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Differential regulation of *Tetraodon nigroviridis Mx* gene promoter activity by constitutively-active forms of STAT1, STAT2, and IRF9☆

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ABSTRACT

Induction of interferons (IFNs) produces an innate immune response through activation of the JAK-STAT signaling pathway. Type I IFN signaling activates downstream gene expression through the IFN-stimulated gene factor 3 (ISGF3) complex, while type II IFN (IFN-γ) signaling is mediated through active STAT1 protein. The IFN target gene *Mx* is involved in the defense against viral infection. However, the mechanism by which *Tetraodon* (pufferfish) *Mx* is regulated by IFN signaling has not been identified. In this study, we describe the cloning and expression of *Tetraodon* STAT1, STAT2, and IFN regulatory factor 9 (IRF9). By combining constitutively-active STAT1 (STAT1-JH1) and STAT2 (STA2-JH1) fusion proteins with IRF9, we demonstrate that a constitutively-active ISGF3 complex increases the transcriptional activity of the *Tetraodon Mx* promoter via direct binding to two IFN-stimulated response element (ISRE) sites. In addition, a constitutively-active TnIRF9-S2C containing a fusion of the C-terminal region of STAT2 and IRF9 also activated the *Mx* promoter through binding to the ISRE sites. Furthermore, constitutively-active STAT1-JH1 elevates *Mx* promoter activity through two IFN gamma-activated sequence (GAS) elements. The *Mx* promoter is also activated by constitutively-active TnIRF9-S2C and STAT1-JH1 protein, as determined using an *in vivo* luciferase assay. We conclude that the *Tetraodon Mx* gene is activated via Type I (IFN-1) and Type II (IFN-γ) signaling. These results provide mechanistic insights into the role of IFN signaling in teleosts, and the *in vivo* luciferase assay may be suitable as a tool for studying induction and regulation by IFNs in teleost fish.

1. Introduction

The mammalian innate immune system is composed of type I, type II, and type III interferons (IFNs). Type I IFNs consist of IFN-α, IFN-β, IFN-ω, IFN-ε, and IFN-κ, type II IFNs of IFN-γ alone, and type III IFNs of IFN-λ1, IFN-λ2, and IFN-λ3. Type I and type III IFNs are often grouped together as virus-induced IFNs, while type II IFNs are active against bacteria [1]. In zebrafish, innate immunity involves type I and type II IFNs. The four type I IFNs are also divided into group I, which includes IFN-Φ1 and IFN-Φ4, and group II, which includes IFN-Φ2 and IFN-Φ3. Type II IFNs can be divided into IFN-γ1 and IFN-γ2 [2,3]. The zebrafish IFN genes cannot be classified as orthologues of mammalian IFN-α or IFN-β, due to low identity at the amino acid sequence level. However, IFN-Φ1 (also called IFN1) is regulated by IRF3 and thus resembles IFN-β, while IFN-Φ3 (also called IFN3) is regulated by IRF7 and thus resembles IFN-α. Both IRF3 and IRF7 are activated by IFNs during viral infection [4,5].

In mammals, type I IFNs (IFN-alpha and beta) bind to a receptor complex formed from IFNAR1 and IFNAR2, while type III IFNs (IFN-

☆ The sequence data in this paper were submitted to the EMBL/GenBank Data Libraries under the accession numbers AAL09414 (TnSTAT1), JQ406641 (TnSTAT2), and JQ412058 (TnIRF9).

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lamda1-3) bind to a receptor complex formed from IL10R2 and IFNLR1. The binding of IFNs to their receptor complex results in cross-phosphorylation and activation of TYK2 and JAK1, which is followed by formation of the active IFN-stimulated gene factor 3 (ISGF3) complex from phosphorylated-STAT1, phosphorylated-STAT2, and IFN regulatory factor 9 (IRF9); the ISGF3 complex subsequently translocates into the nucleus, where it activates transcription of interferon-stimulated genes (ISGs) by binding to IFN-stimulated response elements (ISREs) in promoter regions. These ISGs, which include Mx, encode products that defend against viral infection [6]. In fish, group I IFNs (IFN-phi1and phi4) bind to a receptor complex composed of CRFB1 and CRFB5, while group II IFNs (IFN-phi2 and phi3) bind to a different receptor complex composed of CRFB2 and CRFB5. As in mammals, binding of fish IFNs to their receptor complex is followed by cross-phosphorylation and activation of TYK2 and JAK1, which in turn results in the formation of the active ISGF3 complex.

In mammals, type II IFN (IFN-gamma) binds to a receptor complex consisting of a tetramer of IFNGR1 and IFNGR2. In zebrafish, type II IFN-gamma1 binds to a specific receptor complex, which consists of a tetramer of CRFB13, CRFB17, and two CRFB6 proteins, while IFN-gamma2 binds to a receptor complex formed from a tetramer of two CRFB17 proteins and two unknown proteins. After binding to their specific receptors, JAK1 and JAK2a are activated by cross-phosphorylation, and in turn, phosphorylate STAT1; the resulting homodimer translocates into the nucleus to activate transcription of target genes through binding to IFN-gamma-activated sites (GAS) in promoter regions [6].

