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A CXC chemokine sequence isolated from the rainbow trout Oncorhynchus mykiss resembles the closely related interferon-gamma-inducible chemokines CXCL9, CXCL10 and CXCL11

Abstract

A sequence encoding a CXC - type chemokine from rainbow trout was found to most resemble members of the CXCL9/CXCL10/CXCL11 sub-family. In mammals,

all 3 chemokines are regulated by IFN-g and are chemotactic for activated T lymphocytes. The trout chemokine (gIP1), with a message of 787 nucleotides, contains 100 amino acids in a typical non-ELR CXC chemokine arrangement. A second sequence (gIP2), with 6 nucleotide differences in the coding region when compared to the first, was also identified although it is not known whether this is a second functional gene or a second allele. The gene is separated onto 4 exons, and the introns intervene in conserved positions according to the mammalian equivalents. The sequence encoded by the second exon shares the highest amino acid identity (37%) with CXCL10, with lower values of identity to other CXC chemokines (17-31%). Furthermore, phylogenetic analysis groups the trout chemokine with mammalian CXCL9, CXCL10 and CXCL11 peptides. Constitutive expression of gIP is seen in trout gill and low level expression in spleen, head kidney and liver. In RTS-11 cells, gIP expression can be induced with poly I:C, but not by LPS, suggesting virus-mediated regulation of gIP. Intraperitoneal injection of recombinant trout TNF-a caused elevation in gIP mRNA levels in trout head kidney.

Full text

Introduction

A large family of structurally related chemotactic cytokines (the chemokines) is responsible for controlling the migration of leucocytes during an inflammatory response or infection. Within this family are four subgroups that are characterised depending on the arrangement of the cysteine residues occurring near the *N*-terminus of their mature peptides. These groups are commonly known as CXC (a), CC (b), C (g) and CX_3C (d) [1]. The three dimensional structures of many chemokines belonging to the CXC and CC groups have been determined and appear similar, comprising three b-strands in a Greek key formation and an overlying a-helix stabilised by two disulphide bonds between the four conserved cysteines [2, 3]. CXC chemokines can be further classified depending on whether or not they possess a Glu-Leu-Arg (ELR) motif adjacent to the CXC motif. Those CXC chemokines in possession of an ELR sequence (e.g. interleukin-8) show neutrophil-attracting ability [4, 5], whereas those lacking an ELR motif specifically attract lymphocytes [6, 7].

Three non-ELR CXC chemokines that are closely related according to their peptide sequences are monokine induced by interferon-g (Mig), interferon-

inducible protein 10 (IP-10) and interferon-inducible T-cell alpha chemoattractant (I-TAC) known, under the new nomenclature, as CXCL9, CXCL10 and CXCL11 respectively [1]. These three chemokines selectively share a common receptor, CXCR3, that is expressed on T cells, B cells and natural killer cells with preferential expression apparent on Th1 type T cells. Activity of these ligands and expression of the CXCR3 receptor is intensely increased by T cell activation suggesting a role in inflammatory processes for these molecules [1]. Furthermore, interferon-g (IFN-g), but not TNF-a or IL-1b alone, nor Th2-type cytokines (IL-4, IL-10, and IL-13), has been shown to greatly increase the expression of CXCL9, CXCL10 and CXCL11 consistent with the association of these chemokines with Th1 responses [7, 8, 9]. Of this small subset of chemokines, CXCL10 was the first identified. Modulation of CXCL10 mRNA expression by IFN-g has been observed chiefly in endothelial cells, monocytes, fibroblasts, astrocytes, keratinocytes and neutrophils [10, 11, 12, 13]. Later, CXCL9 was identified as a chemokine expressed in IFN-g-induced THP-1 cells, PBMCs, endothelial cells, keratinocytes, and fibroblasts with 37% amino acid identity to CXCL10 [8, 14]. CXCL11, a chemokine expressed by activated monocytes and astrocytes, was discovered most recently and is approximately 40% identical to CXCL9 and CXCL10 at the amino acid level [7]. This latter chemokine appears to have the highest affinity of the three for the CXCR3 receptor.

Little is known about the cytokine network of fish relative to that known for mammals. However, recent progress has been made in isolating the genes for important cytokines from several species of fish, including interleukin-1b (IL-1b) [15, 16], tumour necrosis factor-a (TNF-a)[17, 18] and three isoforms of transforming growth factor-b [19, 20, 21, 22]. In addition, several chemokine genes have been isolated from fish which are homologous to CXC [23,24,25,26] and CC chemokines [27, 28]. The presence of similar cytokine genes in fish and mammals suggests some evolutionary conservation of the complexities of the immune system although further investigation into the activity of such genes within fish is required.

Although it is known that fish possess chemokines from both the CC and CXC subgroups there are no published data concerning the CXCL9, CXCL10 or CXCL11 chemokines in non-mammalian vertebrates. However, whilst searching for sequences with similarity to chemokines in the EST databases, a sequence with highest amino acid identity to IP-10 (39%) was identified from a catfish

Ictalurus punctatus brain cDNA library (Accession No. BE212851). Sequence information from this EST was used to isolate a similar sequence from the rainbow trout *Oncorhynchus mykiss* that is reported here. Analysis of the mRNA expression of the trout gene was investigated in various tissues and the effect of poly I:C, and LPS upon levels of expression studied. The implications for the presence of a chemokine that controls the trafficking of activated T cells during an inflammatory response in fish are discussed.

Methods and materials

Isolation of a gIP-10-like sequence from a trout cDNA library

The full cDNA sequence of trout g-interferon inducible protein (gIP) was isolated from a rainbow trout kidney leucocyte cDNA library, prepared as described previously [20]. An oligonucleotide primer (IP10-R2) (Table 1) specific for an EST sequence of the catfish was initially used in an anchored polymerase chain reaction (PCR) with the vector-specific T3 primer. The sequence derived from the resulting product was used to design trout specific primers that were used to amplify the remainder of the molecule. A second product was amplified by semi-nested PCR using an initial round of amplification with the IP10-67-F2 primer with the vector-specific primer T7, followed by a further round of PCR using the T7 primer with the IP10-67-F1 primer. All primers are listed in Table 1 and their relative positions depicted in Figure 1. To ensure that both fragments were derived from an individual sequence, a third product was amplified from the cDNA library using the IP10-67-F2 primer and IP10-67-R2 primer that spanned the full coding sequence of the putative trout gIP cDNA, and most of the 3' and 5' UTRs. The latter PCR was repeated to amplify gIP cDNA from a rainbow trout macrophage (RTS-11) cell-line that was derived from the spleen of sexually immature trout [29].

Isolation of the genomic sequence for trout gIP

Genomic DNA was isolated from RTS-11 cells using a proteinase K method as described previously [30]. An extraction mixture containing 500 mL 10X TNE buffer [0.01 M Tris-HCl (pH 8.0); 0.1 M NaCl; 0.01 M EDTA; all Sigma, UK], 50 mL 1M Tris-HCl (pH 8.0) and 20 mL 25% sodium dodecyl sulphate (SDS; Sigma, UK) was added to cell pellets and the mixture incubated at 55¡C in the presence of proteinase K (20 mg.mL $^{-1}$; Promega, UK) with regular vortexing. After 3 h,

reactions were incubated at 37¡C with RNase (10 mg.mL $^{-1}$; Sigma, UK) for 1 h and two phenol-chloroform extractions were performed. Fifty mL sodium acetate solution (3M; Sigma, UK) and 1 mL cold ethanol (100%; Sigma, UK) were added to the DNA fraction. Samples were allowed precipitate overnight at - 20¡C and DNA recovered by centrifugation at 10000 « g for 15 min. The DNA pellet was washed with 200 mL cold ethanol (70%) at 10000 « g for 5 min. Excess ethanol was removed and the dried DNA pellet dissolved to a concentration of 250 ng.mL $^{-1}$ in sterile milliQ H_2O. PCR was performed under the conditions described above where 2 mL of genomic DNA was used as template utilising primers designed in the 5' (IP10-67-F2) and 3' (IP10-67-R2) ends of the rainbow trout gIP cDNA sequence (see Table 1 and Figure 1). The IP10-67-F1 and IP10-67-R1 primers were used to assist the sequencing through the central portion of the trout gIP genomic clone.

