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Short communication

Differential response of the Senegalese sole (*Solea senegalensis*) Mx promoter to viral infections in two salmonid cell lines

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ABSTRACT

Mx proteins are main effectors of the antiviral innate immune defence mediated by type I interferon (IFN I). The IFN I response is under a complex regulation; hence, one of the key issues in understanding virus-host interaction is the knowledge of the regulatory mechanisms governing this response. With this purpose, in this study Chinook salmon embryo cells (CHSE-214) and rainbow trout gonad cells (RTG-2) were transiently transfected with a vector containing the luciferase reporter gene under the control of the Senegalese sole Mx promoter. These transfected cells were infected with infectious pancreatic necrosis virus (IPNV), viral haemorrhagic septicaemia virus (VHSV) and epizootic haematopoietic necrosis virus (EHNV) at different doses in order to study the luciferase fold induction in response to viral infections. Transfected CHSE-214 cells infected with EHNV showed significant induction of the luciferase reporter gene, compared to control non-infected cells, at different times post infection (p.i.). The maximum expression was recorded at 24 h p.i. in cells inoculated with 5×10^2 $TCID_{50}$/mL (2.17 folds compared to control cells). In these cells, the infection with IPNV and VHSV did not result in the luciferase expression at any time and doses tested. In transfected RTG-2 cells, VHSV stimulated luciferase expression, obtaining a maximum activity at 48 h p.i. in cells infected with 5×10^2 $TCID_{50}$/mL (2.9 folds compared to control cells), whereas RTG-2 cells infected with IPNV and EHNV did not show significant luciferase activity at any time point. The different induction of the Senegalese sole Mx promoter in CHSE-214 and RTG-2 cells after infection with the same viruses indicates that cell-specific factors are significantly involved in the IFN-signalling response, and, probably, on the success of the strategies of these viruses to escape the IFN mechanisms. The use of these two different cellular systems might be an interesting approach to identify such cellular factors.

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1. Introduction

In vertebrates, type I interferon (IFN I) system triggers specific signalling pathways that lead to the activation of the innate immune defences against viral infections. IFN I molecules are secreted by infected cells causing an antiviral

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state in neighbouring cells by induction of IFN-stimulated genes (ISGs). This expression is under a complex spatial and temporal regulation, which seems to be responsible for the control of the IFN I antiviral response (Zou and Secombes, 2011).

Mx proteins are one of the main ISGs, playing a main role in the IFN I response. Mx proteins belong to the dynamin superfamily of high molecular weight GTPases, which are involved in intracellular trafficking and membrane remodelling. Although the mechanisms of the Mx antiviral activity are not completely understood, it seems clear that they rely on direct interaction between Mx proteins and viral targets that need to be defined in each case (Haller and Kochs, 2011). Fish Mx proteins have been intensively studied, especially those from cultured species, since to know pathogen–host interaction mechanisms might be essential to develop strategies focused on enhancing fish natural resistance to viral infections (Magnadottir, 2010). Although the antiviral activity of Mx proteins has been largely reported in several fish species (Caipang et al., 2002, 2003; Larsen et al., 2004; Kibenge et al., 2005; Lin et al., 2006; Wu and Chi, 2007; Chen et al., 2008; Fernandez-Trujillo et al., 2013), the regulatory mechanisms of piscine Mx genes are poorly understood, since only a few Mx promoters have been cloned and characterized to date, such as those from pufferfish (Yap et al., 2003), zebrafish (Altmann et al., 2004), rainbow trout Mx1 (Collet and Secombes, 2001), Japanese flounder (Ooi et al., 2006), orange-spotted grouper (Chen et al., 2006), channel catfish (Plant and Thune, 2008), gilthead seabream (Gonzalez-Mariscal et al., 2014), and Senegalese sole (Alvarez-Torres et al., 2013b). Likewise, the study on the regulation of fish Mx gene transcription has been stressed by the observation that Mx genes can be induced by a wide range of viruses, which has been related to early viral protection (reviewed in Langevin et al., 2013).

Senegalese sole (*Solea senegalensis*, Kaup) is a marine flatfish of growing importance in the Southern European aquaculture (APROMAR, 2013), which can be affected by several viruses, such as lymphocystis disease virus (LCDV, *Iridoviridae* family), infectious pancreatic necrosis virus (IPNV, *Birnaviridae* family), and viral nervous necrosis virus (VNNV, *Nodaviridae* family) (Rodriguez et al., 1997; Alonso et al., 2005; Cutrin et al., 2007). Furthermore, viral haemorrhagic septicaemia virus (VHSV, *Rhabdoviridae* family) has been reported to be pathogenic to this fish species under experimental conditions (Lopez-Vazquez et al., 2011).

The Senegalese sole Mx protein (SsMx) displays antiviral activity against IPNV and VHSV, but it does not inhibit the replication of the European sheatfish virus (ESV, *Iridoviridae* family) (Fernandez-Trujillo et al., 2008; Alvarez-Torres et al., 2013a). As a first approach in disclosing the regulation of the SsMx gene expression, the structure of the SsMx promoter has been described and its response to poly I:C has been characterized in transfected CHSE-214 and RTG-2 cells, showing induction in both cell lines with some differences regarding the kinetics and magnitude of the induction (Alvarez-Torres et al., 2013b).

