Fish & Shellfish Immunology 38 (2014) 311–317

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Cloning and characterization of the Mx1, Mx2 and Mx3 promoters from gilthead seabream (*Sparus aurata*)

J.A. González-Mariscal, J.B. Gallardo-Gálvez, T. Méndez, M.C. Álvarez, J. Béjar*

Department of Genetics, University of Málaga, Spain

ARTICLE INFO

Article history:
Received 18 November 2013
Received in revised form
6 March 2014
Accepted 26 March 2014
Available online 3 April 2014

Keywords:
Mx protein
ISRE
Promoter
Gilthead seabream
Sparus aurata

ABSTRACT

Mx proteins are main effectors of the antiviral innate immune response mediated by type I interferon (IFN I). Actually, diverse Mx proteins from fish proved highly active against fish viruses, standing out among them the Mx1, Mx2 and Mx3 from gilthead seabream (*Sparus aurata*), a species exhibiting a natural resistance to viral diseases. In this study, the structure and functional activity of their corresponding promoters (pMx1, pMx2 and pMx3) have been assessed. The three promoters present an identical 3′ region of 157 bp, exhibiting a single canonical interferon-stimulated response element (ISRE), which is indispensible for the poli:IC induction of pMx1 and pMx3, while not for that of pMx2. In the remaining part of the three promoters other regulatory motifs were identified, as gamma IFN activated sites in variable number (1, 4 and 2 in pMx1, pMx2 and pMx3, respectively), as well as several independent GAAA elements or ISRE core sequences (13, 15 and 12 in pMx1, pMx2 and pMx3, respectively). The structural dissimilarities shown by the three promoters parallels with the differences observed in their response profiles, in terms of the time course of the induction, and basal and induced expression levels of each promoter. Altogether, these findings indicate that the expression of Mx1, Mx2 and Mx3 genes from the gilthead seabream might be specifically regulated, in accordance with the functional role of each Mx protein in the successful antiviral response shown by this species.

1. Introduction

The vertebrate innate immunity has an effective antiviral response mediated by type I interferon (IFN I). Hence, one of the key issues in understanding virus–host relationship is the knowledge of the regulatory mechanisms governing IFN response. Fish appear to trigger IFN I in a similar way to that in mammals [1], where its activation is well characterized [2–4]. Briefly, in virus-infected cells, type I IFN response is initiated through recognition of viral products. Such recognition events trigger signalling pathways that activate the transcription of type I IFNs. After binding of IFNs to their receptors in neighbouring cells, the JAK-STAT signalling pathway is activated, and the transcription factor complex ISGF3 (IFN-stimulated gene factor 3) is formed. Finally, the ISGF3 complex specifically binds to the IFN-stimulated response elements (ISRE), located in the promoters of IFN-stimulated genes (ISGs). The expression of ISGs generates an antiviral state in cells. It is also known that the expression of ISGs genes is under a complex spatial and temporal regulation, which seems to be responsible for the control of the antiviral response [5].

Among the ISGs, those of Mx proteins play a main role in the IFN I response [6]. Mx proteins belong to the dynamin superfamily of high molecular weight GTPases, which are involved in intracellular membrane remodelling and intracellular trafficking [7]. Though the basic mechanism of the antiviral activity of Mx proteins is not completely understood, it seems clear that it relies on a direct interaction between the Mx protein and a viral target that needs to be defined in each case [8,9]. The antiviral activity of Mx proteins against a wide range of viruses has been largely reported in several fish species [8–19]. For that reason, fish Mx proteins have been intensively studied, especially in aquacultured species, where knowing pathogen–host interactions might be essential to develop strategies aimed at enhancing fish natural resistance to viral infections [20].

