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Atlantic salmon possesses two clusters of type I interferon receptor genes on different chromosomes, which allows for a larger repertoire of interferon receptors than in zebrafish and mammals

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ABSTRACT

Mammalian type I interferons (IFNs) signal through a receptor composed of the IFNAR1 and IFNAR2 chains. In zebrafish two-cysteine IFNs utilize a receptor composed of CRFB1 and CRFB5, while four-cysteine IFNs signal through a receptor formed by CRFB2 and CRFB5. In the present work two CRFB clusters were identified in different chromosomes of Atlantic salmon. Genes of three CRFB5s, one CRFB1, one CRFB2 and the novel CRFB5x were identified, cloned and studied functionally. All CRFBs were expressed in 10 different organs, but the relative expression of CRFBs varied. Mx-reporter assay was used to study which CRFBs might be involved in receptors for salmon IFNa, IFNb and IFNc. The results of Mx-reporter assays suggest that IFNa signals through a receptor composed of CRFB1a as the long chain and either CRFB5a, CRFB5b or CRFB5c as the short chain; IFNc signals through a receptor with CRFB5a or CRFB5c as the short chain while IFNb may signal through a receptor with CRFB5x as a short chain. Taken together, the present work demonstrates that Atlantic salmon has a more diverse repertoire of type I IFN receptors compared to zebrafish or mammals.

1. Introduction

Interferons are cytokines, which induce antiviral activity in vertebrate cells and are classified into three types according to sequence homology and receptor specificity. Mammalian type I IFNs (IFNα, IFNβ, IFNε, IFNκ, IFNω) and type III IFNs (IFNλ1, IFNλ2, IFNλ3) play important roles in innate immunity against viruses being induced upon host cell recognition of viral nucleic acids, while type II IFN (IFNγ) is a major product of T-cells and has a key role in adaptive immunity against intracellular bacterial pathogens (Kotenko et al., 2003; Pestka et al., 2004; Schoenborn and Wilson, 2007; Takeuchi and Akira, 2007). The three IFN types bind to different receptors, which are structurally and genetically related, and signal through overlapping JAK/STAT signaling pathways. Type I IFNs bind to a heterodimeric receptor composed of a class II cytokine receptor family member (CRFB) with a long cytoplasmic chain and a CRFB with a short cytoplasmic chain (Lutfalla et al., 2003). The mammalian type I IFN receptor is composed of the IFNAR1 and IFNAR2 chains (Uze et al., 2007). The IFN-γ receptor is composed of IFNGR1 and IFNGR2 while the IFN-λ receptor is composed of IFNLR1 and IL10 receptor 2 (IL10R2) (Kotenko et al., 2003; Pestka et al., 2004). IFNAR1, IFNAR2, IFNGR2 and IL10R2 are thought to be derived from a common ancestor gene because they are clustered in the same genomic region (Lutfalla et al., 2003; Pestka et al., 2004).

IFNAR1 and IFNAR2 associate with the Janus family tyrosine kinases Tyk2 and Jak1, respectively, which are activated upon binding of IFN to the receptor (Uze et al., 2007). Subsequently, Jak1 and Tyk2 phosphorylate several tyrosine residues on the intracellular parts of IFNAR1 and IFNAR2, which provide the docking site for phosphorylation of STAT1 and STAT2 transcription factors. Phosphorylated STAT1 and STAT2 form a dimer that associates with IRF9 forming the transcription factor ISGF3, which translocates into the nucleus and activates transcription of hundreds of IFN-stimulated genes (ISGs) by binding to the interferon-stimulated response element (ISRE) in their promoters (Der et al., 1998; Uze et al., 2007). Teleost fish are thought to have a similar type I IFN mediated signaling since STAT1, STAT2, Jak1 and Tyk2 have been identified in fish and ISRE is well conserved in vertebrate ISGs such as Mx (Collet and Secombes, 2001; Collet et al., 2009; Leu et al., 1998, 2000; Skjesol et al., 2010; Sobhkhez et al., 2013; Stein et al., 2007).

Abbreviations: CRFB, class II cytokine receptor family member; IFN, interferon; IFNAR, mammalian type I IFN receptor.

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Teleost fish possess at least four type I IFN subtypes named IFNa, IFNb, IFNc and IFNd. Atlantic salmon (*Salmo salar*) possesses all four subtypes while zebrafish (*Danio rerio*) possesses IFNa (IFNϕ1/IFN1), IFNc (IFNϕ2/IFN2 and IFNϕ3/IFN3) and IFNd (IFNϕ4/IFN4), and Tetraodontiformes (pufferfish) appear to possess only IFNd (Chang et al., 2009; Sun et al., 2009; Svingerud et al., 2012). IFNa and IFNd contain two cysteines and IFNb and IFNc contain four cysteines forming one and two disulfide bridges, respectively (Chang et al., 2009; Hamming et al., 2011; Zou et al., 2007). Otherwise, the four IFN subtypes show relatively low sequence identity. The search for type I IFN receptors in fish was initially based on the demonstration of synteny between the pufferfish *Tetraodon nigroviridis* CRFB genes and the IFN receptor gene cluster in mammals (Lutfalla et al., 2003). The *Tetraodon* CRFB gene cluster contains CRFB1, CRFB2, CRFB3, CRFB4, CRFB5 and CRFB6. Sequence analysis and gene synteny suggested that CRFB1 and CRFB2 were IFNAR2 homologs while CRFB5 is an IFNAR1 homolog. Zebrafish does apparently not have a complete CRFB cluster since only CRFB1 and CRFB2 are linked while CRFB4 and CRFB5 are present on the same chromosome (chr. 9), but are not closely linked (Stein et al., 2007). Moreover, zebrafish CRFB6 is located on chr. 5 (GenBank accession no. NM_001077627). Zebrafish also lacks CRFB3. Gene knockdown of CRFB1, CRFB2 and CRFB5 in zebrafish suggested that the receptor for the 2-cysteine IFNs (IFNa and IFNd homologs) is composed of CRFB1 and CRFB5 while the receptor for the 4-cysteine IFNc homologs is composed of CRFB2 and CRFB5 (Aggad et al., 2009; Levraud et al., 2007). On the other hand, recent work in rainbow trout suggested CRFB3 to be an IFNAR2 homolog and CRFB5 an IFNAR1 homolog (Chang et al., 2013). In Atlantic salmon, IFNa and IFNc display similar antiviral activities in cell lines. They induce similar levels of ISGs and show similar time kinetics in induction of Mx transcription, which seems in disagreement with the zebrafish IFN receptor model (Svingerud et al., 2012). Moreover, in contrast to zebrafish, Atlantic salmon possesses IFNb, which is a 4-cysteine IFN subtype that shows lower antiviral activity and induces lower and later expression of antiviral genes than IFNa and IFNc (Svingerud et al., 2012). Atlantic salmon IFNd does not show antiviral activity (Svingerud et al., 2012). To add more light on type I IFN functions in Atlantic salmon, we have in this work identified CRFB1, CRFB2 and CRFB5 homologs in the Atlantic salmon genome and done initial functional studies. Expression of the CRFBs in organs and cells were studied by quantitative RT-PCR. Mx reporter gene assays suggest that IFNa, IFNb and IFNc have different preferences for the short CRFBs.

2. Materials and methods

2.1. Identification of genomic CRFB sequences

Atlantic salmon IFNGR2 cDNA (GenBank accession no. DY734720) was used as a probe for screening an Atlantic salmon BAC clone library and resulted in identification of one positive clone, BAC882E11 (GenBank accession no. KF845942), which was sequenced. Other genomic regions containing CRFBs were identified by *in silico* screening of genomic sequences generated by the International Collaboration to Sequence the Atlantic Salmon Genome (ICSASG) (Davidson et al., 2010). The gene screening was done by TBLASTN using zebrafish CRFB1 (accession no. NP_001073149) and CRFB2 (accession no. NP_001071094) as queries and resulted in identification of a large CRFB gene cluster in the scaffold scf2339332509p (accession no. KF845941) in addition a sequence containing a CRFB1 gene (CRFB1b) and exons 1 and 2 of CRFB2 (accession no. KF845944) and a sequence containing exons 3–7 of CRFB2 (accession no. KF845943). Annotation of genes in genomic sequences was performed by analysis with the programs FGENESH (http://www.softberry.com) and GENSCAN (http://genes.mit.edu/GENSCAN.html) combined with manual inspections. The first exon of CRFB1 and CRFB2 was identified after identification of the 5′-end by RACE cloning as described below.