In order to continue with the functional characterization of the SsMx promoter, its induction in response to different viral infections has been evaluated in this study. To fulfil this objective, CHSE-214 and RTG-2 cells were transiently transfected with a plasmid containing the firefly luciferase gene under the control of SsMx promoter, and subsequently inoculated with IPNV, VHSV or epizootic haematopoietic necrosis virus (EHNV, *Iridoviridae* family).

2. Material and methods

2.1. Cell culture and virus propagation

Chinook salmon (*Oncorhynchus tshawytscha*) embryo cells (CHSE-214) and rainbow trout (*Oncorhynchus mykiss*) gonad tissue cells (RTG-2) were grown in 75 cm^2 flasks (CellStar) at 22 °C in Leibovitz's medium (L-15, Lonza) supplemented with 10% FBS (Hyclone) and 4 mM L-glutamine (Gibco).

The SsMx promoter activation has been tested in response to the following fish viruses: (1) IPNV (A2 serotype, *Salmo salar* isolate), (2) VHSV (Ip8 isolate, obtained from *Clupea harengus*, Baltic Sea) and (3) EHNV (*Perca fluviatilis* isolate). Viruses were propagated on RTG-2 cells cultured at 20 °C in L-15 medium supplemented with 2% FBS and 4 mM L-glutamine. Supernatants of cultures displaying extensive cytopathic effects (CPEs) were harvested and centrifuged at $4000 \times g$ for 10 min at 4 °C. These virus suspensions were titrated (Reed and Muench, 1938) and stored at −80 °C until used.

2.2. Cell transfection

Monolayers of CHSE-214 and RTG-2 cells were detached by trypsinization, 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 composed of 10 μL of cell suspension, 0.6 μL of the Gateway TurboGFP-N vector (500 ng/μL, Evrogen), 0.5 μL of the pGL4.22-SsMx promoter construct (1 μg/μL) (Alvarez-Torres et al., 2013b) and 0.5 μL of sterile water. CHSE-214 cells were pulsed twice with 1300 v for 20 ms, whereas RTG-2 cells were pulsed once with 1200 v for 40 ms. After transfection, cells were seeded on 48-well plates (CellStar) and incubated at 22 °C for 24 h. At this moment, transfection efficiency was estimated by checking Green Fluorescent Protein (GFP) fluorescence in transfected cells under the inverted microscope. Transfections with efficiencies lower than 70–80% were discarded. CHSE-214 and RTG-2 cells transfected with the promoterless vector (pGL4.22-basic vector, Promega) were used as negative controls.

2.3. Virus inoculation and luciferase activity quantification

Transfected CHSE-214 and RTG-2 cells were grown for 24 h. Afterwards, they were carefully washed twice with D-PBS (Invitrogen), inoculated with each virus at 5×10^3 and 5×10^2 $TCID_{50}$/mL (in L-15 medium supplemented with 10% FBS), and subsequently incubated at 20 °C. Transfected and non-infected cells were used as negative controls.