Cloning and sequencing

All products obtained by PCR were directly cloned into the pGEM-T Easy vector (Promega, UK). Plasmid DNA was isolated from bacterial colonies that had an appropriately sized insert using a Qiaprep spin miniprep kit (QIAGEN, UK). Three randomly selected clones representing each product were sequenced on an ABI 377 Automated Sequencer (Applied Biosystems) using vector or gene-specific primers. Comparisons of nucleotide and amino acid sequences with EMBL and SWISSPROT databases were performed using the FASTA [31] program. Direct comparisons between two sequences were performed using the NEEDLE global alignment program [32] within the EMBOSS suite of programs (http://www.uk.embnet.org/Software/EMBOSS/) from which overall percentage values were recorded. Multiple sequence alignments were generated using CLUSTALW (version 1.74) [33]. Phylogenetic trees were constructed from CLUSTAL generated alignments of CXC chemokine peptide sequences using the Neighbour-joining method [34] within the MEGA 2.1 program [35]. Distance options were set for Poisson Correction and pairwise deletion was used for handling gaps in the alignment. Trees were bootstrapped 1000 times to test phylogeny and then displayed using the TREEVIEW program [36]. The SIGNALIP program [37] was used to predict the position of signal peptide cleavage.

Expression studies

Rainbow trout weighing 300-350 g were obtained from a local fish farm and maintained in a recirculating freshwater system at 14¡C with regular feeding. Three fish were killed and tissues (blood, brain, gill, liver, spleen, heart and head kidney) were isolated immediately and snap frozen in liquid nitrogen. Total RNA was extracted from the frozen tissues using 0.5-1.0 mL RNAzol B (Biogenesis, UK) as previously described [20]. First strand cDNA was synthesised from 5 mg total RNA using Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco, UK) at 42¡C for 50 min using the oligo-dT primer (Gibco) and PCR performed on the resultant cDNAs using the IP10-67-F2 and IP10-67-R1 primers. These primers were specific for trout gIP (Table 1) and spanned intron 1 of trout genomic gIP. PCR reagents were assembled as described above and the cycling protocol was 1 cycle of 94¡C for 2 min, 35 cycles of 94¡C for 1 min, 60¡C for 1 min and 72¡C for 10 s, with a final extension step of 5 min at 72¡C. As a positive control for RT-PCR, b-actin was amplified using trout specific b-actin FOR and b-actin REV primers listed in Table 1 using 30 PCR cycles and an annealing temperature of 55¡C. Amplified products were analysed on a 2% agarose gel containing ethidium bromide (100 ng.mL^{-1}).

In vitro regulation of gIP was also investigated using the RTS-11 cell-line. RTS-11 cells were incubated *in vitro* at 18¡C with poly I:C (50 mg.mL^{-1}; Sigma, UK) or with *E. coli* 0127:B8 LPS (5 mg.mL^{-1}; Sigma, UK) for 2, 4 or 8 h. Control cells were incubated with an equivalent volume of phosphate-buffered saline (PBS, Gibco, UK). The cells were harvested using trypsin-EDTA (Sigma, UK) and total RNA was extracted as above. Semi-quantitative RT-PCR was used to compare the level of the gIP transcript in each sample to the b-actin transcript level. Both gIP and b-actin genes were co-amplified in the same PCR reaction using 30 cycles of 94¡C for 30 s, 55¡C for 30 s and 72¡C for 10 s, with the primer combinations described for expression PCR above. Similarly, the *in vitro* and *in vivo* effects of recombinant trout TNF-a (rtTNFa) [17, 18] on gIP expression in trout cells were investigated using recombinant proteins of both isoforms discovered in this species. Thus, RTS-11 cells were incubated at 18¡C for 5 h in the presence of either rtTNFa1 or rtTNFa2 at 0.1, 1.0, 10.0 or 100.0 ng.mL^{-1} in EB buffer (Qiagen Ni-NTA kit, Qiagen). A control reaction was performed in which an equivalent volume EB buffer was added to RTS-11 cells. Total RNA was extracted and semi-quantitative PCR used to determine changes in gIP expression as described above. To investigate the effect of rtTNF-a *in vivo*, trout were injected intraperitoneally with either rtTNF-a (a mixture comprising 30 mg rtTNF-a1 plus

55 mg rtTNFa2) or PBS. After 24 h the head kidneys were removed and RNA extracted as above. Semi-quantitative RT-PCR for gIP was performed as above.

Results

Cloning and characterisation of rainbow trout gIP

Anchored PCR on a rainbow trout cDNA library, using a primer designed against an EST sequence of the catfish that resembled the CXCL10 chemokine, generated a product containing 268 bp of a cDNA sequence. Comparison of this sequence with the EMBL and Swissprot databases (using the FASTA program) confirmed this sequence as a CXC type chemokine with highest similarity to CXCL10. Further primers, specific for the amplicon, gave rise to a second fragment when used in semi-nested PCR that contained 632 nucleotides. The two products overlapped to allow the deduction of a contiguous sequence containing 787 nucleotides (Figure 2). Of these 787 bp, nucleotides 1-53 and 357-787 formed the 5' and 3' untranslated (UTR) regions respectively whilst the remaining 303 nucleotides formed an open-reading frame (ORF) predicted to encode a peptide of 100 amino acids. A hydrophobic stretch incorporating the first 24 residues was predicted to be a signal peptide using the SignalIP program. Two polyadenylation signal sequences were present within the 3' UTR at nucleotide positions 659-664 and 748-753 and a poly-A sequence continued from nucleotide 769. The third product amplified, beginning in the 5'UTR and ending in the 3'UTR, confirmed the two products were derived from the same message. A similar sequence spanning the entire coding region was amplified from RTS-11 cells. In addition, a second cDNA sequence with 6 nucleotide differences in the coding region (resulting in 3 amino acid changes), and several differences in the 3'UTR (mainly due to the presence of additional repeats of a 11 bp stretch) was discovered in the RTS-11 cell-line. The amino acid substitutions are Gln_{34} to Leu_{34}, Lys_{95} to Asn_{95} and Gln_{96} to Arg_{96}. The 5'UTRs of both transcripts were identical. The original transcript will be referred to as gIP1 and the second transcript identified from RTS-11 cells will be referred to as gIP2.

The genomic sequence of trout gIP was isolated from the RTS-11 cell line using the IP10-67-F2 and IP10-67-R2 primers. A product of 1934 nucleotides was obtained and comparison to the cDNA sequence revealed the presence of 3 introns (200 bp, 240 bp and 657 bp respectively) between 4 exons (Figure 3). The genomic sequence isolated was identical to the gIP2 cDNA sequence within

its exons. Conventional intron splice sites gt-ag mark the boundaries of all three introns.

Comparison of the trout gIP1 propeptide sequence with mammalian CXC chemokines using the NEEDLE program showed approximately 30% amino acid identity to a range of chemokines (CXCL1, CXCL2, CXCL3, CXCL8, CXCL10 and CXCL11), with 14-27% amino acid identity to other mammalian CXC chemokines (*Table 2*). However, when comparing the predicted peptide regions encoded by exon 2, CXCL10 is clearly the most closely related human CXC chemokine to trout gIP, with over 37% amino acid identity; other chemokines having between 17 and 31% amino acid identity within this region. Comparison of the full length trout gIP translation with trout IL-8 [26] revealed only 31% amino acid identity between the two trout chemokines, whereas 44% amino acid identity was shared between the catfish CXCL10-like sequence and trout gIP.2

Multiple alignment of the putative translations of trout gIP1 and gIP2 with human CXCL9, CXCL10, and CXCL11 and the catfish CXCL10-like sequence reveals the conserved positions of the 4 cysteine residues (Figure 4) and the predicted position of signal peptide cleavage that follows a Gly residue in each case. Highest identity was observed in the central portion of the chemokines, with more variation in peptide sequence at both the N-terminal and C-terminal ends.

Phylogenetic analysis using the neighbour-joining program reveals that the trout gIP1 peptide groups with the catfish CXCL10-like molecule and human CXCL9, CXCL10 and CXCL11, although poor bootstrap values are given (Figure 5). The clade of g-IFN inducible chemokines groups distinctly from IL-8 molecules that include trout and flounder IL-8.