2.2. Chromosome linkage analysis

To map scaffolds to chromosomes, BLASTN was performed with the scaffold sequences as queries against the chromosome annotated SNP associated sequences presented in Lien et al. (2011), online additional file 1 (http://www.biomedcentral.com/1471-2164/12/615). Perfect or near perfect hits (>98% identity) to specific chromosome linked SNP sequences were identified. These hits linked scaffolds to chromosome 25 or 21. The mapping to chromosomes 25 and 21 is consistent with homeologous whole genome duplication mapping in Lien et al. (2011).

2.3. cDNA cloning of CRFBs

To confirm the transcriptional start site of CRFB1a, CRFB1b and CRFB2 and the actual stop site of CRFB1b and CRFB2, 5′ and 3′ rapid amplification of cDNA ends (RACE)-PCR was performed using a SMART RACE cDNA amplification kit (Clontech) from head kidney cDNA of poly I:C stimulated fish. All primers used are listed in Table 1. The open reading frames of CRFBs were amplified from head kidney cDNA samples by using *Pfu* polymerase (Promega). PCR conditions were 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. After gel-purification (Qiagen), the PCR products were cloned into the pcDNA3.3-TOPO vector (Invitrogen) for eukaryotic expression.

2.4. Expression of CRFBs in head kidney leukocytes, TO cells and different tissues

Healthy Atlantic salmon (*S. salar*) (400 g) were kept at Tromsø Aquaculture Research Station (Tromsø, Norway) in 300 l tanks containing fresh water at 10 °C and were fed commercial dry food. Prior to treatments, the fish were anesthetized with 0.005% benzocaine (ACD Pharmaceuticals, Norway). All handling of fish was in accordance with the Norwegian "Regulation on Animal Experimentation" and the *in vivo* experiment was submitted to and approved by the Norwegian Animal Research Authority before initiation.

Four Atlantic salmon were used for isolation of primary head kidney leukocytes (HKL). HKL were isolated using 54% Percoll (GE Healthcare) (Svingerud et al., 2012). Isolated cells were stimulated with poly I:C 10 μg/ml or R848 2 μg/ml and RNA was isolated 24 hours after stimulation. Gill, liver, spleen, head kidney, kidney, intestine, muscle, heart, brain and ovary were isolated from five fish, stored in RNAlater (Invitrogen) and isolated with Trizol (Invitrogen). Atlantic salmon TO cells were cultured as described previously (Svingerud et al., 2012), and RNA was isolated with Trizol.

2.5. Quantitative PCR (qPCR) of transcripts in cells and tissues

cDNA was synthesized with the QuantiTect® Reverse Transcription Kit (Qiagen) starting with 200 ng total RNA following standard protocol. qPCR was performed using 6.1 μl 1:10 dilution of cDNA in 15 μl reaction mixture containing 7.5 μl 2X SYBR green PCR Master Mix (Applied Biosystems) and 230 nM of forward and reverse primers (Table 1). Each sample was run in triplicate wells on a 7500 Fast Real Time PCR system (Applied Biosystems). The mixtures were incubated at 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. The absence of primer–dimer artifacts was confirmed by running melting curve step. The relative expression values were normalized against the levels of beta actin and analyzed as

Table 1
Primers used in this study.

Primer name	Accession no.	Primer sequence (5′-3′)	Direction	Use
CRFB1A F1	KF976456	TGGAGAACTTGGAGGTGGGTA	Forward	qPCR
CRFB1A R1		CCAGCTACAACAGCAGGGACT	Reverse	
CRFB1B F1	KF976457	CACCCCCTTCACACAGACTGT	Forward	qPCR
CRFB1B R1		TTCAATGGTTCCCATCTCAGG	Reverse	
CRFB2 F1	KF976455	GCCAAGTACATCGCAGACACA	Forward	qPCR
CRFB2 R1		CTCAGACAGATGAATCCAGTGC	Reverse	
CRFB5A F1	KF976460	CCTCACAGATTCTGATCTGCA	Forward	qPCR
CRFB5A R1		GGTGGGCCCAACGAAGCATCCA	Reverse	
CRFB5B F1	KF976459	CAGCGGAGACTCTGGGATCC	Forward	qPCR
CRFB5B R1		TTTCATGATGACGTCTCTCCC	Reverse	
CRFB5C F1	KF976458	TGGACGTGGTACTGTGTCAGG	Forward	qPCR
CRFB5C R1		AACATGCTACCAGGGAGACCA	Reverse	
CRFBX F1	KF976461	AATCACTCCATGGCCAATCAC	Forward	qPCR
CRFBX R1		TTGTTGAAGCCCAGCTCCTTA	Reverse	
CRFB2 RF1	KF976455	GCTGTGTAGTACTACCCTACT	Forward	RACE-PCR
CRFB2 RF2	KF976455	CGCACTGGATTCATCTGTCTG	Reverse	RACE-PCR
CRFB2 RR1	KF976455	CCTGGTTGTAGTAGGTGTGAC	Forward	RACE-PCR
CRFB2 RR2	KF976455	TACACACCAGAGGGAACTCCA	Reverse	RACE-PCR
CRFB1B RF1	KF976457	CCTGGTTGAGGGCTACCTAGT	Forward	RACE-PCR
CRFB1B RF2	KF976457	CCTCTGGTAACGTCAATCTGT	Reverse	RACE-PCR
CRFB1B RR1	KF976457	GAGAACAGATTGACGTTACCAGA	Forward	RACE-PCR
CRFB1B RR2	KF976457	TGACTAGGTAGCCCTCAACCA	Reverse	RACE-PCR
CRFB1A RR1	KF976456	GGCAGTTGCCACATCCATCCAA	Forward	RACE-PCR
CRFB1A RR2	KF976456	CCACGGGCGACTCTGAGAGGT	Reverse	RACE-PCR
CRFB1A F33	KF976456	GATATGAAACATTTTCTTCGGAC	Forward	ORF cloning
CRFB1A R33		TCAATGTCCCATGTACCCTGAGA	Reverse	
CRFB1B F33	KF976457	GATATGAAACATTTCCTTCTAAC	Forward	ORF cloning
CRFB1B R33		TCAATGTCCCATATAGCCTGAG	Reverse	
CRFB2 F33	KF976455	GTCATGACTCCAGTGATATTGGTG	Forward	ORF cloning
CRFB2 R33		TTACTTCTCTCGTCGCCTCATGTA	Reverse	
CRFB5A F33	KF976460	ATCATGAACGTGGGCTTTGCACTC	Forward	ORF cloning
CRFB5A R33		TCAGACACACACATCCACAACCCC	Reverse	
CRFB5B F33	KF976459	ATCATGAACGTGGGCTTTGCACTC	Forward	ORF cloning
CRFB5B R33		TCAGGACAGATGGTCAGCTTATCA	Reverse	
CRFB5C F33	KF976458	ATCATGAACGTGGGTTTTGCTCTCGTT	Forward	ORF cloning
CRFB5C R33		TCAGACGCACACATCCACAATCAC	Reverse	
CRFBX F33	KF976461	ATCATGATATTACGCCTATTTGCATGG	Forward	ORF cloning
CRFBX R33		TCACCCTGCGCTCCTCTTTCCACT	Reverse	

described earlier (Kileng et al., 2007). Fold up-regulation of the representative genes was calculated by comparison of gene expression in treated vs. untreated cells.