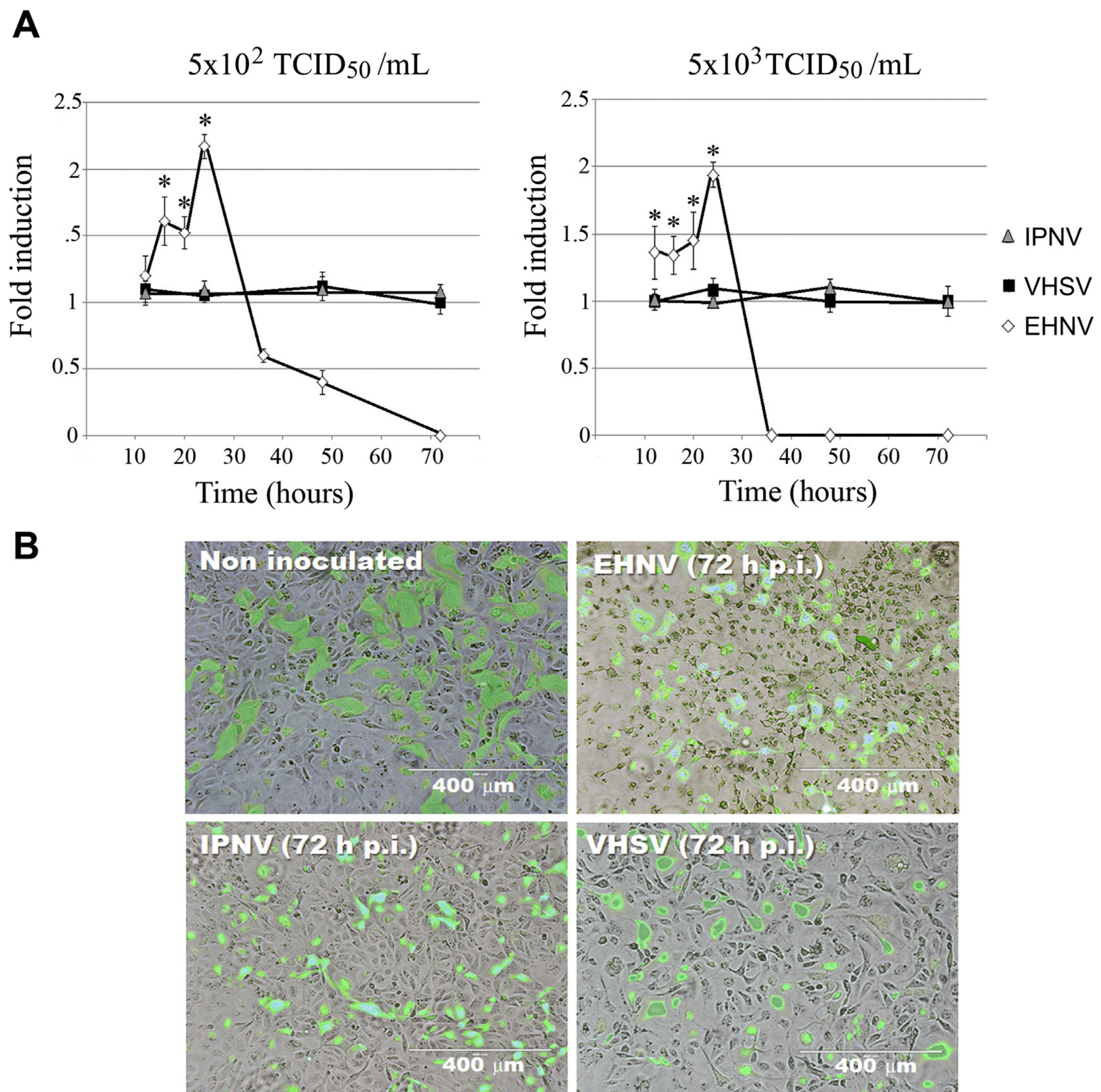

Fig. 1. (A) SsMx promoter fold induction in CHSE-214 cells infected with IPNV, VHSV and EHNV at 5×10^2 and 5×10^3 $TCID_{50}$/mL. Fold induction was calculated as RLU in inoculated cells divided by RLU in non-inoculated cells. Asterisks indicate significant induction compared to transfected non-infected cells ($P < 0.05$). (B) Cytopathic effect evaluation at 72 h p.i. in CHSE-214 cells inoculated with the same viruses at 5×10^3 $TCID_{50}$/mL. Non inoculated cells were used as negative control.

Luciferase activity was measured at 12, 24, 48 and 72 h post-inoculation (p.i.) for IPNV- and VHSV-inoculated cells, or at 12, 16, 20, 24, 36, 48 and 72 h p.i. for EHNV inoculated cells. Three replicates per condition in three independent experiments were carried out.

The luciferase and GFP activity was quantified using the luminometer Victor^3 Multilabel Readers (model 1420, Perkin Elmer). GFP expression was measured at 535 nm after the excitation of living cell monolayers at 485 nm for 1 s. For the subsequent quantification of the luciferase activity, the SteadyGlo^{TM} luciferase substrate (Promega) was added (75 μL/well) and the light emission was measured after 5-min incubation. GFP levels were used to normalize the results of luciferase activity regarding differences in transfection efficiency between samples. In this way, the relative light units (RLUs) were calculated as luciferase activity divided by GFP fluorescence. Data were analysed with the Wallac 1420 workstation version 3.0 software. Fold induction was calculated as RLU in inoculated cells divided by RLU in non-inoculated cells. Differences between RLUs were tested by the two-tailed unpaired Student's *t*-test. Differences of $P < 0.05$ were considered statistically significant.

2.4. Viral multiplication on transfected cells

To confirm the replication of IPNV, VHSV and EHNV on transfected RTG-2 and CHSE-214 cells, the onset of CPEs was regularly checked. When CPEs were not detected (CHSE-214 cells infected with IPNV and VHSV), viral replication was confirmed by quantitative PCR (RT-qPCR) according to the procedure described below.

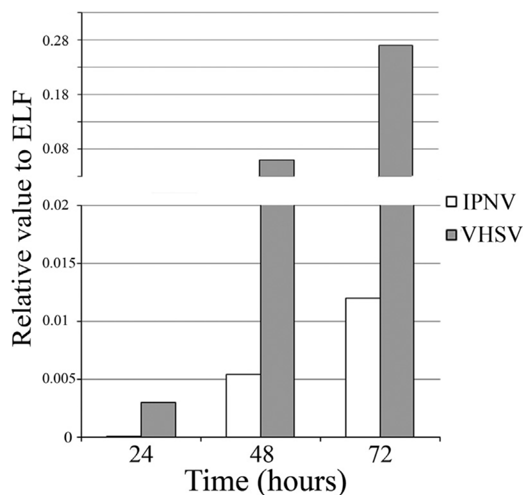

Fig. 2. Relative quantification (RT-qPCR) of viral RNA in CHSE-214 cells at 24, 48 and 72 h after infection with IPNV (VP2) or VHSV (Nucleoprotein). Data are relative to the ELF gene transcription.

CHSE-214 cells were infected with IPNV or VHSV at 5×10^3 $TCID_{50}$/mL, and total RNA was extracted at 24, 48 and 72 h p.i. using the RNeasy-mini kit (Qiagen) following the manufacturer's protocol. The synthesis of cDNA was performed with 0.5 ng of RNA and oligo $d(T)_{16}$ primers using the TaqMan® ABI MultiScribe RT synthesis kit according to the manufacturer's instructions (Applied Biosystems). Amplifications were conducted with the Roche LightCycler 480using Taqman assays described by Lockhart et al. (2007) and Campbell et al. (2011) for the quantification of the viral genome encoding the VP2 protein (IPNV), or the Nucleoprotein (VHSV), respectively. PCR profile was: 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Data were analysed with the comparative Ct method, obtaining relative values (R_v) to the salmon ELF gene transcription (Lester et al., 2012) according to $R_v = 2^{-\Delta Ct}$.

