Intron-Containing Type I and Type III IFN Coexist in Amphibians: Refuting the Concept That a Retroposition Event Gave Rise to Type I IFNs

Zhitao Qi, Pin Nie, Chris J. Secombes and Jun Zou

*J Immunol* 2010; 184:5038-5046; Prepublished online 31 March 2010;
doi: 10.4049/jimmunol.0903374
http://www.jimmunol.org/content/184/9/5038

**References**
This article cites 24 articles, 8 of which you can access for free at:
http://www.jimmunol.org/content/184/9/5038.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Intron-Containing Type I and Type III IFN Coexist in Amphibians: Refuting the Concept That a Retroposition Event Gave Rise to Type I IFNs

Zhitao Qi,*† Pin Nie,* Chris J. Secombes,‡ and Jun Zou‡

Type I and III IFNs are structurally related cytokines with similar antiviral functions. They have different genomic organizations and bind to distinct receptor complexes. It has been vigorously debated whether the recently identified intron containing IFN genes in fish and amphibians belong to the type I or III IFN family or diverged from a common ancestral gene, that subsequently gave rise to both types. In this report, we have identified intron containing type III IFN genes that are tandemly linked in the *Xenopus tropicalis* genome and hence demonstrate for the first time that intron containing type I and III genes diverged relatively early in vertebrate evolution, and at least by the appearance of early tetrapods, a transition period when vertebrates migrated from an aquatic environment to land. Our data also suggest that the intronless type I IFN genes seen in reptiles, birds, and mammals have originated from a type I IFN transcript via a retroposition event that led to the disappearance of intron-containing type I IFN genes in modern vertebrates. In vivo and in vitro studies in this paper show that the *Xenopus* type III IFNs and their cognate receptor are ubiquitously expressed in tissues and primary splenocytes and can be upregulated by stimulation with synthetic double-stranded RNA, suggesting they are involved in antiviral defense in amphibians. *The Journal of Immunology*, 2010, 184: 5038–5046.

Interferons are key cytokines that coordinate the host antiviral response in vertebrates. They consist of three groups, type I, II, and III IFNs, that share low sequence homology but have a similar protein structure consisting of multiple α helices, a structural backbone for IL-10 family cytokines (1). In mammals, it is known that they bind to three different receptor complexes, with IFN-αR1 and 2 interacting with type I IFNs, IFN-γR1 and 2 binding type II IFN, and IFN-AR and IL-10R2 responsible for type III IFN signaling. Type III IFNs, initially identified as IL-28/29 or IFN-λ, were discovered in human chromosome 1 by two different research groups (2, 3). Unlike human type I IFNs, type III IFNs contain four introns, typical of class II helical cytokines. However, type III IFNs possess similar antiviral properties to type I IFNs, despite binding to distinct receptors. To date, it is unclear why vertebrate hosts require two IFN systems with some degree of functional redundancy, although complementary functions between the two systems are beginning to emerge. For example, type I IFNs are able to elicit antiviral responses in all nucleated cells, whereas the type III IFNs are shown to act on a limited number of cell types, such as dendritic and epithelial cells. In addition, it has been suggested that type I and III IFNs could exert their antiviral functions at different stages of infection or against different types of viruses (4, 5).

Recent identification of the IFN genes from fish, amphibians, and reptiles has provided enormous information on IFN gene evolution (6–12). It has been generally accepted that the IFNs originated from an ancient progenitor, which gave rise to the IL-10 family cytokines early in evolution (13). However, the evolutionary origin of the type I and III IFN family has been heavily debated among comparative immunologists, in part because of the multi-intron containing IFN genes identified in fish and amphibians. Based on the protein sequence analysis, we concluded that vertebrate type I IFNs can be divided into two cytoste containing or four cysteine containing subgroups and hypothesized that fish IFNs are more similar to type I IFNs than to type III IFNs, and that the four cysteine containing IFNs, which have a conserved CAWE motif near the C terminus are likely to be the ancestral gene for the type I IFN family (7). However, it can be argued that several features of fish IFNs resemble type III IFNs. For example, unlike type I IFN genes that lack introns in amniotes, fish, and amphibian IFN genes possess the same gene organization as type III IFN genes from birds and mammals (8). From the receptor perspective, Levraud et al. (14) found the class II helical cytokine receptors (zCRFB2 and CRFB5) which zebrafish IFNs bound were structurally similar to type III IFN receptors, although these two receptors have been classified as type I IFN receptors by phylogenetic analysis in a recent study (15). Some have argued that identified fish and amphibian IFN genes could represent the ancestor for type I and III IFN genes in amniotes with modern intronless type I genes having arisen from a multigene containing fish or amphibian IFN gene via a retroposition event (16, 17). However, in this study, we present for the first time conclusive evidence that intron-containing type I and III IFN genes coexist in amphibians, by in silico analysis of the *Xenopus tropicalis* genome.
demonstrating that type I and III IFNs had diverged prior to the retroposition event that was proposed to have led to their divergence. Furthermore, we have identified the putative receptors for type III IFNs and have analyzed their expression after stimulation with polyinosinic-polycytidylic acid (poly(I:C)), a synthetic double-stranded RNA mimicking viral infection. Taken together with previous findings, we conclude that functional multi-intron–containing type I and III IFN genes had diverged by the appearance of early tetrapods during vertebrate evolution, a transition period when vertebrates migrated from an aquatic environment to land.

Materials and Methods

Animal maintenance

Frogs (X. tropicalis) were purchased from the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (Beijing, China) and acclimatized in a freshwater tank at 23˚C under natural photoperiod for 1 wk prior to experiments.