2.6. Reporter gene assay

Chinook salmon embryo cells, CHSE-214, were seeded into 96-well culture plates at a density of 1.7×10^3 cells/well, and grown to 40% confluence overnight at 20 °C in L-15 medium supplemented with 8% fetal bovine serum (FBS), 100 μg/ml streptomycin and 200 U/ml penicillin G. The cells were then co-transfected with different pcDNA3.3CRFB constructs, pRL-SV40 Vector expressing *Renilla* luciferase (Promega) and a construct containing the rainbow trout Mx1 promoter fused to the firefly luciferase gene (Collet and Secombes, 2001). The cells were transfected by adding to each well 10 μl Opti-MEM® (Life Technologies) solution containing 90 ng plasmid DNA (5 ng *Renilla* plasmid and 42.5 ng of each plasmid construct) and 0.25 μl TransIT-LT1 transfecting reagent (Mirus), according to the manufacturer's protocol. The transfected cells were incubated for 48 h before being stimulated with 200 U/ml IFNa, IFNb or IFNc, or left un-stimulated. Recombinant Atlantic salmon IFNa, IFNb and IFNc were produced in HEK293 cells as described (Svingerud et al., 2012). Recombinant IFNa is identical to IFNa1, recombinant IFNb is most similar to IFNb2 (99.5% sequence identity) while recombinant IFNc is most similar to IFNc1 (99.5% sequence identity) (Svingerud et al., 2012). Luciferase activity in the cells was measured 24 h post stimulation using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's protocol. *Renilla* luciferase levels, which are constitutively expressed, were used to normalize firefly luciferase values. The firefly and *Renilla* luciferase activity was measured in a Luminoscan RT luminometer (Labsystems OY) (Martin et al., 1996). All samples for the luciferase assay were set up in five parallels for each treatment and the results are given as relative light units (RLU).

2.7. Statistics

One way ANOVA was used to analyze the significance of data.

3. Results

3.1. Identification of Atlantic salmon CRFB gene clusters

Two clusters of CRFB genes were identified (Fig. 1). The largest cluster (scaffold scf2339332509p) was identified by *in silico* screening of genomic sequences by TBLASTN search using zebrafish CRFB1 (Accession no. NP_001073149) and CRFB2 (Accession no. NP_001071094) as query sequences. Annotation of scf2339332509p (GenBank accession no KF845941) resulted in identification of the following apparently complete CRFB genes: CRFB1a, CRFB3, CRFB4a, CRFB5c, CRFB5x, and a truncated IFNGR2 gene, which was named IFNGR2rel. CRFB3 encodes a 269 amino acid protein, which has previously been deposited in GenBank (Accession number NP001134552), but was named IL-20 receptor alpha chain based on homology without functional confirmation. CRFB4a encodes a protein identical to a salmon IL10R2 sequence previously deposited in GenBank (Accession no. ACN10735) and is herein named

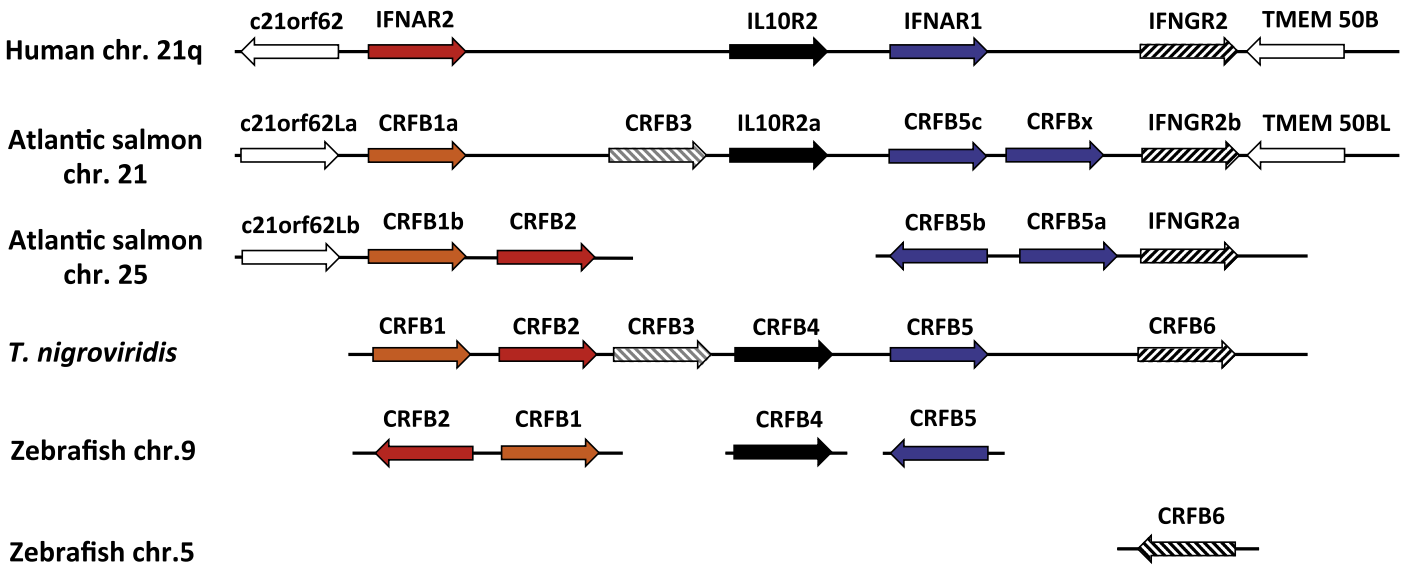

Fig. 1. Genomic organization of Atlantic salmon CRFB genes compared with pufferfish and zebrafish CRFB genes and the human IFN receptor gene cluster. Human, green spotted pufferfish (*T. nigroviridis*) and zebrafish gene organization is shown as described earlier (Lutfalla et al., 2003; Stein et al., 2007). The Atlantic salmon gene clusters were annotated from the genomic sequence scf2339332509p (GenBank accession no KF845941), which is present at chromosome 21 and the genomic sequence in BAC882E11 (GenBank accession no KF845942), which is present in chromosome 25. CRFB1b and CRFB2 exons 1 and 2 were annotated from the genomic sequence scf2339330473 (GenBank accession no KF845944), which is present on chromosome 25 while the remaining CRFB2 exons 3–7 were annotated from the genomic sequence scf2339297394 (GenBank accession no KF845943). The figure illustrates order and orientation (arrows) of genes. C21orf62L, C21orf62 like gene; TMEM50BL, Transmembrane 50B like protein.

SsaIL10R2a. The scf2339332509p scaffold thus contains a cluster of CRFB genes, which shows a high degree of gene synteny with the corresponding human type I IFN receptor cluster and the *Tetraodon* CRFB gene cluster except that scf2339332509p lacks CRFB2. Both the salmon scf2339332509p and the human type I IFN receptor gene cluster contain C21orf62 at one end and IFNGR2 and Transmembrane 50B genes at the other end, which shows that gene synteny exists beyond the IFN receptor clusters. The *in silico* screening also resulted in the identification of an CRFB1gene (SasaCRFB1b) linked to the first three exons of a CRFB2 gene (GenBank accession no. KF845944) while the remaining exons 3–7 of CRFB2 were identified on another genomic sequence (GenBank Accession no. KF845943). CRFB1b and CRFB2 are also linked to a homolog of C21orf62. While CRFB2 appears to be a complete receptor gene, the CRFB1b gene is more difficult to predict due to repeated sequence elements. The first six exons of CRFB1b are of expected size while the last exon(s) may be in one large exon or two to three smaller exons.

The other CRFB gene cluster was identified by screening a genomic BAC clone library using as probe the salmon IFN gamma receptor chain IFNGR2 (Acc. No DY734720) and resulted in identification of one positive clone, BAC882E11. Sequencing and annotation revealed the complete SsaCRFB5b, SsaCRFB5a and SsaIFNGR2 genes (GenBank accession no. KF845942). BAC882E11 thus also displays synteny with the Tetraodon and human IFN receptor gene cluster showing linkage between CRFB5 and IFNGR2/CRFB2.

Linkage analysis showed that scf2339332509p is present on chromosome 21, while CRFB1b and CRFB2, CRFB5b, CRFB5a and SsaIFNGR2 are present on chromosome 25. Henceforth, it is likely that Atlantic salmon contains two separate type I IFN receptor gene clusters, which probably is due to the tetraploid background of salmonids.

The extracellular region of the identified CRFBs possesses two immunoglobulin domain-like subdomains of the fibronectin type III class (FN3) in agreement with the structure of class II cytokine receptors (Bazan, 1990). The number of amino acids in the intracellular domains varies, but the CRFBs can be grouped in short chain CRFBs (similar to IFNAR1) or long chain CRFBs (similar to IFNAR2) as shown in Fig. 2. The exception is CRFB3, which according to the phylogenetic analysis below is homologous to CRFB2, but has a very short intracellular domain of only 24 amino acids.