3. Results

To investigate the response of the SsMx promoter to viral infections, CHSE-214 and RTG-2 cells were transiently transfected with a vector containing the luciferase reporter gene under the control of the SsMx promoter, and subsequently infected with IPNV, VHSV and EHNV at two different doses.

In CHSE-214 cells, no induction of the SsMx promoter was observed after infection with IPNV or VHSV at both viral concentrations tested (Fig. 1A). In addition, CPEs were not observed at any time (Fig. 1B). To confirm the viral replication, RT-qPCR amplifications were conducted using total RNA extracted from infected cells. The results showed an increase in the viral RNA encoding VP2 (IPNV) at 48 and 72 h p.i. Similarly, the RNA encoding the Nucleoprotein (VHSV) increased at 24, 48 and 72 h p.i. (Fig. 2), confirming the replication of both viruses in CHSE-214 cells.

In contrast, a significant induction of the SsMx promoter was detected in EHNV-infected CHSE-214 cells (Fig. 1A). When these cells were inoculated with the lower viral concentration, the promoter activation was recorded at 16, 20 and 24 h p.i., whereas in cells infected with the higher viral dose the induction was at 12 and 24 h p.i. (Fig. 1A). In both cases, maximum luciferase activity was at 24 h p.i. (2.17 and 1.94 fold induction for low and high viral doses, respectively), decreasing afterwards. CPEs were clearly observed, with cells showing advanced CPEs at 36 h p.i. (Fig. 1B).

In RTG-2 cells, the induction of the SsMx promoter was only detected after VHSV infection (Fig. 3A). This induction was significant from 24 h, with a maximum at 72 h p.i. in cells infected with 5×10^2 $TCID_{50}$/mL (2.25 fold induction). CPEs were observed at that time in these cells (data not shown). In cells inoculated with a higher viral concentration (5×10^3 $TCID_{50}$/mL), the promoter activation was recorded as early as 12 h p.i., with the maximum induction at 48 h p.i., decreasing at 72 h p.i. (Fig. 3A), coinciding with the CPE emergence (Fig. 3B). In contrast, the infection with IPNV or EHNV did not result in the induction of the SsMx promoter in RTG-2 cells (Fig. 3A). Cells infected with these two viruses showed advanced CPEs at 72 h p.i. (Fig. 3B), thus confirming the replication of IPNV and EHNV in RTG-2 cells.

CHSE-214 and RTG-2 cells transfected with the promoter-less vector and infected with the viruses did not show induction of luciferase expression (data not shown).

4. Discussion

Due to the absence of a stable cell line derived from Senegalese sole, the characterization of the SsMx promoter was conducted in the salmonid cell lines CHSE-214 and RTG-2. In this way, the activation of the SsMx promoter by poly I:C in transfected RTG-2 and CHSE-214 cells has been previously demonstrated (Alvarez-Torres et al., 2013b), confirming that these two cell lines are appropriate experimental systems to functionally characterize this promoter. In addition, both cell lines derive from different species and tissues, being susceptible to the infection by several fish viruses. For these reasons, they are adequate tools to study the stimulation of the IFN I signalling pathways by viruses, providing two complementary models that may yield a broad understanding of the IFN response in fish. The aim of this study has been to analyse the SsMx promoter response to different viruses. Interestingly, the SsMx promoter was induced only after EHNV infection in CHSE-214 cells, and after VHSV infection in RTG-2 cells. As the three viruses tested (IPNV, VHSV, EHNV) can replicate in both cell lines (Isshiki et al., 2001; Song et al., 2005; Ariel et al., 2009; and the present study), the differential response of the SsMx promoter to the infection by the same viruses in CHSE-214 and RTG-2 cells clearly suggests the major influence of cell-specific factors in the IFN I response.

The EHNV infection induced the SsMx promoter expression only in CHSE-214 cells. The magnitude and kinetics of this induction was similar regardless of the viral dose tested (Fig. 1A); however, the response was earlier (at 16 or 12 h p.i. with low and high viral inoculum, respectively) than that obtained after poly I:C treatment (at 48 h post-induction) (Alvarez-Torres et al., 2013b). In addition, the maximum induction of the SsMx promoter after poly

