues and cell lines. We have also identified three alternative SVs of the trout IL-15 gene and studied their expression. Finally, we have produced the bioactive rtIL-15 and show it can up-regulate the expression of IFN-γ in splenic leukocytes.

Although the translation of the cloned sequence only showed low homology (23–30% sequence identities) with the IL-15s from other species, six lines of evidence presented here suggest that the gene cloned in this study is a fish homolog of IL-15. First, there are seven out-of-frame AUG initiation codons in the 202 bp 5′-UTR of the trout sequence (Fig. 1). IL-15s in mammals contain multiple upstream AUGs (5 in mouse, 12 in human) that have been proposed as negative regulators of gene expression (13). The chicken IL-15 reported also contains at least two upstream AUGs (30). Second, the trout translation has a predicted extraordinarily long LSP of 65 aa. The IL-15s in mammals and chicken also have a LSP of 48 aa (in mammals) or 66 aa (in chicken). In contrast to most signal peptides that are encoded by a single exon, the signal peptides of IL-15 in trout, chicken, and mammals are all encoded across three exons (Figs. 2 and 4). Third, there are well-conserved residues of defined function in the multiple alignment of the IL-15 sequences (Fig. 2), including 10 highly conserved cysteine residues, which have been postulated in mammals to be important in the formation of correctly folded molecules (13), and one of the two IL-15Rα binding sites identified in the human IL-15 molecule (57). Fourth, all the IL-15 proteins, including the trout IL-15, are encoded by six exons, and all the introns in the coding region are phase 0 except the second one that is phase 1 (Table II). Fifth, the trout IL-15 has a broad tissue distribution of expression and could be detected in all the tissues and cell lines examined. The mammalian and chicken IL-15 molecules also show a wide distribution of expression in tissues and cell types (2, 30). Lastly, an unrooted phylogenetic tree (Fig. 3) clearly grouped the trout IL-15 with the IL-15 sequences from other species and separate from the IL-2 molecules.

The unique characteristics of trout IL-15 include a 4-aa repeat (LERL/LERI) encoded by the final exon (Fig. 2) and a putative NLS in the fifth exon. The (LERL/LERI) repeat might be a fish IL-15-specific feature as this repeat can be identified in all the fish IL-15 molecules (Fig. 2). Classical NLS sequences incorporate regions enriched in basic aa and generally conform to one of three motifs (58). The pat4-type NLS consists of a continuous stretch of four basic amino acids (K or R) or three basic amino acids associated with histidine (H) or proline (P). Although there is no NLS found in other IL-15 molecules, a pat4-type NLS (HRKK or RKKH, Figs. 1 and 2) was identified in the trout IL-15 molecule by PSORT. A protein containing NLS sequences may remain cytoplasmic, especially if the NLS sequence is blocked or buried within the protein (61). Hydrophobicity analysis revealed that this NLS is in a hydrophilic region and could be accessed and functional. NLSs are short polypeptide sequences that affect nuclear targeting of the proteins carrying them, and have been identified in cytokines such as IFN-γ (71), IL-5 (72), and other STAT using ligands or receptors (73). It was proposed that putative NLS-bearing ligands and/or their receptors might play a direct role in nuclear translocation of transcription factors such as STATs that do not contain a polycationic NLS (73). The NLS of IFN-γ is involved in complexing STAT1α and nuclear importin α for nuclear translocation of STAT1α, and is crucial for the biological properties of IFN-γ (73). It is known that STAT3/5/6 are involved in IL-15 signaling in mammals (74) and thus the NLS in trout IL-15 might function to assist the translocation of the STAT3/5/6 transcription factors. Multiple SVs have been reported in both human and mouse resulting in two isoforms of IL-15 molecules being translated (75). The LSP isoform of human IL-15 is secreted through the ER/Golgi pathway (76) and is regulated by a different intracellular trafficking pathway (15). In contrast, the SSP isoform is not secreted but rather is stored intracellularly and appears in the nuclear and cytoplasmic components. It is speculated that the intracellular form may serve as a reservoir of IL-15 protein, being released on damage, or that it plays other novel as yet undefined roles within the cells that produce it (16). The NLS in the trout IL-15 might also function to translocate itself to the nucleus after being translated.

RLM-RACE uses T4 RNA ligase to join cellular RNA with a defined ribo-oligonucleotide that marks and preserves the termini of cellular RNA molecules and can be used to analyze the 5′ terminus of a mRNA (77). Using the same batch of cDNA prepared in this article, we have successfully obtained the full-length 5′ end of the trout IL-1β and defined the transcription start site (47). The cloned IL-15 cDNA sequence matched perfectly with the genomic sequence, and Northern blot analysis revealed that a 1.2-kb transcript was detectable (Fig. 9D). This suggests that the cDNA sequence reported in this article is the full-length trout IL-15. Comparison of the cDNA sequence with the genomic sequence obtained, revealed that the trout IL-15 has a unique six exon/five intron organization, compared with IL-15s from other vertebrates which have nine (rat), eight (human and mouse), or seven (chicken) exons. The fish IL-15 gene is the smallest among all the...
known IL-15s, being 6 kb compared with 32 to 97 kb in other species. However, the large sizes of IL-15 genes in other species are mainly contributed by additional exons and large introns in the 5′-UTR. Although the sizes of all the common introns in the coding region are generally larger in other species, all the IL-15s have a transcript with comparable size (Table II).