Isolation of Xenopus splenocytes

Spleen tissue was collected from freshly killed Xenopus under sterile conditions and washed with RPMI 1640 medium containing 5% FCS (Sigma-Aldrich, St. Louis, MO), 10 U/ml heparin, and 100 g/ml penicillin. Splenocytes were prepared according to the protocol described by Koniski and Cohen (18). Briefly, spleen tissue was dispersed by pushing it through a nylon mesh, and the resultant cell suspension carefully layered onto Histopaque 1119 reagent (Sigma-Aldrich) and then centrifuged for 30 min at 400 × g. The splenocytes were then collected from the interface, washed once with 5 ml RPMI 1640 medium and counted. The cells were cultured in 12-well plates for further use.

Gene cloning

Total RNA was extracted from the tissues or cultured cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. First-strand cDNA was synthesized using a Clontech SMART PCR cDNA synthesis kit (Clontech, Mountain View, CA).

The putative open reading frames were initially predicted by in silico analysis of the X. tropicalis genome (www.ensembl.org). Primers were synthesized based on the predicted sequences (Table I), to amplify the full length coding region of the IFN genes and the receptor genes by PCR. Briefly, each 25 µl PCR reaction contained 125 µM of each dNTP, 0.2 µM of each primer, 12 U Ex Taq polymerase (TaKaRa, Shiga, Japan), and 1 µl cDNA template. The PCR program was as follows: 1 cycle of 94˚C for 5 min, 35–40 cycles of 94˚C for 30 s, 54˚C for 30 s, and then 72˚C for 90 s, followed by a cycle of 72˚C for 10 min. The amplified PCR products were checked on an agarose gel and ligated into the pMD18-T vector (TaKaRa Shuzo). The ligation reaction was transformed into competent Escherichia coli DH5a cells (TaKaRa Shuzo) and positive clones were screened by colony PCR using universal vector primers M13F and M13R under the following conditions: 1 cycle of 94˚C for 5 min, 30 cycles of 94˚C for 30 s, 52˚C for 30 s, and then 72˚C for 30 s, followed by a cycle of 72˚C for 8 min. Plasmid DNA was extracted using a plasmid mini-preparation kit and sequenced using the dyeodeoxy chain-termination method on an automatic DNA sequencer (ABI Mode 377, Applied Biosystems, Foster City, CA).

A genome walking approach was applied to amplify the unknown regions of the type I IFN gene, this genomic DNA was from Xenopus laevis using a phenol extraction protocol. Briefly, ~10 mg of the liver was cut into small pieces and resuspended in 600 µl lysis buffer containing 10 mM Tris-HCl, 1 mM EDTA, and 0.1% SDS, pH 8.0. After homogenization on ice, 3 µl protease K was added into the solution and incubated at 55˚C for 4–6 h. RNase was then added and incubated for an additional 30 min. The lysis solution was cooled on ice for 5 min, mixed with 200 µl 250 mM potassium acetate/glacial acetic solution (1:8.7 v/v), and centrifuged at 13,000 rpm at 4˚C for 4 min. The supernatant was collected and DNA precipitated with an equal volume (600 µl) of isopropanol. The precipitated DNA was washed with cold 70% ethanol, dried, and dissolved in water. Genomic DNA (6 µg) was digested at 37˚C overnight with EcoRV, DraI, PvuII, or ScaI, respectively. After checking by electrophoresis, digested DNA was purified and ligated with adaptors (long adaptor: 5'-CTAGTATAGCTGCTAGTATAGCTGCTAGTATAGCTGCTAGTC-3', short adaptor: 5'-ACCAACCCCGCGC-3') at 16˚C overnight. The ligation reaction was terminated by incubation at 70˚C for 5 min. Nested PCR was performed under the following conditions: 1 cycle of 94˚C for 5 min; 6 cycles of 94˚C for 30 s, 68˚C for 30 s, and then 72˚C for 90 s; 30 cycles of 94˚C for 30 s, 68˚C for 30 s, and 72˚C for 90 s, followed by a cycle of 72˚C for 10 min. Primer pairs IFNL2WX1t/AP1 and IFNL2WXin/AP2 were used in the first and the second round PCR, respectively.

Sequence analysis

The protein sequences were deduced from the nucleic acid sequence using the programs on the Expasy Web site (http://ca.expasy.org/tools). Putative transcripts were predicted from the genome sequences using the GenScan program (http://genes.mit.edu/GENSCAN.html) and the Fgenesh program (www.softberry.com). Signal peptides were identified by the SignalP ver- sion 3.0 program (www.cbs.dtu.dk/services/SignalP). Multiple alignments were generated using the ClustalW program and edited with GeneDoc (www.nrbsc.org/gfx/genedoc/index.html). Homology between sequences was calculated using the MegAlign program within the DNASTAR package. Phylogenetic trees were constructed with the Neighbor-joining method in the MEGA4 software package (http://megasoftware.net/omega.html).

Real-time PCR

For in vitro expression analysis, primary splenocytes were prepared as described previously and stimulated for 4 h with 0.1, 1, 10, or 50 µg/ml poly (I:C). Cells were then harvested for RNA extraction and additional real-time PCR analysis. For in vivo expression analysis, each group of three frogs were injected i.p. with an equal volume of PBS or poly(I:C) (2 mg/300 g body weight) and 24 h later, animals were killed and tissues collected for RNA extraction and cDNA synthesis.