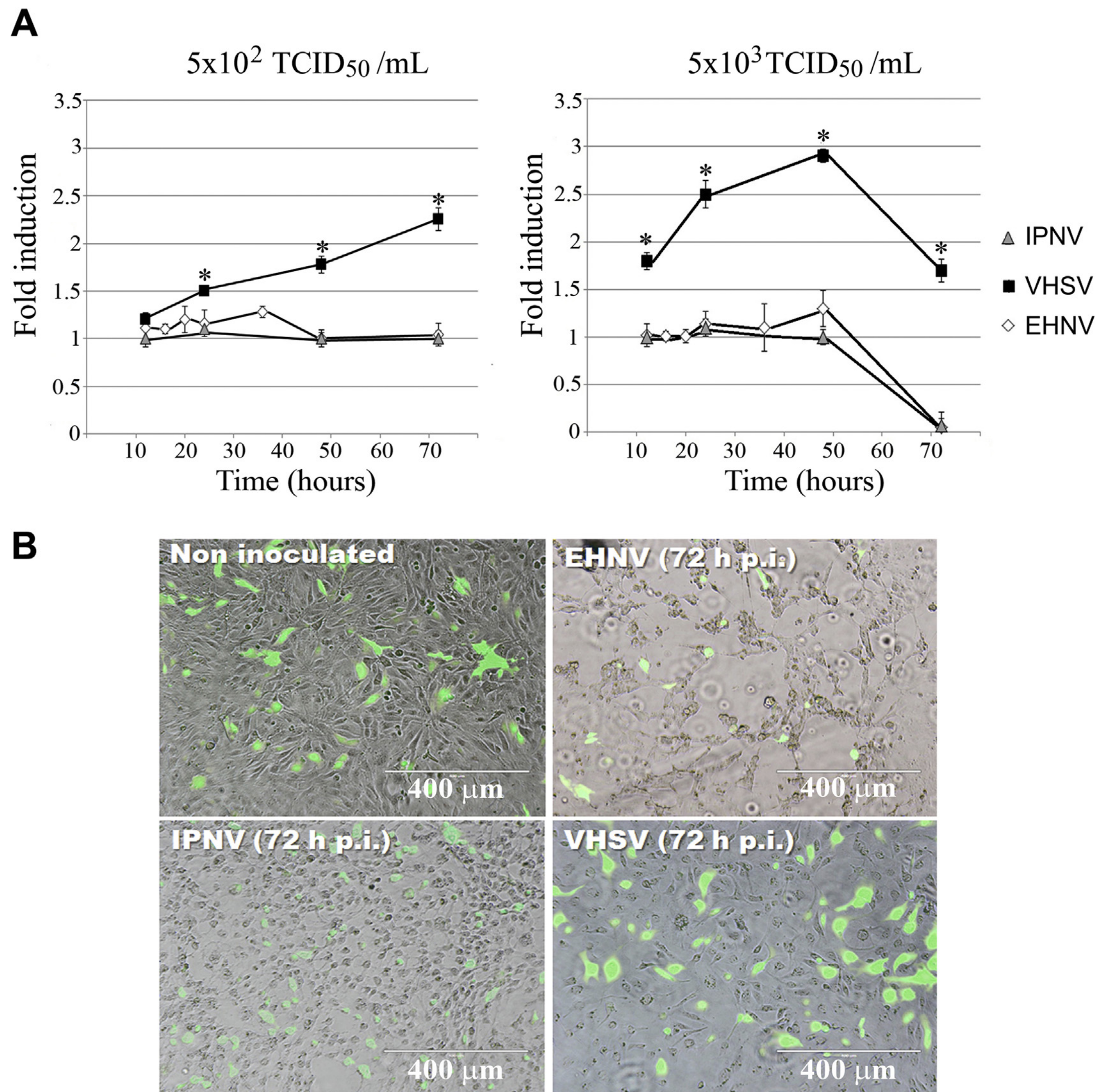

Fig. 3. (A) SsMx promoter fold induction in RTG-2 cells infected with IPNV, VHSV and EHNV at 5×10^2 and 5×10^3 $TCID_{50}$/mL. Fold induction was calculated as RLU in inoculated cells divided by RLU in non-inoculated cells. Asterisks indicate significant induction compared to transfected non-infected cells ($P < 0.05$). (B) Cytopathic effect evaluation at 72 h p.i. in RTG-2 cells inoculated with the same viruses at 5×10^3 $TCID_{50}$/mL. Non inoculated cells were used as negative control.

I:C treatment (5.96 fold induction) (Alvarez-Torres et al., 2013b) was higher than that obtained in this study after EHNV infection (2.17 fold induction). The stimulation of the IFN system in CHSE-214 cells requires poly I:C transfection (Jensen et al., 2002), which may account for the stronger IFN response in comparison with that reported after viral infection, although this response is delayed. Actually, in recombinant CHSE-214 cells expressing luciferase under the control of the rainbow trout Mx1 gene promoter, the induction began 16 h after treatment with rainbow trout IFN α/β (Jørgensen et al., 2007), showing similar kinetics to that recorded in the present study for the SsMx promoter after EHNV infection.

EHNV has been selected in this study as a model of fish DNA viruses, since LCDV, the iridovirus pathogenic to Senegalese sole, does not replicate on CHSE-214 or RTG-2 cells. In addition, EHNV has been described as the first iridovirus causing epizootic mortality in finfish (Whittington et al., 2010). In concordance with our results in CHSE-214 cells, several fish iridoviruses, such as the orange-spotted grouper virus (TGIV), LCDV, turbot reddish body iridovirus (TRBIV), or ESV, have been shown to induce Mx protein expression (Wu and Chi, 2007; Wu et al., 2010; Hu et al., 2011; Alvarez-Torres et al., 2013a). However, Mx proteins did not protect the cells against the infection by these iridoviruses (Caipang et al., 2002; Wu and Chi, 2007; Lester et al., 2012; Alvarez-Torres et al., 2013a), except for gilthead seabream Mx1 and Mx2 proteins, which confer partial protection to CHSE-214 cells against ESV and LCDV (Fernandez-Trujillo et al., 2013). Regarding iridovirus strategies to evade the host immune response, it has been reported that they have evolved

effective immunosuppressive mechanisms by capturing and exploiting host genes, or by presenting inhibitors of specific antiviral genes (reviewed in Williams et al., 2005). This kind of mechanisms has been reported for the ranavirus RCV-Z (rana catesbiana virus Z) (Rothenburg et al., 2011), or the iridovirus SGIV (Singapore grouper iridovirus) (Huang et al., 2008), and has been proposed for LCDV (Pontejo et al., 2013). Since EHNV does not induce the SsMx promoter in RTG-2 cells, it is tempting to suggest some kind of IFN suppression mechanism of this virus against the RTG-2 IFN system, which needs to be confirmed with further experiments.