Expression studies

RT-PCR showed tissue-specific expression of gIP mRNA in various tissues of trout (Figure 6). Expression of gIP was particularly noted in gill, with some low level expression detected in the spleen, head kidney and liver. The brain and heart did not appear to express gIP. Using a semi-quantitative RT-PCR, it was apparent that poly I:C induced high expression levels of gIP in RTS-11 cells after 4 h, which continued to be expressed at high levels at 8 h (*Figure 7*). No induction was observed above basal levels after only 2 h incubation with poly I:C. By contrast, no induction of gIP was observed in RTS-11 cells that were

incubated over the same time range in the presence of LPS when compared to control cells (Figure 7). No induction of gIP was observed in response to rtTNFa1 or rtTNFa2 in the RTS-11 cell-line even at relatively high concentrations (100 ng.mL $^{-1}$) (Figure 8A). However, *in vivo* expression of gIP could be induced in trout, as seen in the head kidney following intraperitioneal injection with rtTNFa (Figure 8B). In all RT-PCR experiments, b-actin bands were successfully amplified.ÊÊ

Discussion

A sequence of 787 nucleotides, isolated from a rainbow trout leucocyte cDNA library, was found to resemble CXCL10. This sequence (gIP1) translated in a single open reading frame (ORF) to produce a putative peptide of 100 amino acids that contained four cysteine residues, characteristic of most chemokines, and essential to formation of the tertiary structure. The first two cysteines were separated by one residue (Gln), thus adopting the CXC-type chemokine arrangement. There was no ELR motif preceding the CXC motif, consistent with observations of mammalian CXCL9, CXCL10 and CXCL11 and other non-ELR CXC chemokines that are inactive toward neutrophils [1]. Signal peptide cleavage was predicted to occur following residue 24 (Gly), which is in accord with the observed signal peptide cleavage position of CXCL9, CXCL10 and CXCL11 [38, 39], and suggests that the trout chemokine is also a secreted molecule. Within the 431 nucleotides of the 3' UTR, two potential polyadenylation signal sequences (AATAAA) [40] were observed, one 16 nucleotides and the other 105 nucleotides upstream from the poly-A sequence. Whether both could be functional is unclear, however, no transcripts ending at the first polyadenylation signal sequence were isolated. An 11 nucleotide stretch within the 3'UTR (gaagactgcct) is repeated 11 times, sometimes inexactly, and has been observed, in other isolates of gIP1, to vary in frequency thus producing transcripts of differing lengths. This phenomenon appears to be common for rainbow trout cytokines, also occurring for both IL-8 and TNF-a molecules (Laing, personal observation). A second copy of this sequence (gIP2) was isolated from the RTS-11 cell-line in addition to a sequence identical to gIP1 isolated from the leucocyte cDNA library. With 6 nucleotide differences in the coding region, gIP2 potentially had 3 amino acid substitutions within its peptide structure, all in positions that tend to be relatively non-conserved between mammalian CXCL9, CXCL10 and CXCL11. Although the complete 3'UTR was not obtained for this transcript (it ends in the region of the IP10-67-R2 primer that resides 10 bp

upstream from the first polyadenylation signal sequence), it was apparent that the length of the 3'UTR was substantially longer in gIP2 than in gIP1. This was mainly due to the presence of more copies (27 versus 11) of the 11 nucleotide repeat sequence mentioned above, but various other differences between the Ôpolymorphic region' and the IP10-67-R2 primer position were also observed. The short 5'UTR sequences (containing 53 bp) of both transcripts were identical. Whether these two different gIP sequences represent different functional genes or merely two alleles, still requires verification. However, two genes for IL-1b and TNF-a have been observed in rainbow trout [41, 42] that appear to be regulated via different mechanisms, perhaps reflecting different functions of these cytokines. Indeed, it has been noted that fish exhibiting polyploidy (as is the case for rainbow trout), commonly possess multiple copies of some genes [43] that do not become deleted from the genome because they adopt new, fundamental roles within the organism. Single amino acid substitutions in mammalian CXC chemokines have been shown to alter the specificity and/or potency of a chemokine to a particular receptor [44], potentially altering the action of the chemokine. Thus, the 3 different amino acids between gIP1 and gIP2 could result in functional differences between these molecules.

The genomic sequence of gIP2 was sequenced from RTS-11 cells, and was shown to contain 4 exons and 3 introns. The introns (which contain 200, 240 and 657 nucleotides in introns 1-3 respectively) divide the coding sequence in conserved positions when compared to mammalian CXCL10 and CXCL11 genes. Exon 1 encodes the signal peptide region, containing 70 nucleotides in trout gIP2 and 61 nucleotides in mouse CXCL10 and human CXCL11 (Figure 9) accounting for the signal peptide of trout gIP containing 3 more amino acids than both mammalian CXCL10 and CXCL11. Exon 2 of mammalian CXCL10 and CXCL11, in both cases, contains 127 nucleotides, identical to the number of nucleotides present in the second exon of trout gIP. The lengths of the final two exons are more variable, reflecting the difference observed in the C-terminal ends of CXC chemokines. Mouse CXCL10 contains 90 nucleotides in exon 3 and 19 in exon 4, whereas exons 3 and 4 of human CXCL11 are 73 and 25 bp long respectively. Exon 3 and 4 of trout gIP more closely resemble those of CXCL10, with 93 nucleotides and 12 nucleotides in each respectively. The partial genomic sequence was also obtained, from RTS-11 cells, for gIP1, revealing differences in the lengths of introns between the two trout sequences in addition to differences already observed in the cDNA sequences. The 2nd intron of gIP1 contains

253 nucleotides compared to the 240 bp of intron 2 of gIP2. This finding confirms that differences observed between the two cDNA sequences could not be the result of sequencing error.ÊÊÊ

Comparisons of trout gIP1 with mammalian CXC chemokines were made using the NEEDLE program. When the complete peptide sequences were compared, no individual chemokine displayed an amino acid identity that was clearly higher than others - human CXCL1, CXCL2, CXCL3, CXCL8, CXCL10 and CXCL11 all displayed approximately 30% amino acid identity to trout gIP1 whilst the rest exhibited identity values of 14-27%. Amino acid sequences of the predicted regions encoded by exon 2 were also compared. This region has been shown to be the essential portion for CXC chemokine activity, as removal of the other regions does not inhibit activity [5]. This comparison revealed that, trout gIP1 clearly showed highest identity to human CXCL10 (37%) with the values of amino acid identity to exon 2 of other CXC chemokines being lower by approximately 6% or more. These values suggest the functional portion of trout gIP is more like CXCL10 than any other chemokine. When comparing trout gIP1 to the trout IL-8 precursor, only 31% amino acid conservation occurred between the 2 molecules. However, the translated catfish CXCL10-like EST sequence shared 44% amino acid identity to gIP1. This is indicative of a closer relationship between the catfish sequence than trout IL-8 to trout gIP.

Alignment of trout gIP1 and gIP2 and the catfish CXCL10-like EST sequence with the peptide sequences of human CXCL9, 10 and 11 revealed conservation of the cysteine residue positions and the position of signal peptide cleavage. The CXCL9 sequence is much longer at the C-terminal end (it contains 125 amino acids compared to 100 residues of trout gIP) [45], and this is reflected in the low values of overall identity relative to those of CXCL10 and CXCL11 when compared to trout gIP. From the alignment it can be seen that most conserved residues reside in the central portion of the chemokine, with relatively less conservation at the N-terminal (signal peptide) and C-terminal ends. This central portion corresponds to the "loop" region speculated to contain the second binding site of CXC chemokines (the 3 amino acids immediately upstream of the CXC motif being the first binding site) that is more ordered than the N-terminus and contains residues essential to creating the correct tertiary structure of the chemokine [2]. As most of this region is encoded by exon 2, it follows that the sequence encoded by this exon would be likely to remain highly conserved for

each particular chemokine, as suggested by the values for amino acid identity described from global alignments above.

Phylogenetic comparison of mammalian and fish CXC chemokines provides further evidence of the relationship between trout gIP and the CXCL9/CXCL10/CXCL11 group of chemokines. The putative trout precursor peptide (together with the catfish EST) groups with human CXCL9, CXCL10 and CXCL11 in one clade, branching more closely to CXCL11, although the bootstrap values are too low to allow a definite statement as to which of the human chemokines is most related. The trout IL-8 molecule groups along with other published IL-8 peptide sequences in a separate clade, supporting that gIP and IL-8 of trout are distinct CXC chemokines. Analysis of the data using the PROTPARS program also grouped trout gIP with CXCL10 and CXCL11, although bootstrap support was once again rather poor (data not shown), increasing the confidence that the correct grouping of this molecule has been obtained. The low bootstrap values most likely result from the high divergence rate that is common for chemokines and their receptors [46]. After the discovery that mouse CXCL11 resided in a tandem arrangement with the CXCL9 and CXCL10 genes it was speculated that CXCL9, CXCL10 and CXCL11 arose by gene duplication from a common precursor early in mammalian evolution [39]. If this is the case, perhaps there is only one representative of this subset of chemokines in fish (i.e. trout gIP may have evolved from the precursor to CXCL9, CXCL10 and CXCL11). This question may only be answered when the full genome of a fish species has been sequenced.

