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Structural and functional characterization of the Senegalese sole (*Solea senegalensis*) Mx promoter

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ABSTRACT

Mx proteins are one of the most studied interferon-stimulated genes (ISGs). The antiviral activity against different fish viruses has been demonstrated for diverse fish Mx proteins, including the Senegalese sole (*Solea senegalensis*) Mx protein (SsMx). The aim of the current study is to characterize the structure and functional activity of the SsMx promoter. Several polyclonal cell populations expressing the luciferase reporter gene under the control of the SsMx promoter have been used to determine the ability of this promoter to drive the expression of the luciferase gene after poly I:C stimulation. In addition, the implication of each interferon-stimulated response element (ISRE) in the activation of the promoter has also been analysed. The genomic structure of the Senegalese sole and Japanese flounder Mx promoters (containing three ISREs) differs from the rest of the fish Mx promoters described to date. The ISRE1, the one closest to the start codon, is the main ISRE involved in the SsMx promoter activity, whereas ISRE2 and ISRE3 show a minor additive effect on this activity. Another feature differing SsMx promoter from the rest of the fish Mx promoters is the presence of a 24-bp GC island close to the ATG codon, including one Sp1 binding site, which may constitute the transcriptional start site. Furthermore, the SsMx promoter contains a gamma interferon activation site (GAS) element.

1. Introduction

The interferon (IFN) system is the first line of defence against viral diseases in vertebrates. Type I IFN is secreted by infected cells and binds neighbouring cell receptors, triggering the JAK-STAT signalling pathway, which is followed by the activation of the STAT1 and STAT2 proteins. These transcription factors associate with IRF-9 to form the interferon-stimulated gene factor 3 (ISGF3). In the nucleus, the ISGF3 complex binds interferon-stimulated response elements (ISREs) in the promoter of interferon-stimulated genes (ISGs), activating their transcription [1].

Mx proteins are the most studied ISGs in fish, having been used as a marker of type I IFN activity. Mx proteins are GTPases with antiviral activity against a broad range of viruses, such as rhabdovirus, birnavirus or nodavirus in a variety of fish species, differing in their antiviral spectrum and intracellular location [2–8]. A complex spatial and temporal regulation of ISG expression seems to be responsible for the control of host defences against viral infection [9]. Thus, one of the main issues in understanding virus–host relationship is the knowledge on the regulatory mechanisms governing IFN response.

The detailed regulatory mechanisms of fish Mx transcription are poorly understood. Fish Mx promoters characterized to date are those of pufferfish (*Takifufu rubripes*), zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*) Mx1, Japanese flounder (*Paralichthys olivaceus*), orange-spotted grouper (*Epinephelus coioides*) and channel catfish (*Ictalurus punctatus*) [10–15]. Rainbow trout and channel catfish Mx promoters contain only one ISRE, whereas pufferfish, zebrafish and grouper Mx promoters contain two close ISREs, and Japanese flounder Mx promoter contains three ISREs. The diversity of fish Mx promoters prompts for characterizing these regions in every species of interest, since Mx regulation might be related with the susceptibility of different fish species to specific viruses.

Therefore, the aim of the present study is to determine the regulatory mechanisms that control the transcription of the Senegalese sole (*Solea senegalensis*) Mx protein (SsMx), which has been previously reported to have antiviral activity against two RNA viruses, the Infectious Pancreatic Necrosis Virus (IPNV, *Birnaviridae*

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family, dsRNA), and the Viral Haemorrhagic Septicaemia Virus (VHSV, *Rhabdoviridae* family, ssRNA) [6]. For that purpose, a structural and functional characterization of the SsMx gene promoter has been performed using a reporter system that expresses the luciferase gene under the control of the SsMx promoter, which is an optimal system to discern regulatory pathways of fish Mx expression *in vitro* [10–17].

2. Material and methods

2.1. SsMx promoter cloning and sequencing

Genomic DNA was purified from three Senegalese sole larvae (0.03–0.06 g) by saline precipitation [18]. Genomic DNA was resuspended in double-distilled water and stored at 4 °C. DNA concentration was measured at 260 nm using the ND-1000 system (NanoDrop Thermo Scientific).

GenomeWalker™ Universal Kit (Clontech) was used to clone the SsMx promoter. Briefly, genomic DNA (0.1 μg/μL) was independently digested with three blunt-end restriction enzymes, *EcoRv*, *PvuII* and *StuI* at 37 °C overnight. Each purified reaction was ligated to the GenomeWalker adaptor (containing AP1 and AP2 primer sequence sites) (Table 1), at 4 °C overnight, using the T4 DNA ligase (Promega) and following manufacturers' instructions. In parallel, two reverse primers, PromSsMxRev1 and PromSsMxnested (Table 1), were designed from the 5′-end region of the SsMx gene (GenBank accession no. EU717076).

Each ligation mixture was amplified by PCR using the Go Taq DNA Polymerase (Promega) and the primers AP1 (forward) and PromSsMxRev1 (reverse) (Table 1). PCR reactions were carried out under the following conditions: 95 °C for 2 min, 35 cycles of 95 °C for 1 min, 60 °C for 30 s and 72 °C for 2 min, and a final step at 72 °C for 5 min. Subsequently, a nested-PCR was conducted using the AP2/PromSsMxnested primers (Table 1) and the PCR conditions previously described. Nested-PCR products were sequenced (ABI PRISM 3130, Applied Biosystem), and multiple alignments were performed with the sequences obtained using the MegAlign software (DNAstar). A 1.3-kb consensus sequence of the SsMx promoter was obtained. To clone the promoter, a PCR amplification was performed using the primers PromSsMxF1 (designed from the 5′-end region of the obtained sequence) and PromSsMxR1 (Table 1). The reaction was conducted in a 50-μL mixture containing 300 ng of genomic DNA, 5 μL of 10× buffer, 2 μL of each primer (15 μM) and 0.2 μL of Taq DNA Polymerase High Fidelity (Life Technologies). The amplification profile was: one step at 95 °C for 2 min, followed by 35 cycles consisting of 30 s at 95 °C, 30 s at 55 °C and 90 s at 68 °C, and a final step at 72 °C for 5 min. Gel purified PCR product was ligated into the pGEM-T Easy vector (Promega) and confirmed by sequencing. In order to determine the presence of ISG motifs, SsMx promoter sequence, as well as Mx promoter sequences from other fish species, were analysed using the EditSeq software (Lasergene DNAstar, Version 7.0.0).