The stimulation of the SsMx promoter after VHSV infection was recorded only in RTG-2 cells inoculated with both viral doses tested, although the response was slightly higher (2.89–2.25 fold) and earlier (12–24 h p.i.) in cells inoculated with the higher viral inoculum. In a previous report (Alvarez-Torres et al., 2013b) luciferase activity in RTG-2 cells after poly I:C stimulation was similar (2.28 fold), although it started earlier, at 6 h after the poly I:C treatment. Therefore, poly I:C induction seems to be faster than VHSV induction in this cell line.

The lack of activation of the SsMx promoter in VHSV-inoculated CHSE-214 cells is in concordance with the absence of induction of the endogenous CHSE-214 Mx after VHSV infection reported by Alvarez-Torres et al. (2013a), which suggests a putative antagonistic effect. Actually, Collet et al. (2004) suggested a suppression mechanism to explain the limited response of the rainbow trout Mx1 promoter to VHSV infection in comparison with the induction triggered by poly I:C in the stable line RTG-P1. Furthermore, it has been recently reported that the VHSV NV protein interferes with the IFN signalling pathway, resulting in a poor induction of the Japanese flounder Mx promoter in vitro and in vivo (Kim and Kim, 2012). Thus, our results support the existence of VHSV antagonistic activity against the Chinook salmon IFN response, adding cell specific factors as important players in VHSV-host interaction.

Finally, the well-established antagonistic effect of IPNV is probably responsible for the absence of the SsMx promoter induction in CHSE-214 and RTG-2 cells. These results reveal the IPNV strategy, which is to inhibit the production of Mx protein by alteration of its gene induction (Collet et al., 2007; Jørgensen et al., 2007; Skjesol et al., 2009), since IPNV replication is affected by the accumulation of Mx protein (Lester et al., 2012). Interestingly, both cell lines respond similarly to IPNV infection, even though several studies suggest that IFN production and/or IFN signalling following infection with IPNV may depend on the cell type infected or may be a complex cell-virus interaction (Collet et al., 2007; Skjesol et al., 2009). In fact, our results are in concordance with those reports showing that IPNV does not induce endogenous Mx gene transcription in CHSE-214 and RTG-2 cells (Collet et al., 2007; Jørgensen et al., 2007; Fernandez-Trujillo et al., 2008, 2011; Alvarez-Torres et al., 2013a). Likewise, the IPNV infection failed in the induction of the Mx gene transcription in TO cells, a cell line derived from Atlantic salmon head kidney cells (Jensen and Robertsen, 2002). Therefore, despite the role of cell-specific factors in the IPNV antagonistic activity, this mechanism might be conserved among salmonid species.

In summary, our results demonstrate the induction of the SsMx promoter in response to EHNV and VHSV infection, highlighting the relevance of specific cellular factors on the IFN signalling pathway control, and, probably, on the success of the strategies of these viruses to escape the IFN mechanisms. For further experiments, it would be especially interesting to identify such cell-specific factors, to characterize the molecular mechanisms behind these viral-host interactions and to compare the effect on the promoters activity of viral isolates with different pathogenicity.

Acknowledgments

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References

Alonso, M.C., Cano, I., Garcia-Rosado, E., Castro, D., Lamas, J., Barja, J.L., Borrego, J.J., Bergmann, S.M., 2005. Isolation of lymphocystis disease virus from sole, *Solea senegalensis* (Kaup), and blackspot sea bream, *Pagellus bogaraveo* (Brünnich). J. Fish Dis. 28, 221–228.

Altmann, S.M., Mellon, M.T., Johnson, M.C., Paw, B.H., Trede, N.S., Zon, L.I., Kim, C.H., 2004. Cloning and characterization of an Mx gene and its corresponding promoter from the zebrafish, *Danio rerio*. Dev. Comp. Immunol. 28, 295–306.

Alvarez-Torres, D., Garcia-Rosado, E., Fernandez-Trujillo, M.A., Bejar, J., Alvarez, M.C., Borrego, J.J., Alonso, M.C., 2013a. Antiviral specificity of the *Solea senegalensis* Mx protein constitutively expressed in CHSE-214 cells. Mar. Biotechnol. 15, 125–132.

Alvarez-Torres, D., Bejar, J., Collet, B., Alonso, M.C., Garcia-Rosado, E., 2013b. Structural and functional characterization of the Senegalese sole (*Solea senegalensis*) Mx promoter. Fish Shellfish Immunol. 35, 1642–1648.

APROMAR, 2013. La acuicultura marina de peces en españa. Asociación empresarial de productores de cultivos marinos, ⟨https://docs.google.com/file/d/0B4_4E-v9oqL_X1ZjQUVPOFphUDA/edit⟩.

Ariel, E., Nicolajsen, N., Christophersen, M.-B., Holopainen, R., Tapiovaara, H., Jensen, B.B., 2009. Propagation and isolation of ranaviruses in cell culture. Aquaculture 294, 159–164.

Caipang, C.M.A., Hirono, I., Aoki, T., 2002. Viral resistance of a recombinant Japanese flounder Mx-transfected fish cell. Fish Sci. 68, 1217–1218.