Expression studies reveal a widespread expression of gIP in trout tissues, with constitutive expression mainly in the gill, and some low level expression in spleen, head kidney and liver. Not all tissues expressed gIP, though, as shown by an absence of this transcript in the brain and heart. Some degree of variation could be observed in the levels of expression between different fish individuals, although the exact reasons for this are unclear. Primers used for these expression studies would amplify both sequences reported in this study, hence differences are unlikely to result from differential expression/polymorphic factors. Constitutive expression of CXCL10 has also been observed in mammalian lymphoid organs, such as spleen and thymus [47], although expression can be induced in a wide array of tissues following an immune stimulus (e.g. brain, eye, lung, intestine, liver, heart, muscle, blood vessels and lymph nodes) [48]. The CXCL11 message does not appear to be constitutively

expressed yet is highly expressed in the lung, heart, small intestine and kidney in humans during endotoxemia, differing from expression patterns of CXCL9 and CXCL10 [39]. Endotoxemia-induced CXCL9 expression is greatest in liver.

The regulation of trout gIP expression in response to various stimuli was compared to the effects similar factors had on levels of mammalian CXCL9, CXCL10 and CXCL11 expression. The effects of LPS and poly I:C upon gIP expression in a rainbow trout macrophage cell-line (RTS-11) were investigated. Semi-quantitative RT-PCR showed that gIP expression levels (relative to b-actin levels) were increased dramatically above basal levels in RTS-11 cells after 4 h of exposure to poly I:C (50 $mg.mL^{-1}$) and was maintained at high levels after 8 h. This is consistent with observations in cultured mammalian macrophages as poly I:C caused a dramatic increase in CXCL10 expression in murine macrophages [49]. However, no effect of LPS (5 $mg.mL^{-1}$) was observed in the RTS-11 cells up to 8 h post-stimulation. This lack of response cannot be attributed to a lack of receptors for LPS in this cell-line since previous studies have shown induction of IL-1b expression in RTS-11 cells following LPS challenge [29]. In contrast, there have been some reports of LPS induction of CXCL10 in mammalian macrophages [50, 51] that are believed to be mediated via the release of IFN-a/b from macrophages [48]. Peak expression of CXCL10 could be obtained by 4 h, declining rapidly by 8 h and returning to basal levels after 16 h [51]. LPS does not induce the expression of all three IFN-g-inducible chemokines in all mammalian cells, however. LPS failed to induce CXCL9 release from human monocytes after 8 h [45], and only a small increase in CXCL11 expression was observed in the mouse RAW (macrophage) cell-line. *In vivo* expression also varies in mammals following challenge with LPS. Expression of human CXCL9 and CXCL11 in the lung, following injection of LPS, is delayed compared with that of CXCL10 [39]. CXCL10 expression rises rapidly and peaks 4 h after LPS injection, whereas CXCL9 and CXCL11 mRNA levels continue to increase between 4 and 8 h.

Various cytokines and mitogens have been investigated as to their effect on CXCL9, CXCL10 and CXCL11 expression levels *in vitro*. In murine macrophages, IFN-a, IFN-b and IFN-g have been shown to greatly induce the level of CXCL10 expression [49]. With IFN-g, this induction peaked between 3-6 h post-stimulation. Indeed, murine CXCL9, CXCL10 and CXCL11 are all strongly induced by IFN-g in RAW macrophage cells [39]. However, the three genes have differing responses to IFN-a, IFN-b and to LPS in mammals. For example, murine CXCL11 is

weakly induced by IFN-a, IFN-b and LPS relative to induction by IFN-g [39]. CXCL9 can be upregulated in human monocytes after 8 h and in peripheral blood mononuclear cells by IFN-g, but not by IFN-a or LPS [45]. CXCL11 levels increased in human keratinocytes following exposure to IFN-g for 6-8 h, yet, as for CXCL10, no induction was induced by TNF-a [52]. Whilst some cytokines alone appear to have little effect on CXCL9, CXCL10 and CXCL11 expression (including IL-1a, IL-3, IL-4, GM-CSF, CSF-1 and TNF-a), the combination of IFN-g with LPS, IL-1b, or TNF-a produces synergistic increases in induction of all three chemokines in a wide variety of cell types [39]. Similarly, combining TNF-a with IFN-g induced expression of murine CXCL11 in Swiss 3T3 cells > 50-fold higher than either cytokine alone. The *in vivo* effect of TNFa on gIP expression in trout head kidney was observed to show an increase of gIP expression above control levels after 24 h. Whilst no similar experiments have been reported for mammalian systems, evidence from *in vitro* studies suggest TNF-a alone has no effect of CXCL10 or CXCL11 expression. This is consistent with the lack of gIP induction by TNF-a in the RTS-11 cell-line observed in this study and suggests that TNFa alone has little or no effect on gIP production by macrophages in trout. Perhaps the effects seen *in vivo* result from the activation of other cell types either in the production of gIP itself or the secretion of other cytokines that may induce gIP in trout macrophages. Thus, it would be interesting to determine which mechanisms and cells are involved in the increase of gIP expression observed in response to TNFa injection in trout. To date, no IFN-g peptide (or nucleotide sequence) has been isolated from a fish species, hence the effect of this molecule upon the expression of the gIP chemokine could not be assessed during this study.

CONCLUSION

In conclusion, a second trout CXC chemokine gene has been discovered that appears to be a homologue of the subfamily of non-ELR chemokines containing CXCL9, CXCL10 and CXCL11. That this gene may be virally regulated, as with its mammalian counterparts, was shown by the ability of poly I:C (a viral mimic) to greatly increase its expression levels in a trout macrophage cell-line. To verify the biological effects of the trout chemokine, however, will require production of the recombinant protein, which should help establish whether trout gIP, like CXCL9, CXCL10 and CXCL11, is chemotactic for activated T lymphocytes or some other target.

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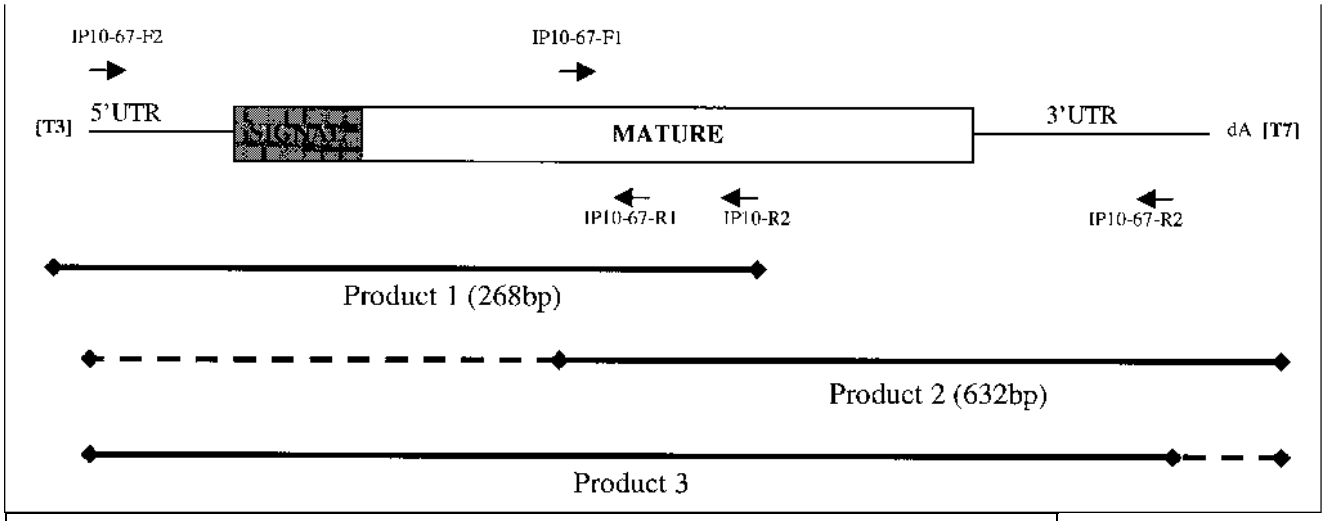
Figures



Schematic diagram showing the relative positions of primers used to isolate trout gIP1 and gIP2 and the derived products.