To generate the templates used to establish the standard curve for IFN-α, IFN-α1, IL-10R2, IFN-β, and β-actin, the cDNA fragments were amplified by PCR using primers listed in Table I and β-actin primers (GenBank accession no. NM_213719, www.ncbi.nlm.nih.gov/nucleotide/47498067). Amplicons were cloned into the pMD18-T vector and sequence confirmed. The concentrations of plasmid DNA were measured by spectrophotometry. The standard curve method for real-time PCR was performed according to the standard protocol of the ABI sequence detection system (version 1.9) using a Chromo 4 Continuous Fluorescence Detector from MJ Research (Foster City, CA). Briefly, serial 10-fold dilutions, for example, ranging from 10^{-8} to 10^{-2} input cDNA copies, were used in PCR for establishing a standard curve. Amplifications were carried out in a final volume of 20 µl containing 1 µl DNA sample, 10 µl 2× SYBR green I Master Mix (Toyobo, Osaka, Japan), 0.5 µl of each primer and 8 µl H2O. The PCR cycling program was: one cycle of 3 min at 95˚C, followed by 45 cycles of 30 s at 94˚C, 25 s at 56˚C, and then 25 s at 72˚C. Each sample was run in triplicate. The real-time PCR data were analyzed using Student t test in Microcal origin 6.0 software with p < 0.05 considered as significantly different between samples from control and treated groups.

Production of recombinant protein and biotesting

The cDNA encoding the predicted mature peptide of Xenopus IFN-α1 was amplified by PCR and inserted into the pQE30 vector (Qiagen, Hilden, Germany) at the BamHI and HindIII restriction enzyme sites. The recombinant protein was expressed in E. coli M15 cells and purified as described previously (7). The protein was quantified by the Bradford assay. The splenocytes were isolated as described previously and cultured in RPMI 1640 medium at 27˚C for 2 h. The cells were incubated with 10 µg/ml cycloheximide for 0.5 h to inhibit synthesis of endogenous IFNs and stimulated with 0.001, 0.01, 0.1, and 1 µg/ml IFN-α1 for 6 h. The cells, both attached and in suspension, were harvested for extraction of total RNA. A Xenopus homolog for IFN-induced protein with tetra- tricopeptide repeats 5 (IFTT-5) known to be induced by type I IFNs in mammals (19), was retrieved from the Xenopus genome database (Scaffold 271, Ensembl no. ENSTXETG00000002265, www.ensembl.org/Xenopus_tropicalis/Gene/Summary?g=ENSXETG00000002265) by homology analysis and used as a marker to assess the effect of the recombinant protein by real-time PCR.

Results

Cloning and sequence analysis of Xenopus IFN-α homologs

In silico analysis of the X. tropicalis genome predicted five tandem linked IFN-α genes (termed IFN-α1–5) in Scaffold 389. Cloning and sequencing of the PCR products spanning the full-length coding regions using primers listed in Table I revealed that IFN-α1, 2, 3, and 5 contained open reading frames, whereas IFN-α4 was a pseudogene with an early stop codon. The nucleotide sequences have been deposited in GenBank under accession numbers: FJ581033, FJ581034, FJ581035, and FJ581036 (www.ncbi.nlm.nih.gov/nucleotide/
Table I. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-L1-fullIF</td>
<td>GAAGCCATGGGAAAATTCTCTATTC</td>
<td>Gene cloning</td>
</tr>
<tr>
<td>IFN-L1-fullIR</td>
<td>GTTGTTGATGGATATCAT</td>
<td></td>
</tr>
<tr>
<td>IFN-L2-fullIF</td>
<td>AGCGATGGAAATTTCCCATTC</td>
<td></td>
</tr>
<tr>
<td>IFN-L2-fullIR</td>
<td>GTTGTTGATGGATATCAT</td>
<td></td>
</tr>
<tr>
<td>IFN-L3-fullIF</td>
<td>GAACGGATGGAAATTCTCTATTC</td>
<td></td>
</tr>
<tr>
<td>IFN-L3-fullIR</td>
<td>GAACGGATGGAAATTCTCTATTC</td>
<td></td>
</tr>
<tr>
<td>IFN-L5-fullIF</td>
<td>CCAAGACCCATGGAAATTTCC</td>
<td></td>
</tr>
<tr>
<td>IFN-L5-fullIR</td>
<td>CCAAGACCCATGGAAATTTCC</td>
<td></td>
</tr>
<tr>
<td>IFN-L1-fullIF</td>
<td>AAATGTTGGCCTGTGGTCC</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>IFN-L1-fullIR</td>
<td>GGAGGATCCATTTATGGGCA</td>
<td></td>
</tr>
<tr>
<td>IL-10R2-fullIF</td>
<td>CACGCAATGGTTCCTCTCGC</td>
<td></td>
</tr>
<tr>
<td>IL-10R2-fullIR</td>
<td>CACGCAATGGTTCCTCTCGC</td>
<td></td>
</tr>
<tr>
<td>IFN-L1-F1</td>
<td>CATGACGATGAAATTCTCTATTC</td>
<td>Genome walking</td>
</tr>
<tr>
<td>IFN-L5-F1</td>
<td>TACGCAGTGGGGAAATAAGTG</td>
<td></td>
</tr>
<tr>
<td>IFN-L5-R1</td>
<td>AGATCTACCTTCCAGCTATGG</td>
<td></td>
</tr>
<tr>
<td>IFN-L1-F1</td>
<td>TTCAATTTAGGCCAACACTTAG</td>
<td></td>
</tr>
<tr>
<td>IFN-L1-R1</td>
<td>GGGTGGATGGGCTGATT</td>
<td></td>
</tr>
<tr>
<td>IFN-3-F</td>
<td>CCACCGACCTCTCTTAATAC</td>
<td></td>
</tr>
<tr>
<td>IFN-4-R</td>
<td>GAAGCTCTCGTGGAACCTTTC</td>
<td></td>
</tr>
<tr>
<td>xactin-F</td>
<td>TTGTGTTGGTCTCTCTATGC</td>
<td></td>
</tr>
<tr>
<td>xactin-R</td>
<td>AGATGCTCTGGGAGATCTC</td>
<td>Production of recombinant protein</td>
</tr>
<tr>
<td>IL-10R2-F1</td>
<td>CGCGTGGAGAGCAGCATGG</td>
<td></td>
</tr>
<tr>
<td>IL-10R2-R1</td>
<td>TACGCTCTTGGTCTCTCTTA</td>
<td></td>
</tr>
<tr>
<td>IFTS-F</td>
<td>TACCATTGATGCTCTCCATC</td>
<td></td>
</tr>
<tr>
<td>IFT5-R</td>
<td>TCTGCTCTTGGTCTCTCTTA</td>
<td></td>
</tr>
<tr>
<td>IFN-L2w1out</td>
<td>GAAGCTGTTGGAGTCTCTCTAC</td>
<td></td>
</tr>
<tr>
<td>IFN-L2w1in</td>
<td>CACGGATCATGCTCCTCTCT</td>
<td></td>
</tr>
<tr>
<td>IFN-L2w2out</td>
<td>CAAGCATAGTGCTCCTCTCTA</td>
<td></td>
</tr>
<tr>
<td>IFN-L2w2in</td>
<td>TACGCTCTTGGTCTCTCTTA</td>
<td></td>
</tr>
<tr>
<td>IFN-L2w3out</td>
<td>CAGCTGAGCCAAAAGGAACAGAG</td>
<td></td>
</tr>
<tr>
<td>IFN-L2w3in</td>
<td>TTTCATCTCTTTGTGGTCTCT</td>
<td></td>
</tr>
<tr>
<td>AP1</td>
<td>GTATACACTCTACTATAGGG</td>
<td></td>
</tr>
<tr>
<td>AP2</td>
<td>ACTATGAGGACACCTGCTGT</td>
<td></td>
</tr>
<tr>
<td>xeExIFN-L-F</td>
<td>GAAGCTTACCTGGAATTTCTCTATTC</td>
<td></td>
</tr>
<tr>
<td>xeExIFN-L-R</td>
<td>GTCACGCTGGGCAAACACATATGCT</td>
<td></td>
</tr>
</tbody>
</table>