Caipang, C.M.A., Hirono, I., Aoki, T., 2003. In vitro Inhibition of fish rhabdoviruses by Japanese flounder, *Paralichthys olivaceus* Mx. Virology 317, 373–382.

Campbell, S., McBeath, A., Secombes, C.J., Snow, M., Collet, B., 2011. Interferon response following infection with genetically similar isolates of viral haemorrhagic septicaemia virus (VHSV) exhibiting contrasting virulence in rainbow trout. Fish Shellfish Immunol. 30, 287–294.

Chen, Y.-M., Su, Y.-L., Lin, J.H.-Y., Yang, H.-L., Chen, T.-Y., 2006. Cloning of an orange-spotted grouper (*Epinephelus coioides*) Mx cDNA and characterisation of its expression in response to nodavirus. Fish Shellfish Immunol. 20, 58–71.

Chen, Y.-M, Su, Y.-L., Shie, P.-S., Huang, S.-L., Yang, H.-L., Chen, T.-Y., 2008. Grouper Mx confers resistance to nodavirus and interacts with coat protein. Dev. Comp. Immunol. 32, 825–836.

Collet, B., Secombes, C.J., 2001. The rainbow trout (*Oncorhynchus mykiss*) Mx1 promoter: structural and functional characterization. Eur. J. Biochem. 268, 1577–1584.

Collet, B., Boudinot, P., Benmansour, A., Secombes, C.J., 2004. An Mx1 promoter–reporter system to study interferon pathways in rainbow trout. Dev. Comp. Immunol. 28, 793–801.

Collet, B., Munro, E.S., Gahlawat, S., Acosta, F., Garcia, J., Roemelt, C., Zou, J., Secombes, C.J., Ellis, A.E., 2007. Infectious pancreatic necrosis virus suppresses type I interferon signalling in rainbow trout gonad cell line but not in Atlantic salmon macrophages. Fish Shellfish Immunol. 22, 44–56.

Cutrin, J.M., Dopazo, C.P., Thiery, R., Leao, P., Olveira, J.G., Barja, J.L., Bandin, I., 2007. Emergence of pathogenic betanodaviruses belonging to the SJNNV genogroup in farmed fish species from the Iberian Peninsula. J. Fish Dis. 30, 225–232.

Fernandez-Trujillo, M.A., Garcia-Rosado, E., Alonso, M.C., Borrego, J.J., Alvarez, M.C., Bejar, J., 2008. In vitro inhibition of sole aquabirnavirus by Senegalese sole Mx. Fish Shellfish Immunol. 24, 187–193.

Fernandez-Trujillo, M.A., Garcia-Rosado, E., Alonso, M.C., Borrego, J.J., Alvarez, M.C., Bejar, J., 2011. Differential antiviral activity of Mx1, Mx2 and Mx3 proteins from gilthead seabream (*Sparus aurata*) against infectious pancreatic necrosis virus (IPNV). Mol. Immunol. 49, 107–114.

Fernandez-Trujillo, M.A., Garcia-Rosado, E., Alonso, M.C., Castro, D., Alvarez, M.C., Bejar, J., 2013. Mx1, Mx2 and Mx3 proteins from the gilthead seabream (*Sparus aurata*) show in vitro antiviral activity against RNA and DNA viruses. Mol. Immunol. 56, 630–636.

Gonzalez-Mariscal, J.A., Gallardo-Galvez, J.B., Mendez, T., Alvarez, M.C., Bejar, J., 2014. Cloning and characterization of the Mx1, Mx2 and Mx3 promoters from gilthead seabream (*Sparus aurata*). Fish Shellfish Immunol. 38, 311–317.

Haller, O., Kochs, G., 2011. Human MxA protein: an interferon-induced dynamin-like GTPase with broad antiviral activity. J. Interferon Cytokine Res. 31, 79–87.

Hu, G.-B., Xia, J., Lou, H.-M., Chen, X.-L., Li, J., Liu, Q.-M., 2011. An IRF-3 Homolog that is up-regulated by DNA virus and poly I:C in turbot, *Scophthalmus maximus*. Fish Shellfish Immunol. 31, 1224–1231.

Huang, X., Huang, Y., Gong, J., Yan, Y., Qin, Q., 2008. Identification and characterization of a putative lipopolysaccharide-induced TNF-α factor (LITAF) homolog from Singapore grouper iridovirus. Biochem. Biophys. Res. Commun. 373, 140–145.

Isshiki, T., Nishizawa, T., Kobayashi, T., Nagano, T., Miyazaki, T., 2001. An outbreak of VHSV (viral haemorrhagic septicaemia virus) infection in farmed Japanese flounder *Paralichthys olivaceus* in Japan. Dis. Aquat. Organ. 47, 87–99.

Jensen, I., Robertsen, B., 2002. Effect of double-stranded RNA and interferon on the antiviral activity of Atlantic salmon cells against infectious salmon anemia virus and infectious pancreatic necrosis virus. Fish Shellfish Immunol. 13, 221–241.

Jensen, I., Larsen, R., Robertsen, B., 2002. An antiviral state induced in chinook salmon embryo cells (CHSE-214) by transfection with the double-stranded RNA poly I:C. Fish Shellfish Immunol. 13, 367–378.

Jørgensen, J.B., Johansen, A., Hegseth, M.N., Zou, J., Robertsen, B., Collet, B., Secombes, C.J., 2007. A recombinant CHSE-214 cell line expressing an Mx1 promoter–reporter system responds to both interferon type I and type II from salmonids and represents a versatile tool to study the IFN-system in teleost fish. Fish Shellfish Immunol. 23, 1294–1303.