PCR was performed using 0.25 ml of DNA polymerase mix (50:1 v/v) of *Taq* DNA polymerase (5 $U.uL^{-1}$; Promega, UK) and *Pfu* DNA polymerase (2 $U.uL^{-1}$, Promega, UK), in 50 mL reactions containing the following components: 2 mL each of forward and reverse primers (each 10 mM), 2 mL dNTP mix (10mM each; Promega, UK), 5 mL *Taq* Buffer (10X; Promega, UK), 5 mL $MgCl_2$ (25 mM; Promega, UK), 31.75 mL sterile H_2O and 2 mL cDNA template (10 $ng.mL^{-1}$). The cycling protocol was 1 cycle of 94¡C for 2 min, 35 cycles of 94¡C for 1 min, 50¡C for 1 min and 72¡C for 1 min, with a final extension step of 5 min at 72¡C. Amplified products were analysed on a 2% agarose gel containing ethidium bromide (100 $ng.mL^{-1}$; Sigma, UK). Products obtained were cloned and sequenced as described below.

5'UTR

```
     1 acttgactgagctgtccatccagactctgaaagaaactgaagaagcagtatcc 53
```

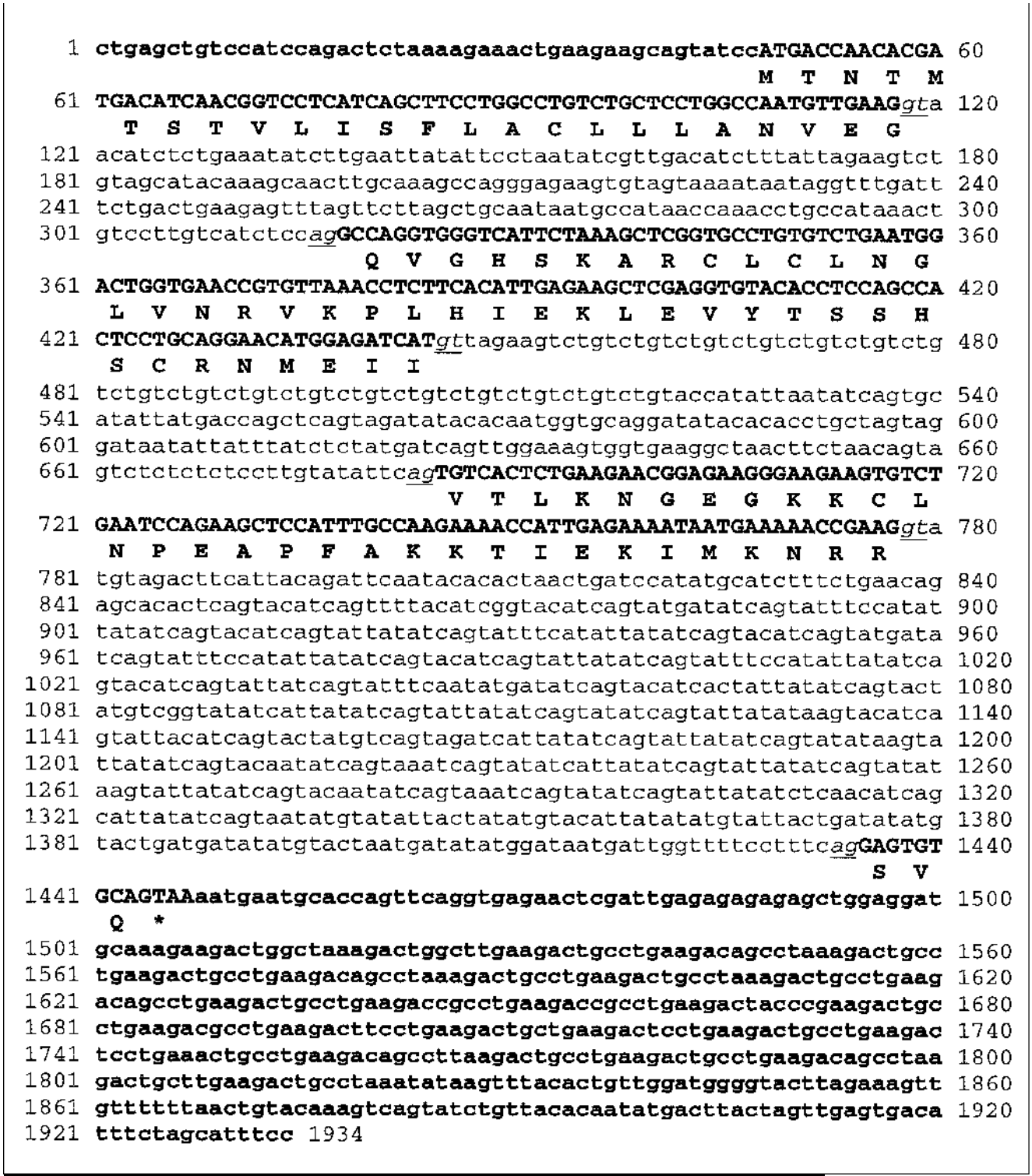
Coding region

```
    54 atgaccaacacgatgacatcaacggtcctcatcagcttcctggcctgtctgctcctggcc 113
     1 M  T  N  T  M  T  S  T  V  L  I  S  F  L  A  C  L  L  L  A   20

   114 aatgttgaaggccaggtgggtcattctaaagctcggtgccagtgtctgaatggactggtg 173
    21 N  V  E  G  Q  V  G  H  S  K  A  R  C  Q  C  L  N  G  L  V   40

   174 aaccgtgttaaacctcttcacattgagaagcttgaggtgtacacctccagccactcctgc 233
    41 N  R  V  K  P  L  H  I  E  K  L  E  V  Y  T  S  S  H  S  C   60

   234 aggaacatggagatcattgtcacctgaagaacggagaagggaagaagtgtctgaatcca 293
    61 R  N  M  E  I  I  V  T  L  K  N  G  E  G  K  K  C  L  N  P   80

   294 gaggctccatttgccaagaaaaccattgagaaaataatgaaaaaacaaaggagtgtgcag 353
    81 E  A  P  F  A  K  K  T  I  E  K  I  M  K  K  Q  R  S  V  Q  100

   354 taa
       *
```