At the moment, the regulatory mechanisms of the fish Mx expression are poorly understood, although few Mx promoters have been cloned and functionally characterized: pufferfish, *Takifufu rubripes* [21], zebrafish, *Danio rerio* [22], rainbow trout, *Oncorhynchus mykiss*, Mx1 [23], Japanese flounder, *Paralichthys olivaceus*

* Corresponding author. Department of Genetics, Faculty of Sciences, University of Málaga, Campus de Teatinos, 29071 Málaga, Spain. Tel.: +34 952136625; fax: +34 952132001.
E-mail address: bejar@uma.es (J. Béjar).

http://dx.doi.org/10.1016/j.fsi.2014.03.031
1050-4648/

[24], orange-spotted grouper, *Epinephelus coioides* [25], channel catfish, *Ictalurus punctatus* [26], and Senegalese sole, *Solea senegalensis* [17]. Otherwise, the interest for studying the regulation of Mx transcription in fish has been stressed by studies reporting: i) the use of Mx expression to test the effects of a knocked-out recombinant virus [27]; ii) the response of rainbow trout and Atlantic salmon Mx promoters to both type I and II IFNs [28,29]; and iii) the apparent blocking of Mx activation by several viruses [28,30,31].

The study of Mx genes in the farmed fish gilthead seabream has special interest, since this species displays a unusually high natural resistance to viral diseases [32], and is an asymptomatic carrier and/or reservoir of several viruses pathogenic to other species, such as viral nervous necrosis virus, VNNV [33], infectious pancreatic necrosis virus, IPNV [34] and viral haemorrhagic septicaemia virus, VHSV [35]. Three independent Mx genes (Mx1, Mx2, and Mx3) have been identified in gilthead seabream [36]. The three Mx proteins possess antiviral activity with a wide antiviral spectrum that includes RNA and DNA viruses, and show interesting differences in their antiviral specificities [37,16]. Additionally, the three Mx genes showed different patterns of induction, in terms of tissue, time course, and level of expression, after an experimental infection with VNNV, which indicates a differential modulation of each Mx gene transcription over the immune response to VNNV [36]. Therefore, assessing the regulatory mechanisms controlling the transcription of the three Mxs can give light to understand the successful antiviral strategies developed by this species. As a first approach in disclosing the regulation of seabream Mx genes, in this study, the promoters of Mx1, Mx2 and Mx3 (pMx1, pMx2 and pMx3) have been cloned; their regulatory motifs have been identified; their responses to poly I:C analysed; and the role of the ISRE motif found screened.

2. Material and methods

2.1. Cloning of gilthead seabream pMx1, pMx2 and pMx3

Genomic DNA was extracted from gilthead seabream fin clips using the saline precipitation method [38]. DNA was resuspended in double-distilled water and stored at 4 °C. DNA concentration and purity were measured by spectrophotometry.

The Genome-WalkerTM Universal Kit (Clontech) was used to clone the three promoters. Briefly, genomic DNA was independently digested with eight different blunt-end restriction enzymes (AfeI, EcoRV, HindIII, HpaI, NruI, PvuII, ScaI, SmaI, and SwaI), purified by phenol–chloroform and ligated to the GenomeWalker adaptor. Two specific reverse primers were designed from intron 1 of each Mx gene, where first sequence differences were detected among them [36]. A first PCR was performed using Go-Taq DNA polymerase (Promega), adaptor primer AP1, and ExtMx1L, ExtMx2L, and ExtMx3L for pMx1, pMx2, and pMx3, respectively (Table 1). The cycling protocol was: 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 2.5 min, and a final step at 72 °C for 5 min. A second round PCR was then carried out with the adapter primer AP2 and IntMx1L, IntMx2L and IntMx3L primers (Table 1), using 1 μL of the first round PCR mix. The cycling protocol was: 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 64 °C for 30 s, 72 °C for 2.5 min, and a final step at 72 °C for 5 min. PCR products were separated on a 0.6% agarose gel. Bands obtained were purified with the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), and directly sequenced. Three new primers were designed from the obtained sequences: PLMx1L, PLMx2L and PLMx3L, and used together with the AP2 primer for a third PCR. Cycling conditions were as in the second PCR round. PCR products were sequenced and analysed using SeqmanII software. A consensus sequence of approximately 1 Kb was obtained from each Mx promoter (Fig. 1S).

Table 1
Primers used in this study. XhoI and BglII sites appear in bold.