The nucleotide sequence of the identified IFN-λR1 was deposited in GenBank under accession number FJ581037 (www.ncbi.nlm.nih.gov/nuccore/256860239). The deduced open reading frame of the Xenopus IFN-λR1 protein contains 1572 bp, encoding a protein of 523 aa with a 21 aa putative signal peptide (Fig. 3A). Six glycosylation sites were predicted using NetNGlyc 1.0. A transmembrane region of 18 aa

256860231, 256860233, 256860235, and 256860237). The deduced peptides for IFN-λ1, 2, and 5 were 179 aa in length, with a predicted signal peptide of 19 aa (Fig. 1). The putative IFN-λ3 peptide was 25 aa shorter than the other three IFN-λs but also had a putative signal peptide of 19 aa. The Xenopus IFN-λs shared 20.0–29.8% identity with human and chicken type III IFNs, higher than that with known type I IFNs (13.4–21.2%) and type II IFNs (14.3–20.1% identity). The amino acid identity between IFN-λs and type I IFNs in Xenopus was 14.2–20.3% (7). The putative mature peptides of IFN-λs have been characterized in humans and are shown to contain two binding chains (IFN-λR1 and two signaling chains (IL-10R2), the latter also shared by IL-10 family members, including IL-10, -19, -20, -22, and -26 (20). Using human IFN-λR1 and IL-10R2 sequences as baits, BLAST analysis of the Xenopus genome database identified two candidate genomic loci within Scaffolds 403 and 1108, respectively. The nucleotide sequences of these two loci were analyzed by the GenScan program, giving rise to two putative transcripts coding for the putative IFN-λR1 and IL-10R2 proteins. Based on the predicted sequences, the full-length mRNA sequences were obtained by PCR using primers listed in Table I, and the deduced protein sequences are shown in Fig. 3. The nucleotide sequence of the identified IFN-λR1 was deposited in GenBank under accession number FJ581037. The deduced open reading frame of the Xenopus IFN-λR1 contained 1572 bp, encoding a protein of 523 aa with a 21 aa putative signal peptide (Fig. 3A). Six glycosylation sites were predicted using NetNGlyc 1.0. A transmembrane region of 18 aa