Multiple transcripts exist for mammalian IL-15 genes. The human IL-15 gene is transcribed into at least four transcripts that encode two functional IL-15 proteins with a LSP and SSP, respectively. Three alternative transcripts have also been identified for the trout IL-15 gene but all the alternative transcripts result in a premature stop of translation, and thus no functional protein is expected. Although expressed at a low level, all the alternative transcripts are detectable in the eight tissues examined, and perhaps represent components of complex regulatory mechanisms to generate the functional protein.

PI3Ks catalyze the phosphorylation of the 3-OH position of the inositol head groups of the PI lipids, namely phosphatidylinositol (PtdIns), PtdIns(4)P, and PtdIns(4,5)P2, resulting in the formation of PtdIns(3)P, PtdIns(3,4)P2, and PtdIns(3,4,5)P3, respectively. PtdIns(3,4)P2 and PtdIns(3,4,5)P3 are generally absent from resting cells but increase intracellularly upon stimulation via a variety of receptors and function as second messengers (78). The PI3K pathway has been well-documented in the signaling of IL-15 (79). Two isoforms of the INPP4 gene, namely INPP4A and B, which encode the inositol polyphosphate 4-phosphatase with similar enzymatic properties, have been isolated in mammals. This enzyme removes the phosphate group at position 4 of the inositol ring, preferentially from PtdIns(3,4)P2 (36). INPP4A has been reported as a regulator of cell proliferation, linked to its ability to dephosphorylate PtdIns(3,4)P2 (80). Despite the difference of intergenic distance, the linkage between IL-15 and INPP4B genes and transcription direction is well-conserved. This conservation suggests that these two genes might be linked functionally, with INPP4B acting as a regulator of the PI3K pathway during IL-15 signaling.

The human BCL10 gene resides on chromosome 1, has a four exon/three intron organization (Ensembl Transcript ID ENST00000271015), and is not linked to IL-15 or INPP4B. BCL10 genes from other species also have multiple exons and no clear physical linkage to IL-15 or INPP4B, as analyzed using the Ensembl database. The trout translation is from a continuous sequence without introns. Although there is a good conservation in the CARD domain with a sequence identity/similarity of ~58%/75%, the overall sequence homology is low, especially in the C terminus, with two deletions apparent. The ESTs only have 96% sequence identities with the genomic sequence and might be transcribed from a related gene at a different locus or from a BCL homolog with multiple exon/intron structure. Thus, it is possible that the trout BCL10 homolog that reside between the IL-15 and INPP4B genes could be due to a retrovirus event and may not be functional.

Computer analysis identified a number of potential sites for transcriptional activators (AP-1 and 3, anti-IFN-2, γ-IRE, GM-CSF, NF-IL-6, NF-kB, c-Myc, and Myb), as well as for repressors (GCF and GR), in the 5′-flanking region and 5′ end of the trout IL-15 gene. Indeed, γ-IRE may be involved in the up-regulation of IL-15 by IFN-γ as seen in this study. Comparative promoter analysis revealed that most of the sites are also present in the promoters of human and zebrafish IL-15 genes, suggesting that these transcription factors and pathways leading to IL-15 expression may be conserved across vertebrates. GCF is a transcriptional regulator that was found to repress transcription of the epidermal growth factor receptor and other genes and binds to GC-rich sequences (SCGSSC) (66). Two GCF-binding sites just upstream of the transcription start site of the trout IL-15 gene imply that GCF could be a repressor of IL-15 transcription in fish also.

Many different cell types express IL-15, although mammalian IL-15 is probably mainly produced by activated macrophages (6, 12). Although IL-15 is constitutively expressed in cells of the monocyte/macrophage lineage, the expression of IL-15 mRNA has been shown to be up-regulated by LPS, poly I:C, as well as bacterial (12, 81, 82) and viral (83) infection. The present results show clearly that IFN-γ is an important immune regulator of IL-15 in fish. rTLIFN-γ selectively increased IL-15 expression but had little effect on other cytokines such as IL-1β and IL-11. In contrast, rTIL-1β had little effect on IL-15 expression while increasing expression of itself dramatically.

Mammalian IL-15 mRNA is broadly expressed, however, it is extremely difficult to detect IL-15 in cell culture supernatants. The production of bioactive membrane bound or secreted IL-15 in mammals appears to be tightly controlled (81, 84). Enhanced IL-15 protein production results from either increased transcription and/or stability of IL-15 mRNA, posttranslational modification, or altered cellular trafficking, and enhanced release of already preformed intracellular IL-15 protein. The specific factors capable of inducing biologically meaningful IL-15 protein production have still to be identified in fish, as IL-15 is mainly regulated in a post-transcriptional manner in mammals. Nevertheless, in this study, we produced the rTL-15 protein and studied its biological activity.

Mammalian IL-15 is a pleiotropic cytokine that has a broad spectrum of biologic activities on different types of cells, including NK, NKT, T, B cells, macrophage/dendritic cells, neutrophils, and mast cells, and other nonimmune cells (10). Thus, it was expected that rTL-15 would affect most mixed leukocyte primary cultures. However, unexpectedly the rTL-15 preferentially stimulated splenic leukocytes from healthy fish, where it induced a large increase in IFN-γ expression, with little, if any, effect on IL-1β expression. This effect was quite long-lived and was still apparent 24 h poststimulation. Although the exact cell types being affected have still to be determined, it is clear that once produced IL-15 will have a profound effect on the ability of the fish immune system to activate antimicrobial defenses and genes induced themselves by IFN-γ.

Disclosures
The authors have no financial conflict of interest.

References