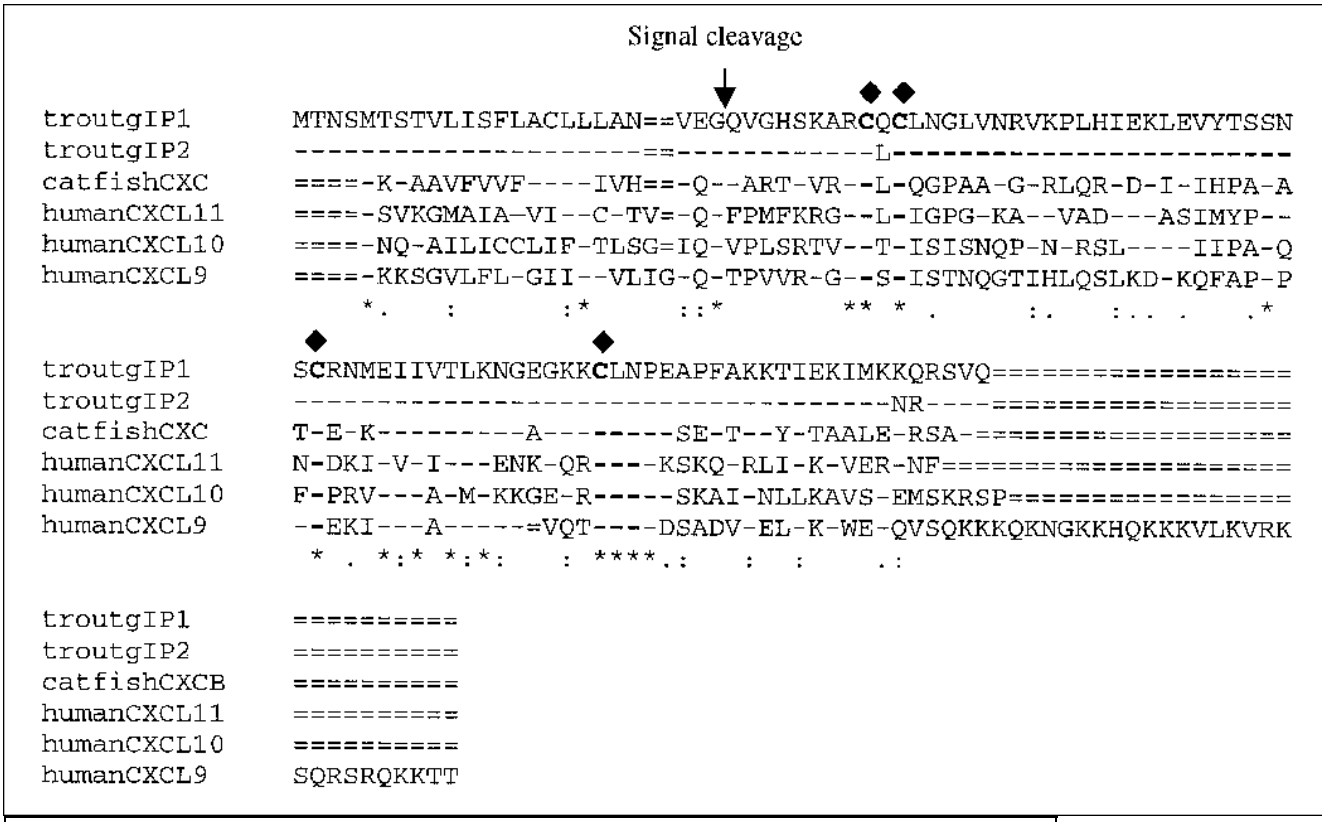
3' UTR

```
   357 aatgaatgacaccagtttaggtgagaactcgatagagagagagctggaggaatgcaaa 416
   417 gaagactgcctgaaaactgcctgaagacagcctaaagactgcctgaagactgcctgaaga 476
   477 ctgcctgaaaactgcctgaagactgcctgaagactgcctgaagactgcc 536
   537 taaatatatgtttacactgttgaatggtacttagaaagttgtttttaactgtacaaagtc 596
   597 agtatctgttacacaatatgacttactagttgagtgacatttctagcatttccaatggaa 656
   657 aaaataaaaagcatgtaaatatgtaaatatacttttttattatgtaatgtcaacaatata 716
   717 tgtatgtatttctcaggttttctatcaaattaataaagaagcatttgactctaaaaaaaa 776
   777 aaaaaaaaaaa 787


   357 aatgaatgacaccagttcaggtgagaactcgatagagagagagctggaggaatgcaaa 416
   417 gaagactgcctaaagactgcctgaagactgcctgaagacagcctaaagactgcctgaaga 476
   477 ctgcctgaagacagcctaaagactgcctgaagactgcctaaagactgcctgaagacagcc 536
   537 tgaagactgcctgaagaccgcctgaagaccgcctgaagactacccgaagactgcctgaag 596
   597 acgcctgaagacttcctgaagactgctgaagactcctgaagactgcctgaagactcctga 656
   657 aactgcctgaagacagccttaagactgcctgaagactgcctgaagacagcctaagactgc 716
   717 ttgaagactgcctaaatataagtttacactgttggatggggtacttagaaagttgttttt 776
   777 taactgtacaaagtcagtatctgttacacaatatgacttactagttgagtgacatttcta 836
   837 gcatttcc 840
```

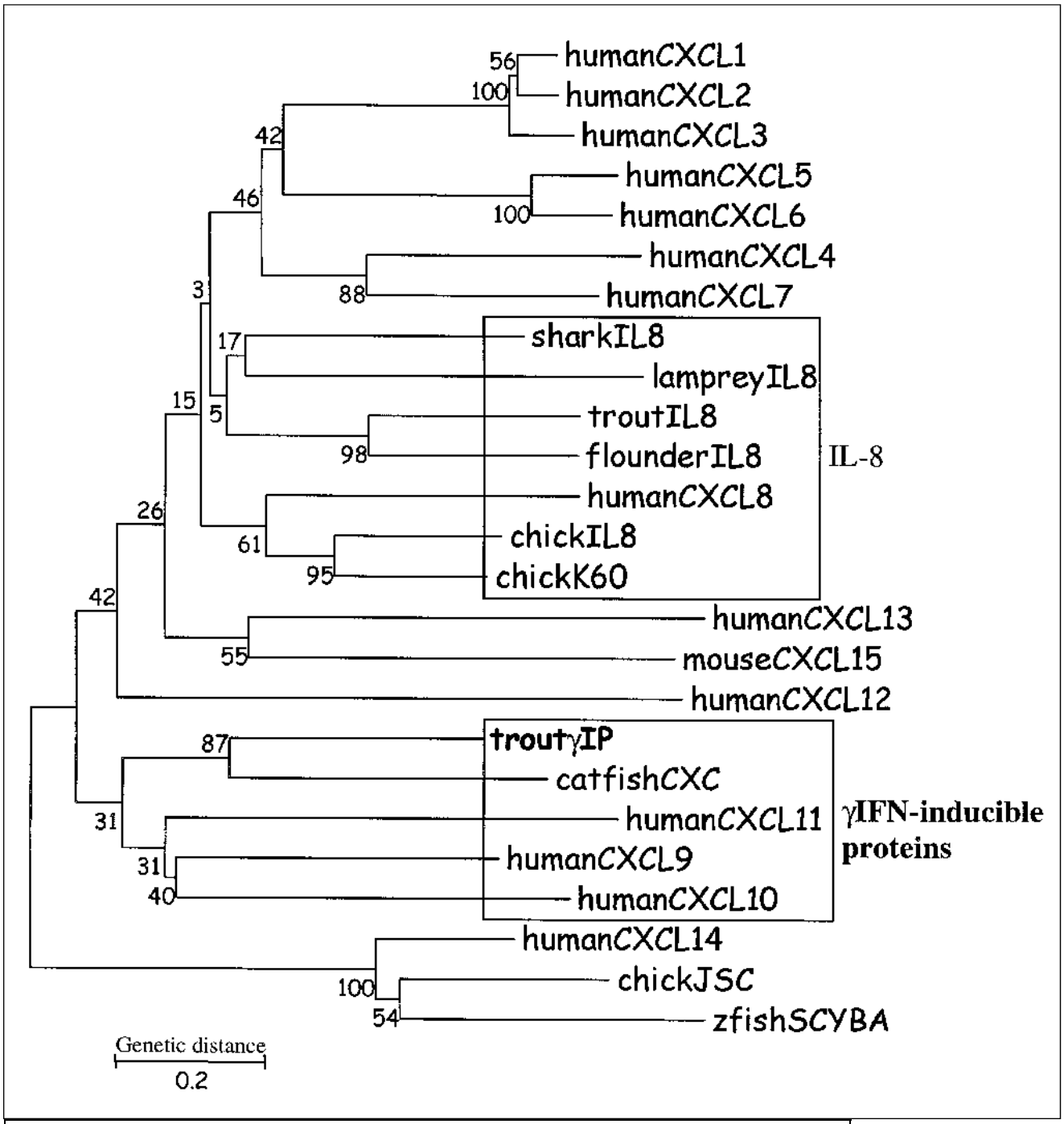
The cDNA sequence of gIP1, separated into 5'UTR, coding sequence and 3'UTR. Sequence underlined in coding region represents the signal peptide and shaded residues show the differences observed between gIP1 and gIP2. The 4 cysteine residues typical of CXC chemokines are shown in bold text, as are the start (ATG) and stop (TAA) codons. Both 3'UTR sequences of gIP1 (top) and gIP2 (bottom) are shown. Boxed residues with alternating shading/non-shading show the 11 bp repeat sequence. Moieties in bold and underlined represent polyadenylation signal sequences. The position of the IP10-67-R2 primer used during the amplification of gIP2 is shown in italics in both 3'UTR sequences. This sequence has been submitted to the EMBL database (Acc. No. AJ303075).