Cloning and sequence analysis of Xenopus IFN-λ receptor homologs

The receptors that bind IFN-λs have been characterized in humans and are shown to contain two binding chains (IFN-λR1) and two signaling chains (IL-10R2), the latter also shared by IL-10 family members, including IL-10, -19, -20, -22, and -26 (20). Using human IFN-λR1 and IL-10R2 sequences as baits, BLAST analysis of the Xenopus genome database identified two candidate genomic loci within Scaffolds 403 and 1108, respectively. The nucleotide sequences of these two loci were analyzed by the GenScan program, giving rise to two putative transcripts coding for the putative IFN-λR1 and IL-10R2 proteins. Based on the predicted sequences, the full-length mRNA sequences were obtained by PCR using primers listed in Table I, and the deduced protein sequences are shown in Fig. 3. The nucleotide sequence of the identified IFN-λR1 was deposited in GenBank under accession number FJ581037 (www.ncbi.nlm.nih.gov/nuccore/256860239). The deduced open reading frame of the Xenopus IFN-λR1 contained 1572 bp, encoding a protein of 523 aa with a 21 aa putative signal peptide (Fig. 3A). Six glycosylation sites were predicted using NetNGlyc 1.0. A transmembrane region of 18 aa
FAVLLFLPILLISGILLY) was seen between residue 253–270, separating the protein into an extracellular region of 229 aa without the leader peptide and an intracellular region of 250 aa. The extracellular region of IFN-β contained six conserved cysteine residues, with Cys96, Cys104, Cys215, and Cys237 potentially forming two disulfide bonds (Cys96 linking to Cys104 and Cys215 with Cys237, respectively). In the intracellular region, one of the two tyrosines (Y517 in human IFN-β) known to be essential for activation of STAT proteins in human IFN-β was also conserved within the C terminus of Xenopus IFN-β, whereas the other (Y343 in human IFN-β) was absent in the corresponding region of the Xenopus IFN-β (21).

The Xenopus IL10-R2 homologous gene contained an open reading frame of 927 bp and translated into a protein of 308 aa (GenBank accession number: FJ581040, www.ncbi.nlm.nih.gov/nuccore/256860245) (Fig. 3B). The IL10-R2 gene has seven exons, where exons 1–5 and the 5′ end region of exon 6 encode proteins in human IFN-α1 were also conserved within the C terminus of Xenopus IFN-α1, whereas the other (Y343 in human IFN-α1) was absent in the corresponding region of the Xenopus IFN-α1 (21).

The Xenopus IL10-R2 homologous gene contained an open reading frame of 927 bp and translated into a protein of 308 aa (GenBank accession number: FJ581040, www.ncbi.nlm.nih.gov/nuccore/256860245) (Fig. 3B). The IL10-R2 gene has seven exons, where exons 1–5 and the 5′ end region of exon 6 encode proteins in human IFN-α1 were also conserved within the C terminus of Xenopus IFN-α1, whereas the other (Y343 in human IFN-α1) was absent in the corresponding region of the Xenopus IFN-α1 (21).

**FIGURE 1.** Multiple alignment of type I and III IFNs from Xenopus, chicken, and human. The alignment was generated by CLUSTALW using full-length protein sequences. The putative signal peptide sequences predicted by SignalP are underlined. Conserved cysteines are shadowed and predicted glycosylation sites are in bold. Intron positions are indicated by arrows and numbered above or below the alignment.

(FAVLLFLPILLISGILLY) was seen between residue 253–270, separating the protein into an extracellular region of 229 aa without the leader peptide and an intracellular region of 250 aa. The extracellular region of IFN-α1 contained six conserved cysteine residues, with Cys96, Cys104, Cys215, and Cys237 potentially forming two disulfide bonds (Cys96 linking to Cys104 and Cys215 with Cys237, respectively). In the intracellular region, one of the two tyrosines (Y517 in human IFN-α1) known to be essential for activation of STAT proteins in human IFN-α1 was also conserved within the C terminus of Xenopus IFN-α1, whereas the other (Y343 in human IFN-α1) was absent in the corresponding region of the Xenopus IFN-α1 (21).

The Xenopus IL10-R2 homologous gene contained an open reading frame of 927 bp and translated into a protein of 308 aa (GenBank accession number: FJ581040, www.ncbi.nlm.nih.gov/nuccore/256860245) (Fig. 3B). The IL10-R2 gene has seven exons, where exons 1–5 and the 5′ end region of exon 6 encode proteins in human IFN-α1 were also conserved within the C terminus of Xenopus IFN-α1, whereas the other (Y343 in human IFN-α1) was absent in the corresponding region of the Xenopus IFN-α1 (21).

The Xenopus IL10-R2 homologous gene contained an open reading frame of 927 bp and translated into a protein of 308 aa (GenBank accession number: FJ581040, www.ncbi.nlm.nih.gov/nuccore/256860245) (Fig. 3B). The IL10-R2 gene has seven exons, where exons 1–5 and the 5′ end region of exon 6 encode proteins in human IFN-α1 were also conserved within the C terminus of Xenopus IFN-α1, whereas the other (Y343 in human IFN-α1) was absent in the corresponding region of the Xenopus IFN-α1 (21).

The Xenopus IL10-R2 homologous gene contained an open reading frame of 927 bp and translated into a protein of 308 aa (GenBank accession number: FJ581040, www.ncbi.nlm.nih.gov/nuccore/256860245) (Fig. 3B). The IL10-R2 gene has seven exons, where exons 1–5 and the 5′ end region of exon 6 encode proteins in human IFN-α1 were also conserved within the C terminus of Xenopus IFN-α1, whereas the other (Y343 in human IFN-α1) was absent in the corresponding region of the Xenopus IFN-α1 (21).

The Xenopus IL10-R2 homologous gene contained an open reading frame of 927 bp and translated into a protein of 308 aa (GenBank accession number: FJ581040, www.ncbi.nlm.nih.gov/nuccore/256860245) (Fig. 3B). The IL10-R2 gene has seven exons, where exons 1–5 and the 5′ end region of exon 6 encode proteins in human IFN-α1 were also conserved within the C terminus of Xenopus IFN-α1, whereas the other (Y343 in human IFN-α1) was absent in the corresponding region of the Xenopus IFN-α1 (21).