3.2. Phylogenetic analyses of the CRFBs

IFNAR1, IFNAR2 and IL10 receptor 2 (IL10R2) sequences from human and mouse, and CRFB1 to CRFB6 sequences from salmon, rainbow trout, green pufferfish and zebrafish were subjected to multiple alignment using the Clustal W program. A phylogenetic tree was then constructed from the alignment using the neighbor-joining method (Fig. 3). The tree illustrates that fish CRFB1, CRFB2, CRFB3 and the mammalian IFNAR2 sequences form one group while fish CRFB5, salmon CRFB5x and mammalian IFNAR1 form another group that are both separated from the CRFB6 /IFNGR2-sequences. The relationships between the fish CRFB4/IL10R2 sequences and mammalian IL10R2 sequences and between fish CRFB6/IFNGR2 and mammalian IFNGR2 sequences were not significant in this analyses, but are consistent with gene synteny and domain analyses. Functional evidence is needed to confirm certain identification of fish CRFB4 as IL10R2 homologs while some functional evidence supports the identification of fish CRFB6 as IFNGR2 (Aggad et al., 2010; Gao et al., 2009).

3.3. Cloning and characteristics of salmon CRFB1 and CRFB2

Based on the genomic sequences, the salmon CRFB1a (Accession No. KF976456), CRFB1b (KF976457) and CRFB2 (KF976455) ORFs were identified by RACE cloning. Sequencing of cDNA clones confirmed protein sequences deduced from the genome. A ClustalW alignment of salmon and zebrafish CRFB1 and CRFB2 compared to human IFNAR2 is shown in Fig. 4. SsaCRFB1 and SsaCRFB2 possess long intracellular regions similar to human IFNAR2 and zebrafish CRFB1 and CRFB2. Otherwise the sequence similarities between fish CRFB1, CRFB2 and mammalian IFNAR2 are very low. SsaCRFB1a has 15.2% and 24.4% amino acid identity with HsaIFNAR2 and DreCRFB1, respectively. SsaCRFB2 has 19.5% and 26.5% identity with HsaIFNAR2

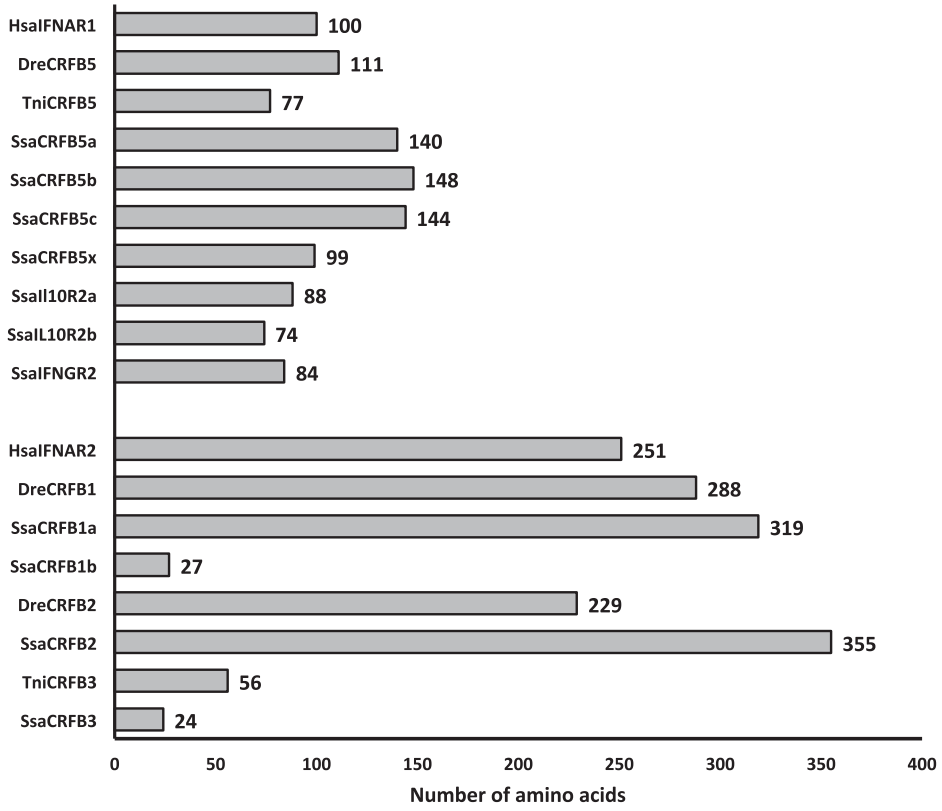

Fig. 2. Length of the intracellular domain of Atlantic salmon and zebrafish CRFBs compared with human IFNAR1 and IFNAR2. The graph shows the number of amino acids. NCBI GenBank accession numbers are listed in Fig. 3.

and DreCRFB2, respectively. In the extracellular region, fish CRFB2 displays strikingly more similarity to HsaIFNAR2 than fish CRFB1. Notably, SsaCRFB2 and DreCRFB2 possess four cysteines at the same positions as the disulfide bridge forming cysteines in IFNAR2 while SsaCRFB1a and DreCRFB1 possess only one of the cysteine pairs.

Full length SsaCRFB1b mRNA was identified from several cDNA clones and confirmed the presence of a stop codon in the middle of the mRNA. This yields a putative CRFB with an external chain and a transmembrane region, but with only a 27 amino acid internal domain. The external region of SsaCRFB1b contains only one of the conserved cysteines and may thus not possess disulfide bonds. The role of SsaCRFB1b is thus uncertain.

In IFNAR2, the last tyrosine Y510 plays a critical role in activation of STAT1 and STAT2, while a second conserved tyrosine Y335 plays a more minor role (Zhao et al., 2008). Y510 is conserved in salmon and zebrafish CRFB1 and CRFB2 sequences, and is located behind of a cluster of negative charged, hydrophilic aspartate (D) and glutamate (E) residues such as in IFNAR2. Fish CRFB1 and CRFB2 also possess a tyrosine in the same region as IFNAR2 Y335.

The human IFNAR2 intracellular domain contains a proline-rich sequence (box 1 motif) close to the transmembrane domain (residues 291–296), which is suggested to be important in JAK1 activation. This motif is, however, not well conserved in neither mouse IFNAR2 nor fish CRFB1 and CRFB2.

A conserved LXSV motif (aa 427–430 in human IFNAR2) surrounded by a D/E – rich cluster is present in fish CRFB1, CRFB2 and IFNAR2, but the function of this motif has not been revealed. In human IFNAR2, phosphorylation of S364 and S384 allows binding to CREB-binding protein (CBP), which then is able to acetylate IFNAR2 on L399. Acetylated L399 is a docking site for IRF9, while phosphorylation of S400 strengthens this interaction leading to the formation of the ISGF3 complex (Tang et al., 2007). However no similar motif could be found in fish CRFB1 and CRFB2 sequences.

3.4. Cloning and characteristics of CRFB5a, CRFB5b, CRFB5c and CRFB5x

The ORFs of SsaCRFB5a (KF976460), SsaCRFB5b (KF976459) and SsaCRFB5c (KF976458) were cloned by RT-PCR based on 3′- and 5′-primers designed from the genomic sequences. Sequencing of cDNA clones confirmed protein sequences deduced from the genome. SsaCRFB5x (KF976461) was cloned based on a cDNA sequence in GenBank (Accession no ACI66319), which encodes a 345 aa protein. SsaCRFB5x deduced from the genomic sequence is, however, 356 aa due to a longer C-terminal end. A Clustal W alignment of salmon and zebrafish CRFB5 compared with human IFNAR1 and salmon CRFB5x is shown in Fig. 5. The phylogenetic analysis suggests that fish CRFB5s are homologs of IFNAR1, although the extracellular region of the fish CRFB5s contains only two FN3 domains in contrast to IFNAR1, which contains four FN3 domains. The external chains of fish CRFB5s all contain the four cysteines, which in IFNAR1 form two disulfide bridges in the inner two FN3 domains. Salmon CRFB5a has 86.4%, 75.9%, 35.7%, 20.6% and 23.2% amino acid identity with SsaCRFB5b, SsaCRFB5c, DreCRFB5, HsaIFNAR1 and SsaCRFB5x, respectively. The intracellular regions of fish CRFB5s and human IFNAR1 are all of the short type, but show very low sequence similarity. This includes the Tyk2-binding region of human IFNAR1 (Fig. 5). A notable exception is the phosphodegron motif DSGXY (aa 534–538 in human IFNAR1), which is conserved in the intracellular region of mammalian IFNAR1