Table 1
Primers used in this study.

Name	Sequence (5′-3′)
PromSsMxRev1	CAATGCAGGGACGAACCTTCTCCTCGATA
PromSsMxnested	ATGGCAGCGTCAAACACAGAGAA
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
PromSsMxF1	ATAGTAAGGAGTATCAACACAATG
PromSsMxR1	TACCATTGTGCGGGCATAGAGGGGTGTGT
PromSsMxF2.2	CTCGAGGACACTACAGAGACAAA
PromSsMxR1.2	AGATCTGAGAAGAGTTCACGGATTGAATGAA
PromSsMxF4.2	CTCGAGCAGAGAAATGAAACTCAAGTGTGGAAAT
PromSsMxF5.1	CTCGAGTGTGCACATTATCCGTTGTTTAGA
PromSsMxR8.1	AGATCTGCACACATAATGTTGTAAGGTGA
MutpromSsMx1F	GAATTCAAAAGTTTCCAAGATAACGAAACTTAACACACGC
MutpromSsMx1R	GCGTGTGTTAAGTTTCGTTATCTTGGAAACTTTTGAATTC

Restriction sites are underlined.

2.2. Construction of SsMx promoter reporter plasmids

Several constructs of the SsMx promoter with different deletions (Fig. 1) were obtained by High Fidelity PCR amplification using forward and reverse primers containing, respectively, the *XhoI* and *BglII* restriction sites at their 5′-ends (Table 1). The PCR products were gel-purified, ligated into the pGEM-T easy vector, and subsequently subcloned between the *XhoI* and *BglII* sites of the pGL4.22-basic vector (Promega). All SsMx promoter constructs were confirmed by sequencing.

In addition, a point mutation on the IFN-stimulated response element 1 (ISRE1) of the SsMx promoter was introduced using the Quickchange II Site-Directed Mutagenesis Kit (Stratagene). The wild type ISRE1 motif (GAAAACGAAA) was replaced by the mutated GATAACGGAAA sequence (mutagenized nucleotide is underlined) using the MutpromSsMx1F/MutpromSsMx1R primers (Table 1, Fig. 1). PCR reaction was conducted using 50 ng of the pGL4+PromSsMx-1324 plasmid (containing the complete sequence of the SsMx promoter, Fig. 1), and the following PCR conditions: 95 °C for 30 s, followed by 18 cycles of 95 °C for 30 s, 60 °C for 2 min and 68 °C for 8 min. After amplification, the product was treated with *DpnI* to digest the parental DNA template. The DNA vector containing the desired mutation was then transformed into competent cells (*Escherichia coli*, DH5α).

2.3. Transient transfections and poly I:C stimulation

Rainbow trout gonad tissue cells (RTG-2) and Chinook salmon (*Oncorhynchus tshawytscha*) embryo cells (CHSE-214) were grown in 75 cm^2-flask (CellStar) at 22 °C in Leibovitz's medium (L-15, Lonza) supplemented with 10% foetal bovine serum (FBS, Hyclone) and 4 mM L-glutamine (Gibco) (growth medium). Cells were trypsinized, resuspended (ca. 10^5 cells) in 10 μL of Neon Resuspension Buffer R (Life Technologies) and immediately transfected using the Neon Transfection System (Life Technologies) following manufacturers' instructions. Each electroporation was performed in a mixture containing 10 μL of the cell suspension, 0.6 μL of the Gateway TurboGFP-N vector (500 ng/μL, Evrogen), 0.5 μL of each pGL4 promoter construct (1000 ng/μL) and 0.5 μL of sterile water. In order to stimulate promoter activity in CHSE-214 cells, poly I:C (Sigma) at 20, 80 or 180 μg/mL (final concentration) was also included into the electroporation reaction, since this cell line needs

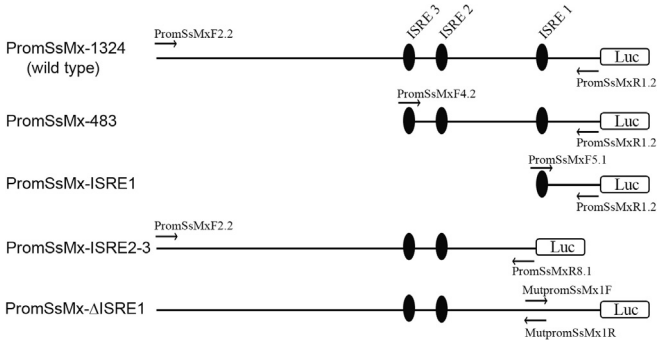

Fig. 1. Schematic representation of the Senegalese sole (SsMx) promoter constructs used in this study. SsMx promoter constructs were cloned into the pGL4 vector, which contains a luciferase reporter gene (Luc). PromSsMx-ΔISRE1 contains a point mutation into the ISRE1. Black circles represent the three ISREs present into the promoter. The PCR primers used to obtain the constructs are represented by arrows.