The Xenopus IL10-R2 homologous gene contained an open reading frame of 927 bp and translated into a protein of 308 aa (GenBank accession number: FJ581040, www.ncbi.nlm.nih.gov/nuccore/256860245) (Fig. 3B). The IL10-R2 gene has seven exons, where exons 1–5 and the 5′ end region of exon 6 encode proteins in human IFN-α1 were also conserved within the C terminus of Xenopus IFN-α1, whereas the other (Y343 in human IFN-α1) was absent in the corresponding region of the Xenopus IFN-α1 (21).

The Xenopus IL10-R2 homologous gene contained an open reading frame of 927 bp and translated into a protein of 308 aa (GenBank accession number: FJ581040, www.ncbi.nlm.nih.gov/nuccore/256860245) (Fig. 3B). The IL10-R2 gene has seven exons, where exons 1–5 and the 5′ end region of exon 6 encode proteins in human IFN-α1 were also conserved within the C terminus of Xenopus IFN-α1, whereas the other (Y343 in human IFN-α1) was absent in the corresponding region of the Xenopus IFN-α1 (21).

The Xenopus IL10-R2 homologous gene contained an open reading frame of 927 bp and translated into a protein of 308 aa (GenBank accession number: FJ581040, www.ncbi.nlm.nih.gov/nuccore/256860245) (Fig. 3B). The IL10-R2 gene has seven exons, where exons 1–5 and the 5′ end region of exon 6 encode proteins in human IFN-α1 were also conserved within the C terminus of Xenopus IFN-α1, whereas the other (Y343 in human IFN-α1) was absent in the corresponding region of the Xenopus IFN-α1 (21).

The Xenopus IL10-R2 homologous gene contained an open reading frame of 927 bp and translated into a protein of 308 aa (GenBank accession number: FJ581040, www.ncbi.nlm.nih.gov/nuccore/256860245) (Fig. 3B). The IL10-R2 gene has seven exons, where exons 1–5 and the 5′ end region of exon 6 encode.
the leader peptide and extracellular region, exon 6 contains the whole transmembrane region, and the 3' end region of exon 6 and exon 7 encode the intracellular region. The transmembrane region is located between residues 230–252, separating the protein into an extracellular region of 278 aa (excluding the leader peptide) and a much shorter intracellular region of 56 aa.

The Xenopus IL10-R2 shares 31.7, 35.3, and 32.1% amino acid similarity with human, mouse and chicken IL-10R2, respectively.

Gene synteny of the chromosomal loci containing the IFN-λ receptors is well conserved (Fig. 4). In the Xenopus genome, IFN-λR1 is situated next to the IL-22R2 gene, as seen in chickens and humans. The gene coding for the IFN-λ signaling chain, IL-10R2, is within the region containing three other receptor genes, including IFN-αR1, IFN-αR2, and IFN-γR2 (Fig. 4B). A comparable region is also conserved in the Xenopus genome (Fig. 4B), although the gene order for IFN-αR1 and IFN-γR2 was reversed in Xenopus and the IFN-γR2 gene appears to be duplicated.

Phylogenetic tree analysis

Type I and III IFNs belong to the α helical cytokine family, which contains IL-10 family members and IFN-γ. To analyze the
evolutionary relationship of the identified *Xenopus* type III IFNs with related cytokines, an unrooted phylogenetic tree was constructed using the neighbor-joining method. As shown in Fig. 5A, four major clades were apparent that were well supported with respect to bootstrap values. These were for IL-10s (100% bootstrap value), IFN-γs (90% bootstrap value), type I IFNs (85% bootstrap value), and type III IFNs (100% bootstrap value). The IL-10 and IFN-γ clades seemed to be evolutionary closer than type I and III IFNs, converging into a clade with 85% bootstrap confidence. Within the type III IFN clade, the four *Xenopus* IFN-λs were constrained into a single distinct branch separate from that containing avian and mammalian IFN-λs, suggesting they were expanded by species/amphibian specific gene duplication events. Two subgroups of *Xenopus* type I IFNs were indicated by the phylogenetic tree, one containing IFN-3, -4, and -5 and the other containing IFN-1 and -2, with the latter appearing to have a relatively closer relationship with the intron lacking type I IFN genes in reptiles, birds, and mammals.

Two major groups of fish type I IFNs were also revealed, as seen previously, with each duplicated into further subgroups, supporting our previous hypothesis that at least some teleost species appear to have undergone two major IFN duplication events (9).

The two putative receptors for *Xenopus* IFN-λs were supported by the phylogenetic tree analysis where the *Xenopus* IFN-AR1 and IL-10R2 grouped with their counterparts from higher vertebrates, respectively (Fig. 5B). The IL-10R2 gene was located within the genomic locus also containing the IFN-αR2, IFN-αR1, and IFN-γR2 genes and the phylogenetic tree analysis showed that the encoded protein had a closer relationship with IFN-γR2 than IFN-αR1, with IFN-αR2 more distant and forming a clade with IL-10R1 and IL-22R1.

**In vitro and in vivo expression analysis**

The tissue distribution of IFN-λs and IFN-ARs was studied in healthy *Xenopus*, to determine whether they are constitutively...