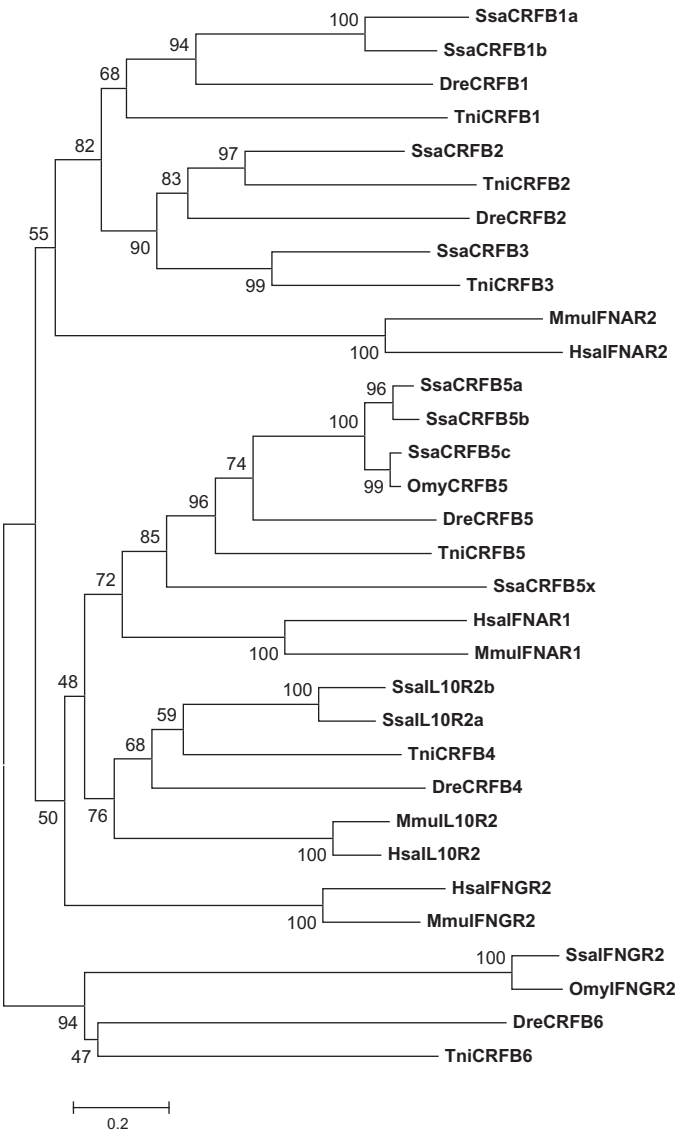

Fig. 3. Phylogenetic tree of fish CRFB1–CRFB6 and mammalian IFNAR1, IFNAR2, IFNGR2 and IL10R2. The evolutionary history was inferred using the neighbor-joining method within the MEGA5 program (Saitou and Nei, 1987; Tamura et al., 2011). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. GenBank accession numbers: Atlantic salmon: SsaCRFB1a (KF976456), SsaCRFB2 (KF976455), SsaCRFB3 (KF845941), SsaCRFB5a (KF976460), SsaCRFB5b (KF976459), SsaCRFB5c (KF976458), SsaCRFB5x (KF976461), SsaIFNGR2 (KF845942), SsaIL10R2a (KF845941), SsaIL10R2b (ACI67546); human: HsaIFNAR1 (NP000620), HsaIFNAR2 (CAA61914), HsaIFNGR2 (NP005525), HsaIL10R2 (NP000619); mouse: MmuIFNAR1 (NP034638), MmuIFNAR2 (NP034639), MmuIFNGR2 (NP032364), MmuIL10R2 (NP032375); Rainbow trout: OmyCRFB5 (CBL94869), OmyIFNGR2 (ABY87189); *Tetraodon nigroviridis* (Tni): TniCRFB1 (CAD67780), TniCRFB2 (CAD79444), TniCRFB3 (CAD79445), TniCRFB4 (CAD79446), TniCRFB5 (CAD67766), TniCRFB6 (CAD79448); zebrafish: DreCRFB1 (NP_001073149), DreCRFB2 (NP_001071094), DreCRFB4 (ABJ97309), DreCRFB5 (NP_001029357), DreCRFB6 (NP_001071095).

and fish CRFB5 including SsaCRFB5a and SsaCRFB5c, while SsaCRFB5b and SsaCRFB5x have the tyrosine replaced with a histidine residue. The DSGXY motif is vital for ubiquitination, endocytosis and degradation of IFNAR1 (Kumar et al., 2007). Although SsaCRFB5x shows low sequence similarity with CRFB5s, it is a putative candidate for IFN-receptor chains since CRFB5x is a short chain CRFB, which groups with IFNAR1 and CRFB5s in the phylogenetic analyses and which possesses the phospho-degron motif DSGXY in the internal domain.

3.5. Functional studies of CRFB chains using Mx reporter assay

To study the involvement of CRFBs in receptors of IFNa1, IFNb and IFNc, we used a reporter assay based on IFN activation of the Mx promoter, which contains a conserved ISRE element (Collet and Secombes, 2001). For this purpose we used CHSE-214 cells, which in antiviral assays respond to IFNa1, show a weak response to IFNc, but do not respond to IFNb (Robertsen et al., 2014, unpublished data). The cells were transfected with single salmon CRFBs (CRFB1a, CRFB2, CRFB5a, CRFB5b, CRFB5c, CRFB5x) or pairs of long and short CRFBs together with a reporter construct containing the Mx promoter linked to a luciferase gene. Forty-eight hours later, the cells were stimulated with IFNa1, IFNb or IFNc for 24 hours and then measured for luciferase activity (Fig. 6). Without IFN stimulation, transfection with single CRFBs gave only minor increases in Mx-response while CRFB1a/CRFB5a, CRFB1a/CRFB5b and CRFB1a/CRFB5x all gave significantly increased Mx-responses compared to cells transfected with control plasmids. The explanation for elevated Mx-responses in non-stimulated cells transfected with CRFB1a/CRFB5 pairs may be that these CRFBs activate each other upon overexpression and pairing.

Transfection with CRFB2 alone or in combination with CRFB5a, CRFB5b, CRFB5c or CRFB5x gave no increase in the Mx-response even after stimulation with IFNa1, IFNb or IFNc (data not shown). The Mx reporter response to IFNa1 was significantly increased for CRFB1a combined with CRFB5a, CRFB5b or CRFB5c and for CRFB5b alone, but not for the other constructs. This suggests that SsaCRFB1a may be the long chain and any of the other CRFB5s may be the short chain in an IFNa receptor. The Mx reporter response to IFNc was significantly increased in cells transfected with CRFB5a and CRFB5c alone and for CRFB1a combined with CRFB5a, but not for the other constructs. This suggests that CRFB5a and CRFB5c are the main short chains in IFNc receptors. CRFB1a is less likely to be part of the IFNc receptor since the IFNc response in CRFB1a/CRFB5a transfected cells were not significantly higher than the IFNc response to cells transfected with CRFB5a alone. Interestingly, the Mx reporter response to IFNb was increased only in cells transfected with CRFB5x, which suggests that IFNb may signal through a receptor containing CRFB5x as the short chain.

The IFN responses induced with single transfected CRFB chains may be due to pairing with endogenous CRFBs, but it cannot be excluded that some of the IFNs may induce signals through only one CRFB chain. The reason why we did not observe increased responses with CRFB2 alone or in combination with CRFB5a, CRFB5b, CRFB5c or CRFB5x is unknown, but might be due to ineffective functional expression of SsaCRFB2 or that endogenous CRFB2 is expressed at relatively high levels in CHSE cells.

Taken together, these data suggests that IFNa, IFNb and IFNc signal through different receptors.

3.6. Expression of CRFBs in tissues

The constitutive expression levels of CRFBs were examined in different tissues including gill, liver, spleen, head kidney, kidney, intestine, muscle, heart, brain and ovary (Fig. 7). CRFB transcript levels are shown relative to CRFB5b, which has the lowest expression in most tissues. While all CRFBs are ubiquitously expressed in all 10 organs, there are notable differences in expression within the groups of CRFBs with long internal chains (CRFB1 and CRFB2) and CRFBs with short internal chains (CRFB5 and CRFB5x). The ratio

Fig. 4. Clustal W alignment of fish CRFB1, CRFB2 and human IFNAR2. Gaps introduced to increase identity are shown by dashes. Identical amino acids among all sequences are indicated by "*", whereas those with high or low similarity are indicated by ":" and ".". The predicted signal sequences in the N-terminus are underlined and the putative transmembrane regions are shaded gray. Cysteine residues forming disulfide bridges in IFNAR2 and their fish counterparts are emphasized in bold. Transmembrane regions are shaded gray. In the internal domain, two putative STAT activating tyrosine (Y) motifs are in bold and shaded gray while the preceding negative charged cluster of aspartate acid (D) and glutamate (E) are in bold. The conserved LxSV motif is in bold and shaded. Hsa: human, Ssa: salmon, Dre: zebrafish. NCBI GenBank accession numbers are listed in Fig. 2.