	Name	Sequence 5′-3′
Genome walking primers	ExtMx1R	ACACAGTGTCAAACAGAAGGAGATG
	IntMx1R	AATACATCTTACATGACAAAAGAGGCCTG
	ExtMx2R	TAGCAGAAATGTTCTTTATGACTGGAG
	IntMx2R	ATCTGCAATACATATCCATATCCGC
	ExtMx3R	TGTTATTAACATATGAATATTTCCGGG
	IntMx3R	TTTTCCTTAATTACCACACCTGTCC
	AP1	GTAATACGACTCACTATAGGGC
	AP2	ACTATAGGGCACGCGTGGT
	PLMx1R	TCCATCTCATCTTTGGCGTTTCG
	PLMx2R	GTATTGTGGCACTCTGTTTGACCTCAG
	PLMx3R	ATGCTGTGGTTGTCCCTGTTCC
Cloning primers	CMVXhoIF	TTACGC**CTCGAG**GCGAAAGG
	CMVBglIIR	CGTTGGG**AGATCT**CCCATATGG
	XhoIMx1F	CTGCAGCTCC**CTCGAG**TGG
	BglIIMx1R	TCGTGATGTAAATCCATC**AGATCT**TTGG
	XhoIMx2F	TGAGA**CTCGAG**TTTTGTTTTTTGTCAG
	BglIIMx2R	CTCAATGTTTCT**AGATCT**TTGAGTTTCC
	XhoIMx3F	CTTTGGTC**CTCGAG**ATTGATTTG
	BglIIMx3R	GTCTATCCATC**AGATCT**GTGGCG
	XhoISREF	TTCGTCCCATTA**CTCGAG**AGAGTAAAGAC
	XhoISREMx2F	CGGCAA**CTCGAG**AAGAAAAAGGAAAG
	ATGBglIIR	GTTCATGCTGCTC**AGATCT**TGTCTGC

To search for possible ISG motives in the corresponding promoters, all sequences were analysed by using EditSeq software (Lasergene DNAstar, version 7.0.0).

2.2. Construction of pMx1, pMx2 and pMx3 reporter plasmids

Complete promoter fragments were generated by PCR with specific primers designed from the consensus sequences, and containing a XhoI restriction site on the forward primers (XhoIMx1F, XhoIMx2F XhoIMx3F for pMx1, pMx2 and pMx3 respectively) and a BglII restriction site on the common reverse primer, AtgBglIIR, that was used to clone the three promoters (Table 1). PCRs were carried out with the Go-Taq DNA polymerase (Promega), and cycling conditions were: 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 64 °C (pMx1), 63 °C (pMx2) or 61 °C (pMx3) for 30 s, 72 °C for 1.5 min, and a final step at 72 °C for 5 min. Amplification products were purified as described above, and digested with XhoI and BglII. Then, these fragments were purified and ligated to pGL4.22 (luc2CP/Puro, Promega), previously digested with XhoI and BglII. The vectors containing the complete promoters were named pMx1Luc, pMx2Luc and pMx3Luc.

For the promoter deletion studies, five vectors were constructed (schemes appear in Fig. 4): three of them including the corresponding 5′ ends up to the ISRE motif; one containing the common 3′ end of the three promoters, including the ISRE motif; and finally, a fifth vector containing the 3′ end of the pMx2 with the common ISRE motif and one of the two close ISRE-like motifs specific of pMx2. For amplifying the vectors containing the 5′ regions, the three constructs with the complete promoters were used as templates and specific primers (XhoIMx1F/BglIIMx1R, XhoIMx2F/BglIIMx2R, and XhoIMx3F/BglIIMx3R, Table 1 and Fig. 1S) were designed to obtain the desired fragments. PCR conditions were: 95 °C for 2 min, 35 cycles of 95 °C for 30s, 64 °C (pMx1), 60 °C (pMx2) or 61 °C (pMx3) for 30 s, 72 °C for 1 min, and a final step at 72 °C for 5 min. Constructs were named pMx1noISRE (−111 to −821), pMx2noISRE (−171 to −1001) and pMx3noISRE (−171 to −1181). The vector pISRE comprised the 3′ extreme of the three promoters (−48 to −111) and was constructed using as template the pMx1Luc vector and the primers XhoIISREF and AtgBglIIR. The vector pISRE2 (−48 to −111) was constructed using as template the pMx2Luc vector and the primers XhoIISREMx2F and AtgBglIIR. For