---

**FIGURE 5.** Phylogenetic tree analysis of the *Xenopus* type III IFNs (A) with other known IFNs and the putative receptors (B) with relevant receptor families. Unrooted phylogenetic trees were constructed using the full-length protein sequences with the neighbor joining method within the MEGA 4 package and bootstrapped 10,000 times. Bootstrap values over 70% are shown.
For in vitro studies, primary splenocytes were isolated from healthy Xenopus and stimulated for 4 h with poly(I:C) at doses of 0.1, 1, 10, and 50 μg/ml, respectively. Xenopus IFN4, one of the five type I IFN genes, was used here as a positive control, to assess the regulatory effects of poly(I:C) on the IFN response, and was shown to be upregulated in a dose-dependent manner (Fig. 7). In addition, a dose-dependent effect on gene expression was seen for IFN-λ1, 2, 3, and 5, with doses above 1 μg/ml resulting in a significant increase of IFN-λ transcripts. Moreover, the increasing levels of the three IFN-λ transcripts were comparable, ranging from 4- to 16-fold. The induced IFN-λ response was confirmed in animals that were injected i.p. with poly(I:C) (Fig. 8). All of the IFN-λ genes were induced significantly in spleen, the major lymphoid organ in Xenopus, where an ~33-, 9-, 11-, and 10-fold increase in expression of the IFN-λ1, 2, 3, and 5 genes was seen, respectively.

In mammals, it has been shown that IL-10R2 is constitutively expressed in tissues. Fig. 6 shows that constitutive expression of IFN-λ, IFN-λR1, and IL-10R2 was detected in a wide range of tissues, including heart, liver, spleen, kidney, intestine, and stomach. Compared with IFN-λ1, IFN-λ3, and IFN-λ5, IFN-λ2 was weakly expressed in liver and lung.

For in vitro studies, primary splenocytes were isolated from healthy Xenopus and stimulated for 4 h with poly(I:C) at doses of 0.1 and 1 μg/ml, respectively. Xenopus IFN4, one of the five type I IFN genes, was used here as a positive control, to assess the regulatory effects of poly(I:C) on the IFN response, and was shown to be upregulated in a dose-dependent manner (Fig. 7). In addition, a dose-dependent effect on gene expression was seen for IFN-λ1, 2, 3, and 5, with doses above 1 μg/ml resulting in a significant increase of IFN-λ transcripts. Moreover, the increasing levels of the three IFN-λ transcripts were comparable, ranging from 4- to 16-fold. The induced IFN-λ response was confirmed in animals that were injected i.p. with poly(I:C) (Fig. 8). All of the IFN-λ genes were induced significantly in spleen, the major lymphoid organ in Xenopus, where an ~33-, 9-, 11-, and 10-fold increase in expression of the IFN-λ1, 2, 3, and 5 genes was seen, respectively.

Characterization of biological activity
To determine whether IFN-λs are functional in antiviral immunity in Xenopus, the recombinant protein of IFN-λ1 was produced and used to stimulate primary splenocytes for 6 h. As shown in Fig. 9, IFN-λ1 significantly induced gene expression of Xenopus IFIT-5, a gene known to be upregulated by type I IFNs in mammals (19), at doses of 0.1 and 1 μg/ml (p < 0.05) in a dose-dependent manner. Doses of 0.001 and 0.01 μg/ml had no significant stimulatory effect on IFIT-5 gene expression.

Discussion
It is generally accepted that type I and III IFNs have a common evolutionary origin. However, it is debatable as to whether type I or type III IFNs may have evolved initially, and subsequently gave rise to the other form, or whether the ancestral IFN gene gave rise to both forms in higher vertebrates. The debate has been influenced by the discovery in fish and amphibians of IFN genes that have higher homology to type I IFN but possess a gene organization typical of type III IFN (i.e., containing four introns and five exons) (7, 10–13). The intronless type I IFNs in amniotes appear to have arisen from a retroposition event, that was generally assumed to have replaced the type I locus, with subsequent gene duplication expanding the family (7, 8). However, a recent paper has proposed that it is also possible type I IFNs were formed from the retroposition event itself, by giving rise to a second IFN locus in the genome at a different location (17). In this hypothesis the newly formed type I locus retained over time much of the original sequence and function of the retroposed gene, whereas the original intron-containing gene(s) diverged more quickly to form type III IFNs. This hypothesis excludes the possibility of intron-containing type I and type III IFNs coexisting. In this study, we report for the first time that intron-containing type I IFN genes do indeed coexist with intron-containing type III IFN genes in amphibians, and hence, had diverged by the emergence of tetrapods.

Five IFN-λ genes have been found within a single locus in the Xenopus genome, all encoding a peptide with a putative signal peptide at the N terminus, although one of the genes (IFN-λ4) appeared to be a pseudogene due to the presence of an early stop codon. Various lines of evidence support the contention that these are authentic type III IFN genes. First, homology and phylogenetic tree analysis show the Xenopus genes are more closely related to type III IFN of birds and mammals than to type I IFN. Second, synteny analysis shows that the type III IFN gene locus is relatively well conserved, with some immediate gene neighbors being present. Last, the type III IFN receptor locus was also identified, and although functional studies remain to be performed, at least shows the potential for a diverged type III IFN in amphibians to be able to signal via a distinct receptor.