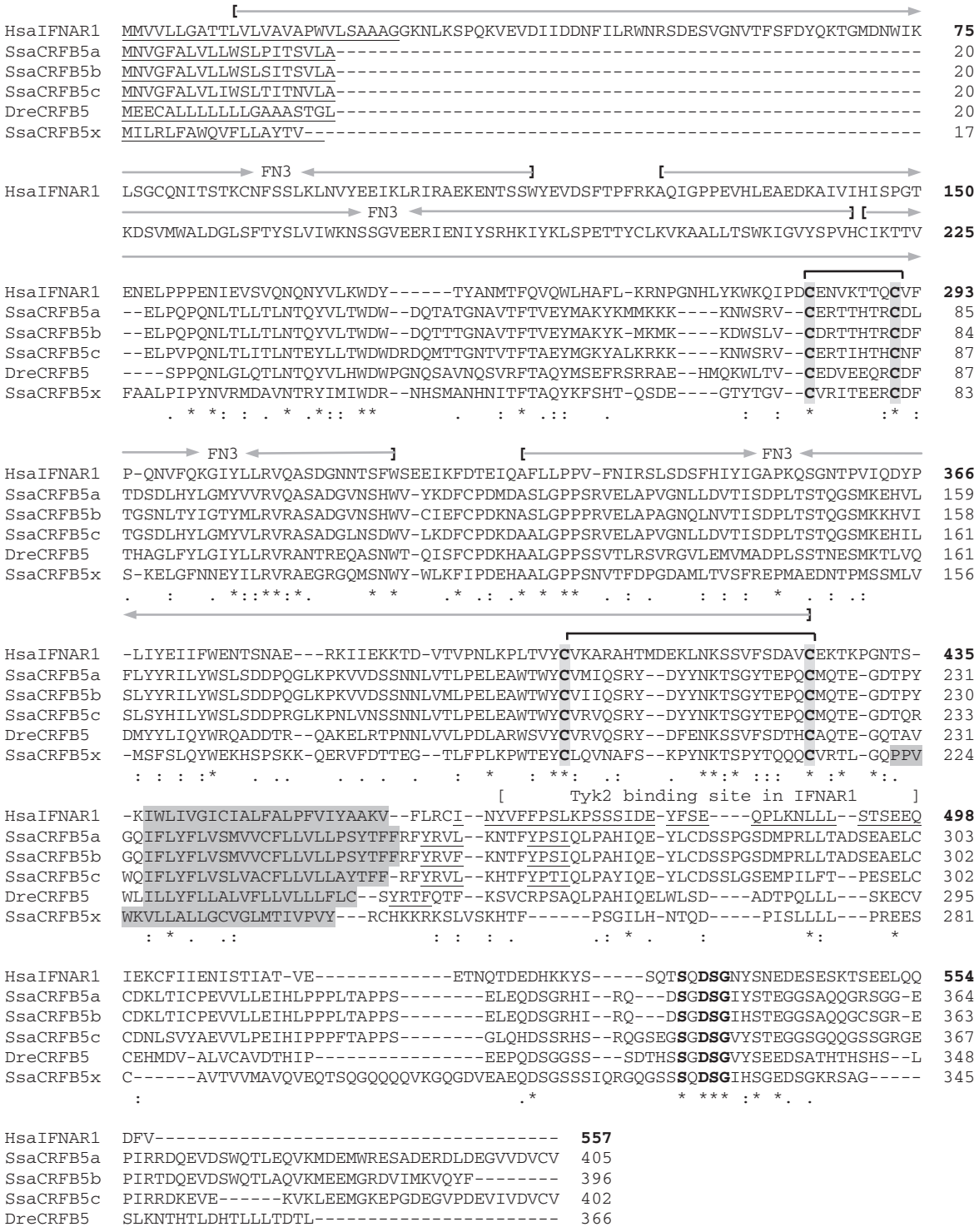

Fig. 5. Clustal W alignment of fish CRFB5, CRFB5x and human IFNAR1. Gaps introduced to increase identity are shown by dashes. Identical amino acids among all sequences are indicated by "*", whereas those with high or low similarity are indicated by ":" and ".". The predicted signal sequences in the N-terminus are underlined and the putative transmembrane regions are shaded gray. Cysteine residues forming disulfide bridges in IFNAR1 and their fish counterparts are emphasized in bold with gray shading. Transmembrane regions are shaded gray. Tyk2 binding site is underlined in human IFNAR1, and the conserved amino acids in mammals are in bold. The putative linear endocytic motif YXXΦ is underlined, and the phosphodegron motif DSGXY in bold. The FN3 domains are marked above the IFNAR1 sequence. Mm: mouse, Hsa: human, Ssa: salmon, Dre: zebrafish. GenBank accession numbers are listed in Fig. 2.

of CRFB2 mRNA to CRFB1a mRNA was approximately two in the kidney, heart and spleen, four in the heart and 5.5 in the ovary. In contrast, CRFB1a is predominant in the intestine and muscle where the ratio of CRFB1a mRNA to CRFB2 mRNA was five in the intestine and 4.5 in the muscle. In head kidney, liver and brain, CRFB1a and CRFB2 had similar transcript levels. In most tissues, CRFB5a and CRFB5c had similar transcripts levels, except that CRFB5a was predominant in the ovary, while CRFB5c was most abundant in the spleen. CRFB5x transcripts were lowest in the ovary and most abundant in the gill.