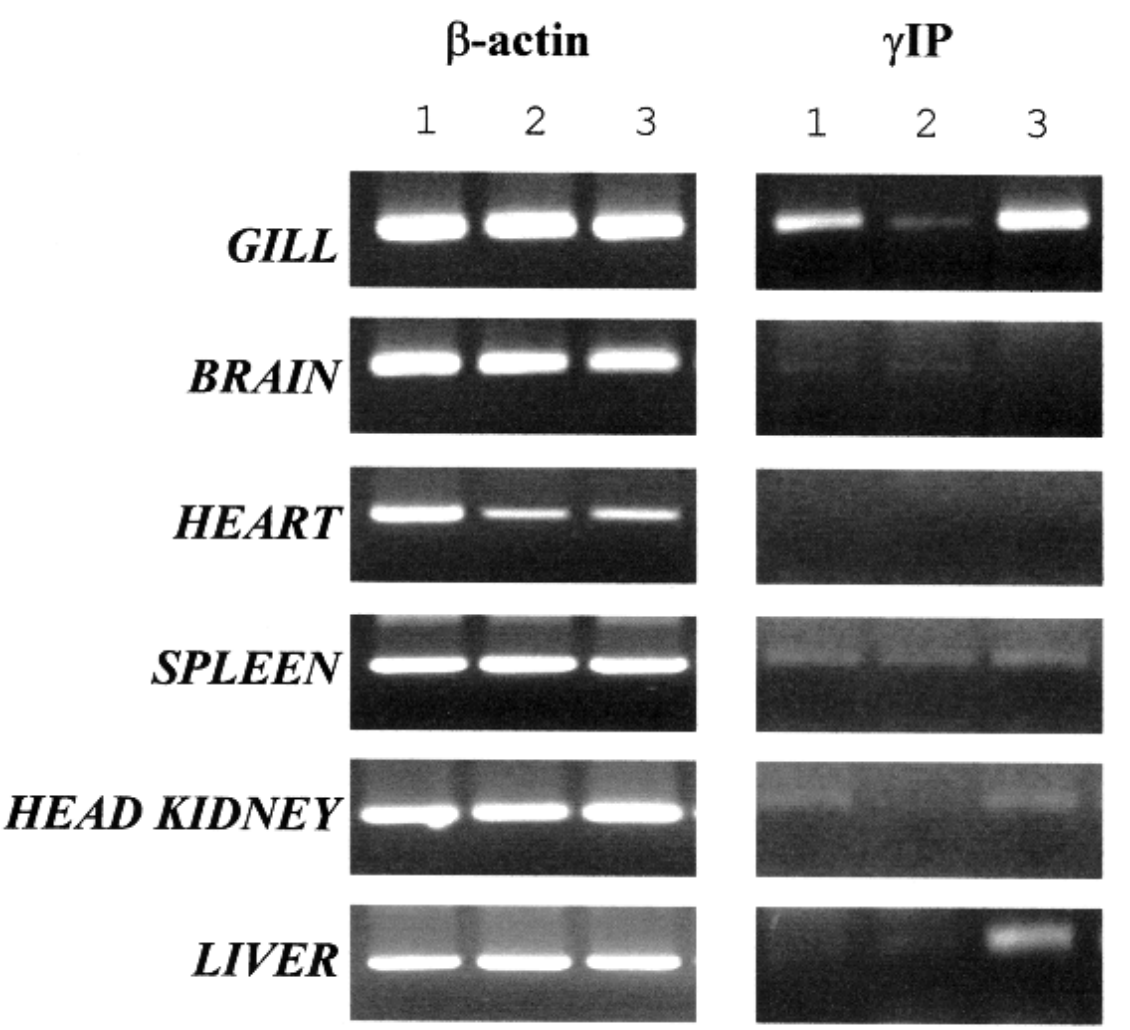
The genomic sequence of gIP2. Exon sequences are shown in bold text and intron splice sites are indicated in italics and underlined. Sequence that encodes for protein is shown in capital letters. This sequence has been submitted to the EMBL database (Acc. No. AJ417078).



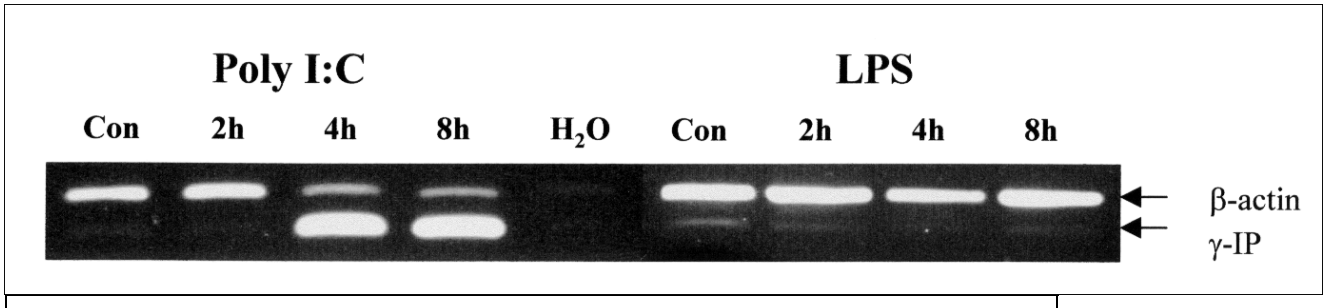
Multiple alignment of trout gIP1 and gIP2 with human CXCL9, CXCL10 and CXCL11 molecules and the putative CXCL10-like peptide of a catfish EST. Dashes (-) represent amino acids that are identical to trout gIP1 and gaps in the alignment are shown with Ô = Ô. Beneath the alignment, Ô*' indicates conserved residues shared between all sequences, whilst Ô:' and Ô.' represent high and low amino acid similarity respectively. The position of signal peptide cleavage is shown with an arrow, and the 4 conserved cysteine residues highlighted with Ô♦'.



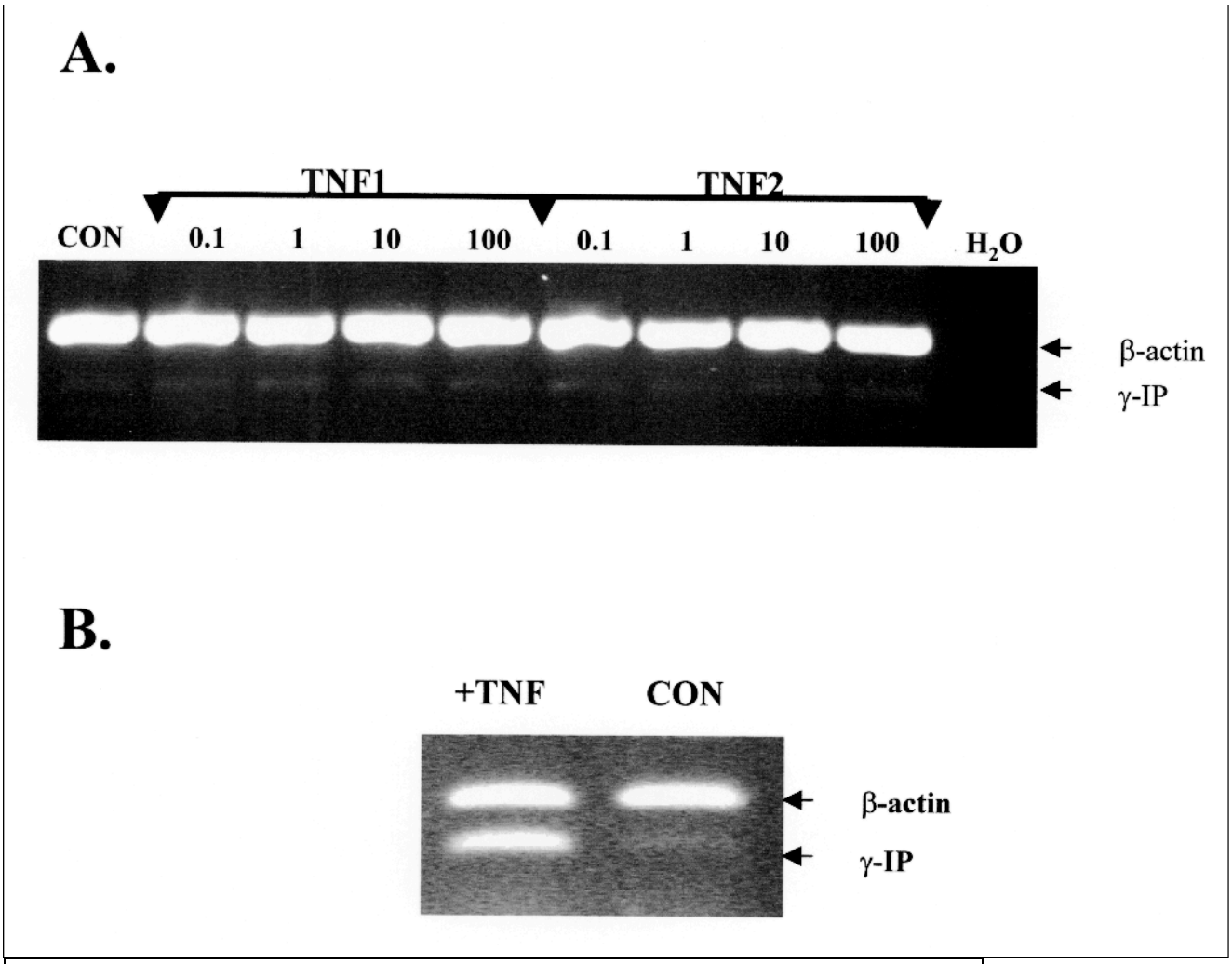
An unrooted phylogenetic tree based on the Neighbour-joining method produced from a CLUSTAL generated alignment of CXC chemokine peptide sequences using the MEGA 2 software. The data was subjected to Poisson correction and gaps were deleted from the alignment using a pairwise method. The resulting tree was bootstrapped 1000 times (shown as percentages). gIFN-inducible proteins (CXCL9, CXCL10 and CXCL11) and IL-8 molecules are boxed in their separate clades.