That multiple type III IFN genes exist in amphibians is similar to the situation in mammals where multiple genes and pseudogenes are known. However, the phylogenetic tree analysis suggests that the Xenopus IFN-λs were expanded by gene duplication events within a species/vertebrate group, and that these genes are not homologous to those known in mammals. Currently, the highest number of IFN-λ genes are present in the shrew (17), where nine genes have been identified, although the draft quality of the genome sequence may ultimately impact on this number. In Xenopus the IFN-λ1, 2, and 5 molecules contain six cysteines with five seen in conserved positions relative to avian and mammalian IFN-λs. In contrast, in IFN-λ3, a pair of cysteines has been deleted. With the exception of the Xenopus IFN-λ4 pseudogene, the other four IFN-λ genes were induced in primary splenocytes after stimulation with poly(I:C), a ligand for TLR3, and similar dose-dependent patterns were observed. An inducible IFN-λ response was also confirmed by in vivo studies using poly(I:C) treatment. These data are in agreement with the findings in mammals, demonstrating type III...
IFNs were activated on virus infection or stimulation with double-stranded RNAs in a wide range of lymphoid, myeloid, and epithelial cell types (2–4). Selective induction of type I or III IFNs has not been reported in higher vertebrates and, in fact, both type I and III IFNs are upregulated in a similar manner. Recent comparison of the regulatory elements in the promoter regions of the type I and III genes suggests they may be activated through common signaling pathways involving STAT2 and Tyk2 (23). The recombinant IFN-α1 protein was produced and shown to activate the IFT-5 gene (Fig. 9), suggesting the IFN-αs are indeed functional in regulating immune responses in Xenopus.

In mammals, type III IFNs are known to bind to a receptor complex consisting of IFN-λR1 and IL-10R2 (2, 3). The IFN-λR1 has high affinity for binding the ligand, whereas IL-10R2 is mainly responsible for signaling on activation. IL-10R2 is not a specific receptor for type III IFNs but is used as a common signaling receptor by several members of the IL-10 cytokine family, including IL-10, -19, -20, -22, and -26 (20). The specificity of IL-10R2 signaling is dependent on activation of the ligand binding chains that are expressed in subsets of cell types (22). By gene synteny analysis, the corresponding homologs of IFN-λR1 and IL-10R2 have been identified in the Xenopus genome. Although interaction of the putative receptors with the Xenopus IFN-λs requires future investigation, sequence alignment and in particular the phylogenetic tree analysis indicates they are homologs of the mammalian IFN-λ receptor chains. Interestingly, we found the Xenopus IFN-λR1 was ubiquitously expressed in tissues and cells, which is in contrast with the findings in mammals where IFN-λR1 expression is restricted to certain cell types (4). Like its ligand, IFN-λR1 was shown to be inducible in most tissues and in primary splenocytes after stimulation with a known TLR3 ligand, poly(I:C), suggesting that an increase of IFN-λR1 receptor expression could contribute to the TLR3 ligand-activated IFN-λ response in amphibians. Indeed, IFN-λR1 knockout mice show impaired antiviral capability even after treatment with TLR3 or TLR9 agonists (4).

So how do these new findings in Xenopus impact on the discussion about the IFN molecules found in fish and IFN evolution in early vertebrates? The close phylogenetic relationship of fish IFNs with Xenopus and amniote type I IFNs suggests that the identified fish IFNs are likely members of the type I IFN family, even though they are divided into subgroups that show receptor divergence (15). It is still possible that type III IFN homologs could be present in fish and awaits further study. Recent studies with salmonids also highlight that it is not necessary to have intronless IFN genes for multiple gene duplications to occur, with the salmon genome consisting of at least 11 intron-containing genes (8, 9). IFN-like genes have been found in cartilaginous fish, such as elephant shark (7), and these predicted proteins possess a CAVE motif that is present in four cysteine containing type I IFNs among all vertebrate orders and also have a close evolutionary relationship with vertebrate type I IFNs (data not shown), supporting our previous hypothesis that type I IFNs could have originated from an ancestor containing four conserved cysteines (7). Recent studies have also demonstrated that functional genes, such as Mx and GBP, which are known to be involved in the IFN response in vertebrates, are also present in nonvertebrate chordates, such as amphioxus (24). This suggests an IFN-like system had been shaped in early chordates, although existence of IFN homologs remains to be determined. In conclusion, the presence or absence of introns within IFN genes does not seem to correlate with IFN type I/III divergence and the timing of the appearance of IFNs has still to be established. Nevertheless, the similar protein structures of type I and III IFNs gives strong evidence supporting a common origin, with both types having the same genomic organization of five exons and four introns, with the position of introns, especially the last two introns, remarkably conserved.

**FIGURE 7.** Expression of IFN-αs, IFN-λR1, IL-10R2, and IFN4 in Xenopus primary splenocytes. The primary splenocytes were stimulated with poly(I:C) at doses of 0.1, 1, 10, and 50 μg/ml for 4 h and RNA was extracted for real-time PCR analysis. The gene expression level was normalized against the housekeeping gene β-actin and fold change of expression obtained by comparing the normalized expression of the experimental group with that of the control group. The mean of three independent experiments is shown and bars indicate the SEMs.

**FIGURE 8.** Expression of IFN-αs, IFN-λR1, and IL-10R2 in Xenopus tissues after stimulation in vivo with poly(I:C). Three animals were injected i.p. with poly(I:C) and 24 h later tissues were collected for RNA extraction. The gene expression level was normalized against the housekeeping gene β-actin and fold change of expression obtained by comparing the normalized expression of the experimental group with that of the control group. The mean of three independent experiments is shown and bars indicate the SEMs.

**FIGURE 9.** Induction of IFT-5 gene expression in primary splenocytes by recombinant IFN-λ1. The primary splenocytes were stimulated with recombinant IFN-λ1 at doses of 0.001, 0.01, 0.1, and 1 μg/ml for 6 h and total RNA was extracted for real-time PCR analysis. The gene expression level was normalized against the housekeeping gene β-actin and fold change of expression obtained by comparing the normalized expression of the experimental group with that of the control group. The mean of three independent experiments is shown and bars indicate the SEMs.
Disclosures
The authors have no financial conflicts of interest.

References