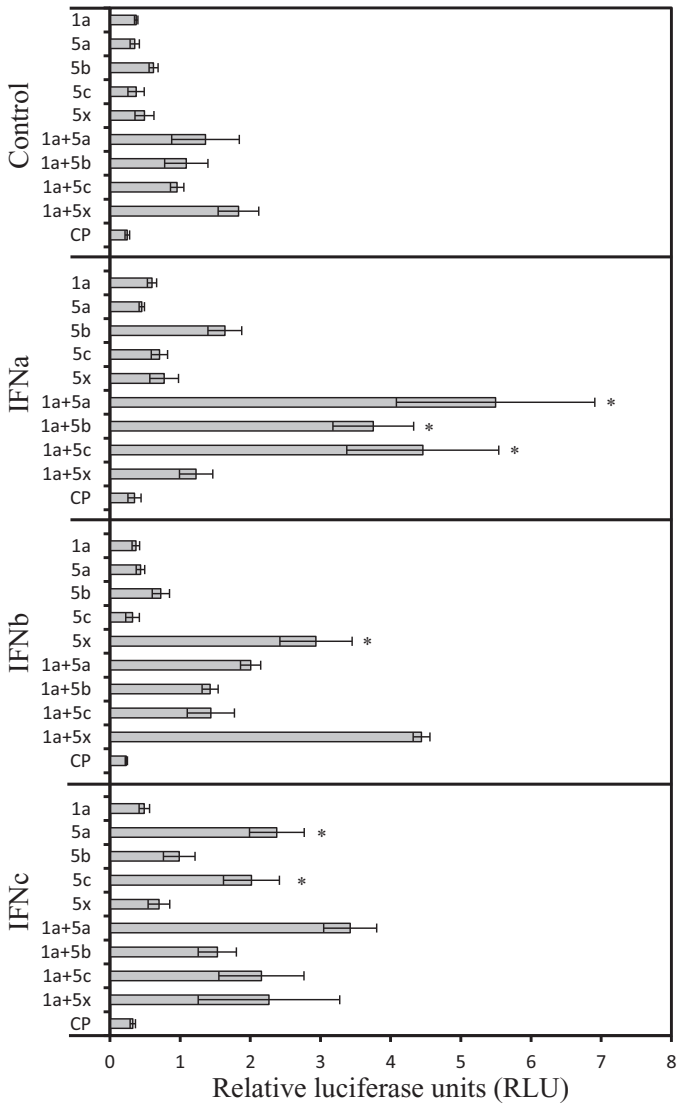

Fig. 6. IFN induced activation of the Mx promoter in CHSE cells transfected with various CRFB constructs. The cells were transfected with single or double pcDNA3.3 CRFB constructs or pcDNA3.3 alone (CP) as indicated on the figure, together with pRL-SV40 Vector expressing *Renilla* luciferase and a construct containing the rainbow trout Mx1 promoter fused to the firefly luciferase gene. The transfected cells were incubated for 48 h before being stimulated with 200 U/ml IFNa, IFNb or IFNc, or left un-stimulated (Control). Luciferase activity in the cells was measured 24 h post stimulation. Firefly luciferase values were normalized with *Renilla* luciferase levels (n = 5) and the data are presented as relative luciferase units (RLU). The results are representative of four independent experiments. Statistics was analyzed by using one way ANOVA. The values from IFN stimulated, single CRFB-plasmid transfected cells were compared with values from IFN stimulated, control plasmid transfected cells and with non-IFN stimulated, single CRFB-plasmid transfected cells. The values from IFN stimulated, double CRFB-plasmid transfected cells were compared with values from non-stimulated, double CRFB-plasmid transfected cells and with the corresponding IFN-stimulated, single CRFB-plasmid transfected cells. Values significantly different from all controls are shown with * ($p < 0.05$).

3.7. Expression of CRFBs in TO cells and leukocytes

To study if the expression of IFN receptors were dependent of cell types, we compared the expression of CRFBs in primary head kidney leukocytes and TO cells, which is a cell line derived from head kidney of Atlantic salmon (Wergeland and Jakobsen, 2001). In TO cells, the level of CRFB2 transcripts was 127 times higher than CRFB1a transcripts while the level of CRFB2 transcripts were twofold higher than CRFB1a transcripts in leukocytes (Fig. 8). CRFB5a, CRFB5b, CRFB5c and CRFB5x displayed similar transcription levels in TO cells, while CRFB5c transcripts were most abundant in leukocytes, and were 200 times more abundant than CRFB5b transcripts. CRFB1b and CRFB5x could be induced by poly I:C and R848 in head kidney leukocytes. CRFB5b and CRFB5a could be induced by poly I:C only while CRFB2 and CRFB5c could be induced by R848 only. CRFB1a transcripts remained unchanged after stimulation.

4. Discussion

The present work demonstrates identification of two CRFB clusters in the Atlantic salmon genome, one on chromosome 21 and the other on chromosome 25, which is in agreement with the tetraploid origin of salmonids. The first cluster contains CRFB1a, CRFB3, CRFB4a (IL10R2a), CRFB5c, CRFB5x and a IFNGR2 related gene and shows a high degree of gene synteny with the *T. nigroviridis* CRFB cluster and the human IFN receptor cluster. In contrast to *T. nigroviridis*, this salmon cluster lacks CRFB2, however. The second cluster, which is present in the BAC882E11 sequence, contains CRFB5a and CRFB5b and an IFNGR2 gene. Chromosome 25 also contains one CRFB1 gene (CRFB1b) and one CRFB2 gene. It is thus quite possible that chromosome 25 also contains a full IFN receptor gene cluster, but the linkage of CRFB1b and CRFB2 genes to the BAC882E11 cluster has not yet been verified.

As IFNAR1/IFNAR2 and most other heterodimeric class II cytokine receptors are formed by a CRFB with a long intracellular domain (IFNAR2) and a CRFB with a short intracellular domain (IFNAR1), it is likely that fish type I IFN receptors have a similar composition. Phylogenetic analysis and gene synteny suggest that fish IFNAR2 homologs exists among CRFB1, CRFB2 and CRFB3 while CRFB5s are IFNAR1 homologs. However, CRFB3 is less likely to be part of a classical type I receptor since it has a very short cytoplasmic domain, which in salmon is only 26 amino acids and lacks potential tyrosine phosphorylation sites. Taken together, the genomic data suggest that Atlantic salmon have a much more extended repertoire of type I IFN receptors than zebrafish since salmon possesses at least three CRFB5 members where CRFB5a and CRFB5b are most similar (89% amino acid identity), while CRFB5c shows only 75% identity with CRFB5a and is even more different in the cytoplasmic domain. In addition, the phylogenetic analyses indicate that CRFB5x, which is a novel CRFB, might be another short-chain IFN receptor candidate. However, CRFB5x shows at most 28% identity with the CRFB5s. Similar to zebrafish, Atlantic salmon appears to have one functional gene each of CRFB1 and CRFB2 since the CRFB1b gene appeared to be non-functional due to the presence of a stop codon in its ORF, which results in a truncated intracellular region. While intracellular isoforms of CRFB5 and CRFB3 were found in rainbow trout (Chang et al., 2013), we did not identify any isoforms of CRFB1, CRFB2 or CRFB5, which lack signal peptides in Atlantic salmon neither by cDNA sequencing nor searching the EST and genomic databases in GenBank. Intracellular IFN receptors thus do not appear to be a general phenomenon in salmonids.

Expression of salmon CRFB1a, CRFB2, CRFB5a, CRFB5b, CRFB5c and CRFB5x was confirmed by cDNA cloning of their ORFs, and their roles as IFN receptor chains were investigated by Mx reporter assay. In this assay we tested if cells transfected with individual CRFBs or pairs of a long-chained CRFB (CRFB1 or CRFB2) and a short-chained CRFB (CRFB5a, CRFB5b, CRFB5c or CRFB5x) would give an increased Mx reporter gene response after stimulation with IFNa, IFNb or IFNc. Firstly, the data showed increased responses to IFNa1 in cells transfected with CRFB1a and CRFB5a or CRFB1a and CRFB5b or CRFB1a and CRFB5c, which suggest that these may all be receptors for IFNa1. Secondly, IFNb only gave an increased Mx response in cells transfected with CRFB5x, suggesting this to be a short chain in an IFNb receptor. Thirdly, IFNc gave increased responses in cells