RT-PCR analysis to detect expression of gIP in trout tissues. Three individual fish were investigated (represented numerically). b-actin and gIP products were amplified using 30 and 35 cycles of PCR respectively.

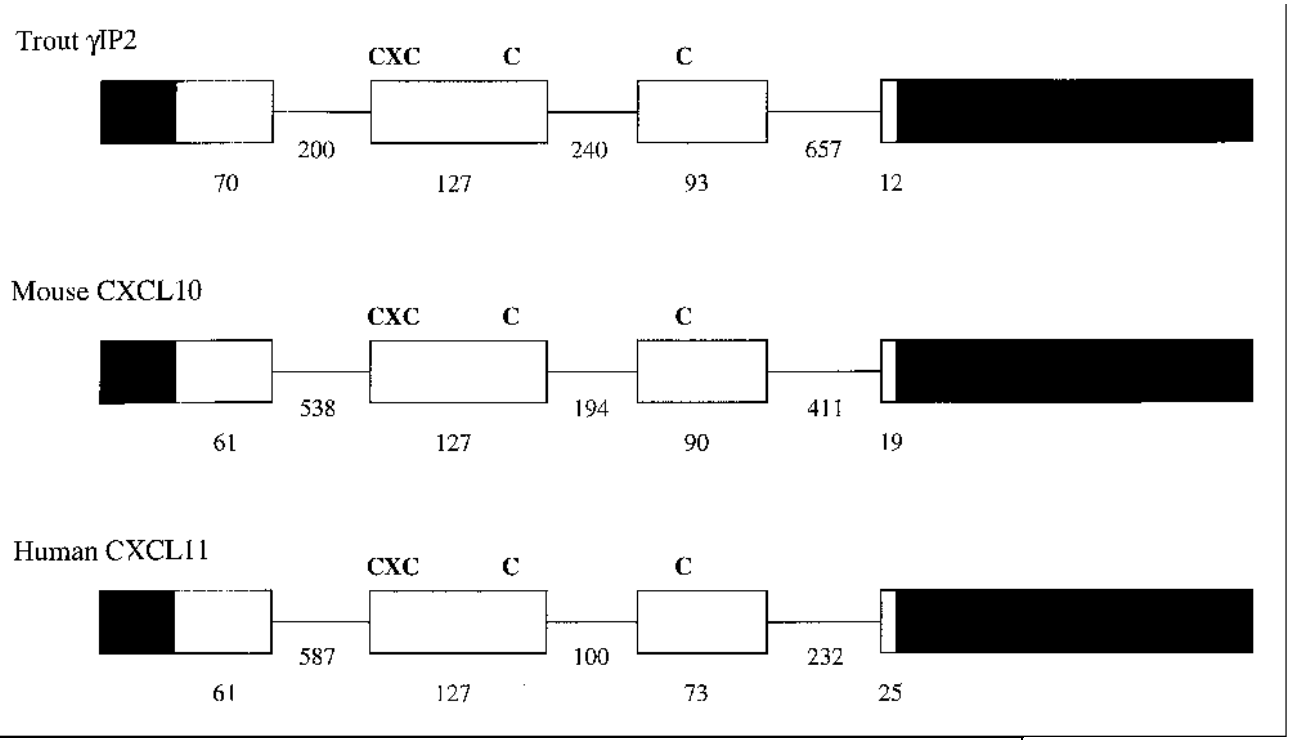


Expression of gIP in RTS-11 cells following exposure to poly I:C (50 ($mg.mL^{-1}$) or LPS (5 ($mg.mL^{-1}$) for 2, 4 or 8 h. Results were compared to an unchallenged control (Con) to which an equivalent volume of PBS was added. Semi-quantitative PCR was performed to amplify b-actin and gIP in the same reactions for 30 cycles. A negative control (using sterile H_2O as template) was included to show that bands corresponding to gIP were not derived from contamination.



Semi-quantitative PCR to determine the *in vitro* and *in vivo* effects of TNF-a on gIP expression in trout. b-actin and gIP were amplified in the same reactions for 30 cycles. [A] Increasing concentrations of trout rtTNFa1 or rtTNFa2 (0.1-100 $ng.mL^{-1}$) were incubated with RTS-11 cells for 5 h prior to RNA extraction. Expression levels of

gIP were compared to that of an unchallenged control (CON) to which an equivalent volume of EB was added. A negative control using sterile water as template in PCR was used to negate any false positives in the reactions. [B] *In vivo* expression of gIP in rainbow trout head kidney cells following intraperitoneal injection with 30 (mg rtTNFa1 plus 55 (mg rtTNFa2 (+ TNF) compared to trout injected with PBS (CON).



Schematic diagram comparing the genomic organisation of trout gIP2 with mammalian CXCL10 and CXCL11 genes. Exons are shown as boxes and introns are represented as straight lines, with numbers below showing the nucleotide size of each. Regions shaded black represent the 5' and 3' UTRs. The relative positions of the 4 conserved cysteine residues of the chemokines are shown above exons 2 and 3.

Name of Primer	Primer Sequence (5'-3')
IP-10-R2	CCG TTC TTC AGA GTG ACA ATG
IP10-67-F2	CTG AGC TGT CCA TCC AGA CTC
IP10-67-F1	TGT CTG AAT GGA CTG GTG AAC
IP10-67-R2	GGA AAT GCT AGA AAT GTC ACT C
IP-10-67-R1	CTC AAT GTG AAG AGG TTT AAC ACG
T7	TAA TAC GAC TCA CTA TAG GG
T3	AAT TAA CCC TCA TAA AGG G
b-ACTIN FOR	ATC GTG GGG CGC CCC AGG CAC C
b-ACTIN REV	CTC CTT AAT GTC ACG CAC GAT TTC

Primers used to isolate trout gIP

Chemokine	Accession No.	Amino acid identity (%) complete	Amino acid identity (%) Exon 2 only
Mammalian CXC chemokines			
CXCL1	J03561	29.91	28.57

CXCL2	M57731	29.91	26.19
CXCL3	M36821	30.19	26.19
CXCL4	M25897	26.73	21.43
CXCL5	X78686	24.56	26.19
CXCL6	U83303	27.19	29.55
CXCL7	M54995	21.09	17.74
CXCL8	Y00787	30.00	31.11
CXCL9	X72755	22.40	28.57
CXCL10	X02530	30.00	37.21
CXCL11	U59286	30.00	30.95
CXCL12	U16752	19.00	26.19
CXCL13	AF044197	22.02	20.43
CXCL14	AF073957	22.00	16.67
CXCL15 (mouse)	Q9WVL7	13.77	19.05
Fish chemokines			
Trout IL-8	AJ279069	31.00	ND
Catfish EST	BE212851	44.00	ND

	Comparison of the trout IP-10-like sequence with known chemokines using the NEEDLE program

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Presented at the 4th symposium on Cytokines and apoptosis in the cardiovascular system, Halle, Germany, June 6-7, 2002

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IL-2, IL-4, B lymphocyte, caspase, proliferation, cell cycle entry

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Prolactin, cytokine, A. castellani, MGC.

CINC-1 mediates the sympathetic component of inflammatory mechanical hypersensitivitiy in rats

Berenice B Lorenzetti, Fabiane H Veiga, Claudio A Canetti, Stephen Poole, Fernando Q Cunha, Sergio H Ferreira

Inflammatory hyperalgesia, CINC-1, tumour necrosis factor alpha, interleukin-1b, interleukin-8, sympathetic mediators, prostaglandin E2

A CXC chemokine sequence isolated from the rainbow trout Oncorhynchus mykiss resembles the closely related interferon-gamma-inducible chemokines CXCL9, CXCL10 and CXCL11

Kerry J Laing, Niels Bols, Christopher J Secombes
Chemokine, rainbow trout, IP10, I-TAC, Mig

Meeting report of the “Club Cytokine” (French Society of Immunology) meeting, in Le Croisic, France

Mariette Lisbonne, Hélène Plun-Favreau

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