Kibenge, M.J.T., Munir, K., Kibenge, F.S.B., 2005. Constitutive expression of Atlantic salmon Mx1 protein in CHSE-214 cells confers resistance to infectious salmon anaemia virus. Virol. J. 2, 75–81.

Kim, M.S., Kim, K.H., 2012. Effects of NV gene knock-out recombinant viral haemorrhagic septicaemia virus (VHSV) on Mx gene expression in epithelioma papulosum cyprini (EPC) cells and olive flounder (*Paralichthys olivaceus*). Fish Shellfish Immunol. 32, 459–463.

Langevin, C., Aleksejeva, E., Passoni, G., Palha, N., Levraud, J.-P., Boudinot, P., 2013. The antiviral innate immune response in fish: evolution and conservation of the IFN system. J. Mol. Biol. 425, 4904–4920.

Larsen, R., Røkenes, T.P., Robertsen, B., 2004. Inhibition of infectious pancreatic necrosis virus replication by Atlantic salmon Mx1 protein. J. Virol. 78, 7938–7944.

Lester, K., Hall, M., Urquhart, K., Gahlawat, S., Collet, B., 2012. Development of an *in vitro* system to measure the sensitivity to the antiviral Mx protein of fish viruses. J. Virol. Methods 182, 1–48.

Lin, C.-H., Christopher John, J.A., Lin, C.-H., Chang, C.-Y., 2006. Inhibition of nervous necrosis virus propagation by fish Mx proteins. Biochem. Biophys. Res. Commun. 351, 534–539.

Lockhart, K., McBeath, A.J.A., Collet, B., Snow, M., Ellis, A.E., 2007. Expression of Mx mRNA following infection with IPNV is greater in IPN-susceptible Atlantic salmon post-smolts than in IPN-resistant Atlantic salmon parr. Fish Shellfish Immunol. 22, 151–156.

Lopez-Vazquez, C., Conde, M., Dopazo, C.P., Barja, J.L., Bandin, I., 2011. Susceptibility of juvenile sole (*Solea senegalensis*) to marine isolates of viral haemorrhagic septicaemia virus from wild and farmed fish. Dis. Aquat. Organ. 93, 111–116.

Magnadottir, B., 2010. Immunological control of fish diseases. Mar. Biotechnol. 12, 361–379.

Ooi, E.L., Hirono, I., Aoki, T., 2006. Functional characterisation of the Japanese flounder, *Paralichthys olivaceus*, Mx promoter. Fish Shellfish Immunol. 21, 293–304.

Plant, K.P., Thune, R.L., 2008. Genomic organisation of the channel catfish Mx1 gene and characterisation of multiple channel catfish Mx gene promoters. Fish Shellfish Immunol. 24, 575–583.

Pontejo, S.M., Sanchez, C., Martin, R., Mulero, V., Alcami, A., Alejo, A., 2013. An orphan viral TNF receptor superfamily member identified in lymphocystis disease virus. Virol. J. 10, 188–193.

Reed, L.H., Muench, H., 1938. A simple method of estimating fifty percent end point. Am. J. Hyg. 27, 493–497.

Rodriguez, S., Vilas, P., Gutierrez, M.C., Perez-Prieto, S.I., 1997. Isolation and preliminary characterization of a birnavirus from the sole (*Solea senegalensis*) in Southwest Spain. J Aquat. Anim. Health 9, 295–300.

Rothenburg, S., Chinchar, V.G., Dever, T.E., 2011. Characterization of a ranavirus inhibitor of the antiviral protein kinase PKR. BMC Microbiol. 11, 56–58.

Skjesol, A., Aamo, T., Hegseth, M.N., Robertsen, B., Jørgensen, J.B., 2009. The interplay between infectious pancreatic necrosis virus (IPNV) and the IFN system: IFN signaling is inhibited by IPNV infection. Virus Res. 143, 53–60.

Song, H., Santi, N., Evensen, O., Vakharia, V.N., 2005. Molecular determinants of infectious pancreatic necrosis virus virulence and cell culture adaptation. J. Virol. 79, 10289–10299.

Williams, T., Barbosa-Solomieu, V., Chinchar, V.G., 2005. A decade of advances in iridovirus research. Adv. Virus Res. 65, 173–248.

Whittington, R.J., Becker, J.A., Dennis, M.M., 2010. Iridovirus infections in finfish critical review with emphasis on ranaviruses. J. Fish Dis. 33, 95–122.

Wu, Y.C., Chi, S.C., 2007. Cloning and analysis of antiviral activity of a barramundi (*Lates calcarifer*) Mx gene. Fish Shellfish Immunol. 23, 97–108.

Wu, Y.C., Lu, Y.F., Chi, S.C., 2010. Anti-viral mechanism of barramundi Mx against betanodavirus involves the inhibition of viral RNA synthesis through the interference of RdRp. Fish Shellfish Immunol. 28, 467–475.

Yap, W.H., Tay, A., Brenner, S., Venkatesh, B., 2003. Molecular cloning of the pufferfish (*Takifugu rubripes*) Mx gene and functional characterization of its promoter. Immunogenet 54, 705–713.

Zou, J., Secombes, C.J., 2011. Teleost fish interferons and their role in immunity. Dev. Comp. Immunol. 35, 1376–1387.