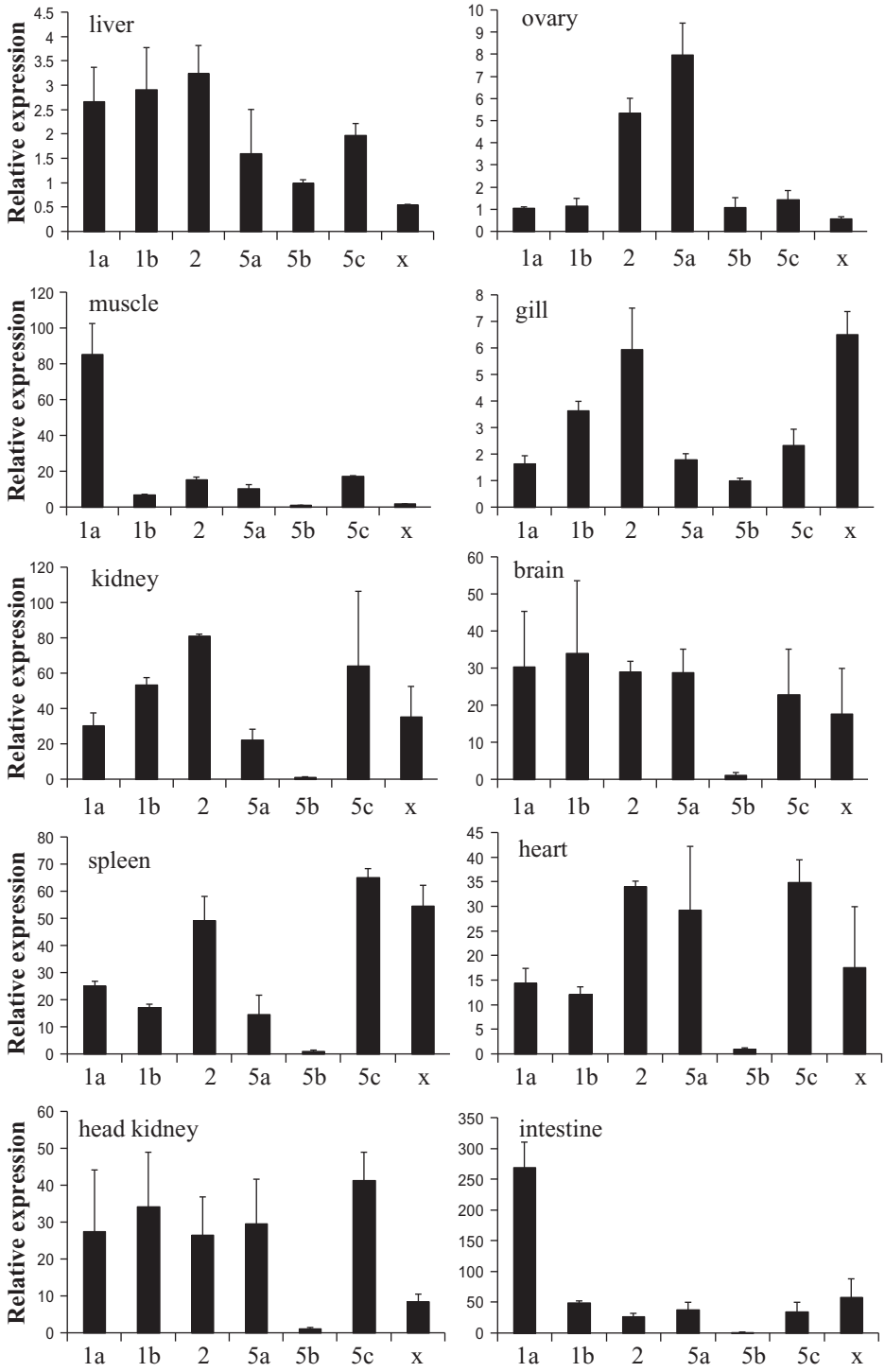

Fig. 7. Expression of CRFBs in different tissues. Transcription of CRFB1a, CRFB1b, CRFB2, CRFB5a, CRFB5b, CRFB5c and CRFB5x were measured by RT-qPCR in liver, ovary, muscle, gill, kidney, brain, spleen, heart, head kidney and intestine (n = 5). Transcription of CRFBs is presented as relative expression compared to CRFB5b, which had the lowest transcript values among CRFBs in most tissues.

transfected with CRFB5a and CRFB5c, suggesting these to be short chains in an IFNc receptor. The nature of the short CRFBs in salmon IFN receptors thus seems more complex compared to zebrafish since salmon has at least three CRFB5 members and CRFB5x, which can fill that role.

None of the IFNs gave increased responses in cells transfected with CRFB2, which might be due to ineffective expression of the transfected CRFB2 or that the intracellular domain of Atlantic salmon CRFB2 is not functional in CHSE cells, which originate from another salmonid species. At present, we have to assume that CRFB2 is part of the IFNb and IFNc receptors based on the zebrafish receptor study (Aggad et al., 2009). Recently, rainbow trout CRFB3 was launched as an IFNAR2 homolog, but this is less likely since the intracellular domain of CRFB3 has only 26 amino acids and thus lacks conserved signaling motifs found in IFNAR2 and CRFB1/CRFB2. CRFB3 was however not tested in the present work.

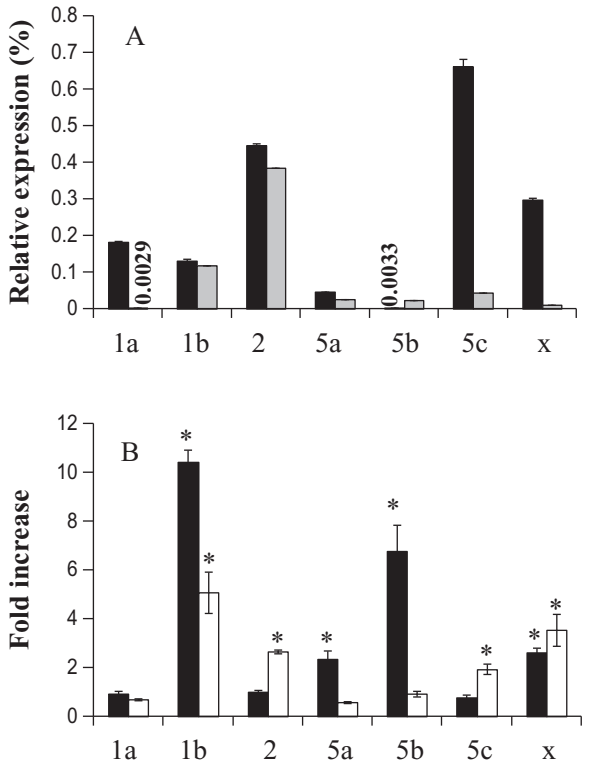

Fig. 8. Expression of CRFBs in TO cells and leukocytes. (A) Transcription of CRFB1a, CRFB1b, CRFB2, CRFB5a, CRFB5b, CRFB5c and CRFB5x were measured by RT-qPCR in TO cells and head kidney leukocytes. Black bar: leukocytes; gray bar: TO cells. Transcription of CRFBs was presented as relative expression (%) by comparing to transcription of beta actin. (B) The transcriptional changes of CRFBs in head kidney leukocytes after poly I:C or R848 stimulation were measured by RT-qPCR. The values show fold increase in transcription (mean ± SD) compared to non-stimulated cells (calculated using the ΔΔCt method). Statistical significant differences ($p < 0.05$) are indicated with *. Black bar: Poly I:C treated cells; white bar: R848 treated cells. ($n = 4$).

Our previous observation that IFNa and IFNc showed similar antiviral activities in cell lines seemed to contradict with the present finding that they use different receptors (Svingerud et al., 2012). This phenomenon may, however, be explained by the fact that all of the CRFBs are constitutively expressed in TO cells and mammalian research has shown that only a small number of receptors per cell is needed for type I IFNs to induce a full antiviral response. The lower response of TO cells to IFNb may be due to low expression of CRFB5x or another CRFB, or that IFNb has less affinity for its receptor compared to IFNa and IFNc.

CRFB1, CRFB2, CRFB5 and CRFB5x were ubiquitously expressed in the tissues examined, although, the relative expression varied between tissues. While CRFB2 was more highly expressed than CRFB1a in most tissues and in TO cells, CRFB1a was much more highly expressed in muscle and intestine compared to CRFB2. CRFB5b showed the lowest expression of all putative IFN receptor members, while CRFB5a and CRFB5c were similarly expressed in most tissues. CRFB5x was expressed at a lower level compared to CRFB5a and CRFB5c. These data give, however, no information about the CRFB expression in different cell types, which is more interesting than the overall tissue expression. The possession of multiple CRFBs with long and short internal chains offers the possibility of many different combinations to form complete type I IFN receptors. It can thus not be excluded that some fish cell types express receptors for only one subtype of IFN while other cells may have receptors for all three subtypes. Differential expression of type I and type III IFN receptors is seen in human where type I IFN receptors are ubiquitously expressed while type III IFN receptors are more highly expressed in specialized cell types such as epithelial cells (Sommereyns et al., 2008).

Like in *Tetraodon* and zebrafish, Atlantic salmon CRFBs show very low sequence similarity to the mammalian IFNAR1 and IFNAR2 not only in the external region, but also in the intracellular region. Jak1 and Tyk2 binding elements could thus not be identified with certainty among the salmon CRFBs. The large divergence in vertebrate IFN receptors may be due to adaption to many different virus species, since development of proteins that antagonize the Jak–STAT pathway is important for the pathogenicity of vertebrate viruses.

Some important sequence elements were, however, found to be conserved in fish and mammalian type I IFN receptor chains. Firstly, the last tyrosine Y510 in IFNAR2, which plays a critical role in activation of STAT1 and STAT2, was in fact also identified in fish CRFB1 and CRFB2. Secondly, two motifs that are important in internalization and degradation of IFNAR1 were identified in the fish CRFB5 internal domains. These motifs are important for the number of active IFN receptors on the cell surface and thus the cell's response to IFN.

In conclusion, the present work has identified two clusters of CRFB genes in Atlantic salmon and has shown that salmon has a larger repertoire of short chain type I IFN receptors compared to zebrafish. Based on the zebrafish type I IFN receptor model and the results of the present Mx reporter assays, we suggest the following preliminary hypothetical model for IFN receptors in Atlantic salmon: IFNa signals through a receptor composed of CRFB1a as the long chain and CRFB5a, CRFB5b or CRFB5c as the short chain; IFNc signals through a receptor composed of CRFB2 as the long chain and CRFB5a or CRFB5c as the short chain and IFNb signals through a receptor composed of CRFB2 and CRFB5x as the short chain.

Acknowledgments

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