The IFNs and IFN receptors of *Tetraodon* (pufferfish) have not previously been described in full. Through comparative genomic analysis, the *Tetraodon* (Tn) genes encoding class II helical cytokine receptors (HCRII) and their ligands were found to be grouped with those of human. The finding that TnCRIFB6 and TnCRIFB7 are grouped with IFNGR1 of human and mouse suggests that IFNGR1 underwent duplication in *Tetraodon*. TnCRFB1, TnCRFB2, and TnCRFB3 are grouped with IFNAR2 of human, mouse, cow, sheep, and chicken, which suggest that three copies of IFNAR2 exist in *Tetraodon*. Finally, *Tetraodon* IFN is grouped with IFN of zebrafish and fugu, suggesting that only one copy of IFN may be present in the genome [7]. These limited reports are, however, insufficient to describe the detailed signaling of IFNs and IFN receptors in *Tetraodon.*

The primary components of downstream IFN-signaling pathways in fish, such as JAKs and STATs, have been isolated in several fish species. We previously identified the genes encoding JAK1, JAK2, JAK3, and Tyk2 [8,9], and subsequently STAT5 and STAT6 [10,11] in *Tetraodon*. In addition, the following genes have been identified and isolated as full-length cDNAs from the following fish species: JAK1, JAK2, STAT1, STAT3, and STAT5 from zebrafish (*Danio rerio*) [12–14]; JAK1 and JAK3 from common carp [15]; STAT1 from crucian carp and olive flounder [16,17]; and four JAK family members and five STAT family members (but not STAT2) from mandarin fish (*Siniperca chuatsi*) [18]. The numbers of identified JAK protein isoforms also differ by species. The mammalian JAK protein family consists of four members: JAK1, JAK2, JAK3, and TYK2 [19]. In the ancestor of zebrafish and green pufferfish, JAK2 underwent duplication to produce JAK2A and JAK2B [12,20]. On the other hand, fruit fly and brine shrimp each encode a single JAK protein [21,22]. Due to the similarities of these proteins with their counterparts in mammals, it is believed that IFN signaling pathways are also similar between fish and mammals.

The most frequently studied mammalian IFN-induced antiviral proteins are protein kinase R (PKR), oligoadenylate synthetases (OAS), and Mx (myxovirus resistance) proteins [23–26]. Mx proteins are dynamin-like GTPases, which contain a tripartite GTP-binding domain required for antiviral activity, and a leucine-zipper motif for oligomerization. These proteins putatively prevent viral replication by inhibiting the intracellular trafficking of viral nucleocapsids [27]. Two *Mx* genes, *Mx1* and *Mx2*, have been identified in the mouse genome [28,29]. Mx1 protein is located in the cell nucleus, while Mx2 protein is in the cytoplasm. Such different patterns of localization mean that Mx1 protein can inhibit replication of influenza virus, but not vesicular stomatitis virus (VSV) [30], while Mx2 protein can interfere with the replication of VSV, but not that of influenza virus [31].

Different isoforms of Mx proteins have been identified in mammals, birds, and fishes. Two Mx isoforms have been described in human (MxA and MxB) and mouse (Mx1 and Mx2), while three Mx isoforms have been described in rat (Mx1, Mx2, and Mx3). Pig, duck, and chicken have been reported to encode only one Mx protein [32-34]. Interestingly, the number of Mx isoforms differs dramatically between fish species. Seven Mx isoforms have been identified in zebrafish [7], five in channel catfish [35], four in European eel [36], three each in Atlantic salmon, rainbow trout, grass carp, rock bream, and gilthead sea bream [37-42], two each in goldfish and European sea bass [43,44], and only one each in orange-spotted grouper, Japanese flounder, rare minnow, pufferfish, and barramundi [45–49].

The Mx proteins of Atlantic salmon and Japanese flounder have been demonstrated to possess antiviral activity [50,51]. Studies on the transcriptional regulation of the *Mx* gene in fish have become important, because such studies may elucidate the susceptibility of different fish species to specific viruses. Therefore, *Mx* promoters have been characterized from several fish species [35,45,47,51–54]. These *Mx* promoters contain one or two ISREs, with the exception of that in Japanese flounder, which contains three ISREs. Although these *Mx* promoters can be activated by poly I:C or purified zebrafish IFN, the detailed regulatory mechanisms underlying fish *Mx* transcription by STAT proteins are unknown. Here, we investigated JAK/STAT signaling in *Tetraodon* by cloning the pufferfish genes encoding STAT1, STAT2, and IRF9, as well as its *Mx* promoter. Previously, Poat et al. reported that cells expressing a chimeric IRF9 protein fused to the trans-activating domain of either STAT1 or STAT2 exhibited an increased response to IFN, which significantly inhibited viral replication and expression of the HCV1b and HCV2a viral proteins, without IFN-α induction [55]. Furthermore, an IRF9-STAT2 hybrid protein, which acts as an ISGF3-like activator, was also able to inhibit infection by DNA and RNA viruses in the absence of IFN signaling [56].

In the present study, we examined the effects of constitutively-active TnIRF9-S2C and STAT1-JH1 on the transcription of *Mx* via the ISRE and GAS sequences in its promoter. Overexpression of zIFN1 and zIFNγ resulted in transactivation of the *Tetraodon Mx* promoter in *in vivo* luciferase assays, providing evidence that this promoter can be activated by type I (zIFN1) and type II IFN (zIFNγ) signaling. These findings indicate that the antiviral *Mx* gene of *Tetraodon* is differentially regulated by constitutively-active forms of STAT1, STAT2, and IRF9.

2. Materials and methods

2.1. Cloning of full-length Tetraodon STAT1, STAT2, and IRF9 complementary DNA (cDNA)

The 5′ and 3′ ends of *Tetraodon STAT1*, *STAT2*, and *IRF9* mRNA were obtained by PCR using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA), as previously described [22]. The following primer pairs were used for PCR: ST1F (5′-AAG CTT ATG TCA CAG TGG GGG C-3′) containing a *Hind*III site and ST1R (5′-GGT ACC TTC ATT CAT CTG AGC T-3′) containing a *Kpn*I site; ST2F (5′-